BINDING AND GATING PROPERTIES OF THE KAINATE IONOTROPIC GLUTAMATE RECEPTOR

Presented by

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DOCTOR OF PHILOSOPHY

À mon meilleur ami,
À l'amour de ma vie,
À mon mari,
Benjamin

À mes parents pour leur constance, leur amour et leur support
À mon frère, David et mes soeurs, Valérie & Christina
pour tous ces précieux moments passés en famille

"In the pioneering days of structure determination,
researchers were driven by the conviction that once they had solved a biological
structure, its function or mechanism would become immediately obvious.
It came as a shock when they found this was not necessarily so
and that the opposite was more frequently true."
(Fersht, 1995)

- *commenting on an impromptu lunch discourse by Francis Crick*

ABSTRACT

Kainate-type ionotropic glutamate receptors (KA iGluRs) are ligand-gated ion-channels that play a key role in regulating both excitatory and inhibitory synaptic transmission. They are implicated in several CNS disorders including neuropathic pain, epilepsy and fear conditioning. Despite this, we have a very limited understanding of their basic structural and functional properties. In fact, much of what we know about the KAR comes from the closely-related AMPAR. Interestingly, the crystal structure of the agonist-binding domain of closely-related AMPARs confirmed the proposed bi-lobed structure and together with functional studies show that the extent of clamshell closure is agonist-dependent and directly correlated to agonist efficacy. Yet, no information was available for KARs. Given this, the main purpose of this thesis is to elucidate the binding and gating properties of KARs. The paucity of KAR agonists available at the beginning of this work, however, did not permit a thorough examination of the proposed relationship between clamshell closure and agonist efficacy. In light of this, using a combination of *in silico* docking and an electrophysiological assay, we have identified a wide spectrum of novel agonists that activate KARs, some of which are endogenous to the central nervous system. Moreover, we developed a methodology to report ligand-evoked conformational changes in the agonist-binding domain of intact KARs. Surprisingly, we showed that closure of the agonist-binding domain does not determine agonist efficacy at KARs suggesting they might not behave as previously thought. Furthermore, previous work has shown that external ions exclusively modulate the response amplitude and decay kinetics of KA, but not AMPA, receptors, through an unknown mechanism. Strikingly, we demonstrated that the neurotransmitter, L-glutamate, is not sufficient to activate KARs but that external ions are uniquely required for activation of KA, but not AMPA receptors. In summary, our research identifies novel structural and gating features of KARs which serves to further delineate them from AMPARs.

RÉSUMÉ

Le récepteur kaïnate (KAR) est un récepteur ionotropique au L-glutamate qui exerce un rôle crucial dans la régulation de la transmission synaptique excitatrice et inhibitrice. Les KARs sont impliqués dans diverses pathologies du système nerveux central (SNC) tel que la douleur neuropathique, l'épilepsie ou la peur conditionnée. Néanmoins, notre niveau de compréhension de leur propriétés structurelles et fonctionnelles reste aujourd'hui encore, extrêmement limitée. De plus, les avancées réalisées dans la connaissance des KARs proviennent pour l'essentiel de données obtenues à partir de son analogue structurel, le récepteur AMPA (AMPAR). En effet, des études cristallographiques combinées à une approche fonctionnelle ont permis de démontrer que le domaine renfermant le site de liaison du AMPAR possède une conformation bilobée dite "à clapet" et que son degré de fermeture est directement corrélé à l'efficacité de l'agoniste. Toutefois, aucune de ces informations n'étaient disponibles pour les KARs. Ainsi, l'ensemble de cette thèse s'est inscrit dans l'élucidation des propriétés structurales et dynamiques des KARs. Au début de ce travail, le peu d'agonistes connus pour les KARs ne nous permettait pas d'étudier en profondeur la corrélation entre le degré de fermeture du clapet et l'efficacité des agonistes. Par conséquent, nous avons premièrement identifié une nouvelle gamme d'agonistes du KAR (certains étant endogènes au SNC), en combinant une approche de docking *in silico* et d'enregistrements electrophysiologiques. Par ailleurs, nous avons développé une méthodologie permettant d'évaluer au sein de KARs intacts, les modifications conformationnelles du domaine de liaison à l'agoniste. De manière surprenante, nos résultats démontrent que contrairement aux AMPARs, le degré de fermeture du clapet n'est pas corrélé à l'efficacité du ligand, ce qui suggère que les KARs n'adoptent pas le comportement préconçu. D'autre part, les ions extracellulaires n'étaient connus jusqu'ici que pour leurs rôles modulateurs de l'amplitude de la réponse et de la cinétique de déclin des KARs, via un mécanisme encore inconnu à ce jour. Or, nous démontrons que le L-glutamate n'est pas suffisant en lui même pour activer le KAR, mais qu'il requiert la présence d'ions extracellulaires. Ce

mécanisme est uniquement observé pour le KAR et non pour le AMPAR. En conclusion, nos travaux de recherche ont permis d'identifier de nouvelles caractéristiques structurales et fonctionnelles des KARs, qui permettent de les discriminer davantage des AMPARs.

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ABBREVIATIONS

γ-GG	γ-D-glutamylglycine
A	agonist
ABD	agonist binding domain
ACBC	1-aminocyclobutane-1-carboxylic acid
ACPC	1-aminocyclopropane-1-carboxylic acid
AMPA	2-amino-3-(3-hydroxy-5-methyl-4-isoxazolyl)propionic acid
AR	agonist and receptor complex (inactive state)
AR*	agonist and receptor complex (open state)
ATP	adenosine triphosphate
ATPA	2-amino-3-(5-tert-butyl-3-hyrdoxy-4-isoxazolyl) propionic acid
cAMP	cyclic adenosine monophosphate
cGMP	cyclic guanosine monophosphate
CASK	calmodulin-associated serine/threonine kinase
CNQX	cyano-7-nitroquinoxaline-2,3-dione
CNS	central nervous system
CNG	cyclic-nucleotide gated
Con-A	concanavalin-A
CTZ	cyclothiazide
DCKA	5,7,-dichlorokynurenic acid
DNQX	6,7-Dinitroquinoxaline-2,3-dione
DRG	dorsal root ganglia
EPSCs	excitatory postsynaptic synaptic currents
ER	endoplasmic reticulum
FRET	fluorescence resonance energy transfer
GABA	gamma amino butyric acid
GDEE	L-glutamic acid diethyl ester
GFP	green fluorescent protein
GlyR	glycine receptor
GPCR	G protein-coupled receptor
GRIP	glutamate receptor-interacting protein
GYKI 53655	1-(4-aminophenyl)-4-methyl-7,8-methylenedioxy-5H-2,3-benzodiazepine
KA	kainate
KARP	kainate receptor regulatory protein
iGluR	ionotropic glutamate receptor
IW	(S)-5-Iodowillariine

KA	kainate
KNF	Kosland-Nemethy-Filmer
LAOBP	lysine-arginine-ornithine binding protein
LBD	ligand-binding domain
LGIC	ligand-gated ion channels
L-Glu	L-glutamate
LIVBP	leucine-isoleucine-valine binding protein
LTP	long-term potentiation
LTD	long-term depression
nAChR	nicotinic acetylcholine receptor
mGluR	metabotropic glutamate receptor
mRNA	messenger ribonucleic acid
MWC	Monod-Wyman-Changeux
Narp	neuronal activity-regulated pentraxin
NBQX	6-nitro-7-sulphamoylbenzo[f]quinoxaline-2,3-dione
NMDA	N-methyl-D-aspartate
NMDG	N-methyl-D-glucamine
NMR	nuclear magnetic resonance
NETO1/NETO2	neuropilin tolloid-like 1/ neuropilin tolloid-like 2
NS-102	5-nitro-6,7,8,9-tetrahydrobenzo[g]-2-3-dione-3-oxime
P_2X	purinergic
PICK1	protein interacting with C kinase 1
PKA	protein kinase A
PKC	protein kinase C
PLC	phospholipase C
PSD-95	post-synaptic desensity-95 protein
QBP	glutamine binding protein
R	receptor
RT-PCR	reverse transcription-polymerase chain reaction
sAHP	slow $Ca2^{+}$-activated K^{+} current
SAP	synapse-associated protein
SCAM	substituted cysteine accessibility method
SYM 2081	(2S,4R)-4-methylglutamate
TARP	transmembrane AMPA receptor regulatory protein
TM	transmembrane domain

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The work in this thesis has been inspiring, often exciting, many times challenging, but always an interesting experience. It has been made possible by many people who have supported me. It is with pleasure and honor that I would like to thank the many people who made this thesis possible.

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CONTRIBUTIONS OF AUTHORS

This thesis is assembled in accordance with the regulations of the Faculty of Graduate studies and Research from McGill University. It is written in a manuscript-based format, and comprises three original published manuscripts in **CHAPTERS 1, 2** and **3.** In addition to the published article presented in **CHAPTER 3**, supplementary data and text have also been added in an updated discussion to further complement the published research findings. All the manuscripts included in this work are co-authored. This section details to what extent the co-authors contributed to these studies:

The Results **CHAPTER 1** entitled "DOES THE AGONIST-BINDING DOMAIN OF INTACT KA RECEPTORS ADOPT LIGAND-DEPENDENT CONFORMATIONS*?*" was published as an article in the Journal of Physiology in 2006:

Fay, A.M. and Bowie, D. (2006) Distinct Agonist-Evoked Conformational Changes in Intact GluR6 Kainate Receptors. ***Journal of Physiology***. 572(pt1):201-213.

- D. Bowie performed preliminary electrophysiology experiments for this paper. I performed most of the electrophysiological experiments and all the mutagenesis work. Additionally, I performed all the data analysis and assembled figures for the manuscript. Both authors designed the experiments and the manuscript was co-authored.

The Results **CHAPTER 2** entitled "DOES CLOSURE OF THE AGONIST-BINDING DOMAIN CORRELATE WITH AGONIST EFFICACY AT KA RECEPTORS?" was published as an article in Molecular Pharmcology in 2009:

Fay, A.M., Corbeil, R.C., Brown, P., Moitessier, N., Bowie, D. Functional Characterisation and *in silico* Docking of Full and Partial GluK2 Kainate Receptor Agonists. ***Molecular Pharmacology***.75(5):1096-1107.

- I performed all the electrophysiology experiments (Figures 2, 3, 4, 6, Table 1) in this manuscript, with the exception of some data dose-response curves in Figures 3. P. Brown provided complementary data for this Figure in panel C and D, including the dose-response data for D-aspartate and some points for L-aspartate. I initiated and provided the rationale for the collaboration with the Moitessier group. I performed the molecular ligand docking experiments using the program FITTED with R.C. Corbeil (Figures 1, 4, 5, 7 and Table 1). I performed all the analysis required, drew and assembled all the Figures and Tables. Both the author and D. Bowie were involved in the design and rational of the experiments and writing of the manuscript. N. Moitessier, R.C. Corbeil and P. Brown also edited the manuscript.

The Results **CHAPTER 3** is entitled "ARE EXTERNAL IONS AN ABSOLUTE REQUIREMENT FOR KA RECEPTOR ACTIVATION?" was published in the Journal of Neuroscience in 2006:

Wong, A.Y.C., **Fay, A.M.,** Bowie, D. (2006). External ions are co-activators of the ligand-gated kainate receptor ion-channel. ***The Journal of Neuroscience***. 26(21):5750-5.

- I contributed 20% of the experimental design, execution and analysis. In regards to the data, I performed (1) pilot experiments evaluating the experimental conditions necessary for Figure 2 and (2) the experiments and analysis of the data reported in Figures 4A, B and C (10 mM only). A. Wong performed all the other electrophysiology and mutagenesis work, analyses and figure-making. All authors were significantly involved in the writing and editing of the manuscript.

Finally, a list of abstracts that were submitted to local, national and international scientific meetings for poster presentations throughout my graduate studies includes:

1. **Fay, A.M.,** Corbeil,C.R., Moitessier, N & Bowie, D. (2008) Endogenous D- and L-amino acids act as inverse agonists at ionotropic kainate glutamate receptors. *6th Annual FENS Meeting. A145.10. Geneva, Switzerland.*

2. **Fay, A.M.,** Corbeil,C.R., Moitessier, N & Bowie, D. (2007) Molecular docking and functional analysis of novel kainate glutamate receptor agonists. *1st Annual CAD-CAN Meeting. Toronto, Canada*

3. **Fay, A.M.** & Bowie, D. (2005) Ionotropic glutamate receptors and the structural basis of drug potency. *McGill Biomedical Graduate Research Day Abstracts.*

4. Wong, A.Y.C., **Fay, A.M**., Bowie, D. (2005) Kainate receptor activation has an absolute requirement for external ions. *Soc. Neurosci. Abstrs., 486.8., New Orleans, USA*

5. **Fay, A.M**. and Bowie, D. (2004). What is the molecular basis of full and partial agonist behaviour at the GluR6 kainate receptor? *Soc. Neurosci. Abstrs., 732.10.*

6. **Fay, A.M.** and Bowie, D. (2004). Full and partial agonists promote distinct conformational changes in the agonist binding domain of GluR6 kainate receptors. *Fed Eur Neurosci., FENS abstr, 2, A184.5.*

7. **Fay, A.M**. & Bowie, D. (2003) State-dependent modulation of GluR6 kainate receptors by concanavalin-A. *Soc. Neurosci. Abstrs, 361.1.*

GENERAL PREFACE

The glutamatergic system is the prominent pathway for excitatory information in the brain, and is thus essential for its normal function. Consequently, this system has been implicated in various CNS pathologies. Essential components of the glutamatergic system are the postsynaptic glutamate receptors (GluRs) which respond to the binding of the amino acid L-glutamate, released from the presynaptic neuron. Despite the fact that the ionotropic GluR family has been identified as a key target for a wide spectrum of neurological disorders (such as autism, depression, Alzheimer's disease and epilepsy), there is currently only one clinical drug (i.e., memantine) that targets ionotropic GluR dysfunction. The obvious question then is: why aren't there more GluR drugs? The main reason underlying this problem is that we still don't have a good understanding of how iGluRs work at a very basic level. When I joined Dr. Bowie's laboratory, there were two main outstanding questions in the field of kainate (KA) ionotropic glutamate receptors that drove my doctoral research studies:

(1) The first main issue was to understand **how changes in the KA receptor structure relate to its gating behavior**. Prior to this work, recent progress in this area had been possible as a result of the key finding that the clamshell-like agonist-binding domain of the closely related-AMPA receptor could be isolated and cristallized with a number of ligands. Although, no crystal was yet available for KA receptors at the beginning of this work, they were thought to operate much like the AMPA receptors, where closure of the ligand binding domain was thought to correlate with agonist efficacy. These findings, however, have generated a number of questions. For example, how does the isolated agonist-binding core of KA receptor relate to the intact receptor? How does the binding of an agonist promote activation and what are the specific requirements for ligands acting at KA receptors? A complicating issue is that upon prolonged agonist application, KA receptors have been shown to exhibit rapid and

pronounced desensitization, a phenomenon yet to be adequately addressed by these studies. This, in turn, has raised additional questions such as: "Does the agonist-binding domain adopt the same or different conformation during activation and desensitization?" and, "Is the conformation elicited during desensitization the same for agonists exhibiting different desensitization profiles or is this also agonist-dependent?" Before the beginning of my thesis, the number of KA receptor agonists was very limited and did not permit a thorough study of the basic mechanism of KA receptor activation and desensitization.

(2) A second important issue to consider was the **structural basis underlying the effects of different pharmacological tools that modulate KA receptor kinetics**. For example, it has been shown that KA receptors are modulated by both large proteins, such as lectins, and by very small charged atoms, curiously external anions and cations alike. How, then do these entirely unrelated molecules exert their effect on KA receptor activation and desensitization? Do these molecules also play a role in governing closure of the agonist binding domain? Recent work from our laboratory has shown that ions modulate the efficacy of L-glutamate responses at KA receptors, yet the basis of this effect cannot be explained by crystallographic data of its agonist-binding domain.

To address these issues, the present doctoral thesis is divided into three main parts:

(1) In the first part, the **REVIEW OF THE LITERATURE,** I introduce the reader to the topic of this thesis by a brief and relevant overview of the state of the field. Specifically, the first sections about the properties of KA receptors cover the breadth of literature until *now*. This applies to issues that did not directly influence my research *per se*, but that nonetheless provide valuable information necessary to obtain an appropriate

understanding of KA receptors. In contrast, the last section of this part presents the state of the field as it was understood *before* I started to do my thesis research, highlighting specific gaps in the literature. This set-up is to allow the reader to fully appreciate the motivation underlying the work performed during this thesis and will be explicitly noted in the text. Following this section, the **RATIONALE AND OBJECTIVES** of this work are explicitly stated.

(2) In the second part, I present my **RESULTS IN THREE DISTINCT MANUSCRIPT-BASED CHAPTERS** which have been published. Additionally, I provided relevant connecting text to ensure that the thesis has continuity and thus integrate the text into a cohesive unit with a logical progression from one chapter to the next.

(3) In the third part, I discuss the broader implications of our research finding in the **DISUCUSSION & CONCLUSIONS** and speculate on the potential role of the novel KA receptor binding and gating properties that we have identified. Experiments to address some of these issues and move this field ahead are also considered here.

PART ONE

REVIEW OF THE LITERATURE

1. GENERAL INTRODUCTION

1.1. What Is a Ligand-Gated Ion-Channel?

Ligand-gated ion-channels (LGICs) are transmembrane receptor proteins with a channel pore that enable rapid and selective ion fluxes across biological membranes upon binding of chemical messengers such as neurotransmitters (Hille, 1992;Krusek et al., 2004). These receptors fulfill a diversity of roles in the nervous system including triggering muscle contraction (Katz, 1966;Colquhoun & Sakmann, 1998;Hogg *et al.*, 2003) and mediating changes in synaptic efficacy such as those required for learning and memory (Bliss & Collingridge, 1993;Whitlock *et al.*, 2006).

Based on their structure, transmitter-gated ion-channels can be divided into three main classes. First, the cys-loop receptors which represent the pentameric LGIC superfamily and include nicotinic acetylcholine receptors (nAChRs), serotonin receptors (5-HT_3Rs), gamma amino butyric acid ($GABA_A$) and glycine (Gly) receptors (Colquhoun & Sivilotti, 2004;Brejc *et al.*, 2001). Secondly, the adenosine triphosphate (ATP)-activated purinergic (P_2X) receptors are classified in a unique category because they are trimers that assemble as homo and heteromeric channels (North, 2002). The third class includes the tetrameric glutamate-gated ion-channels, more commonly referred to as ionotropic glutamate receptors (iGluRs). Based on their pharmacology and functional properties, these receptors are further divided into three main families (Figure 1), including NMDA (N-methyl-D-aspartic acid), AMPA (α-amino-3-hydroxyl-5-methyl-4-isoxazole-propionate), and kainate (KA) receptors, with the latter subfamily being the main subject of this thesis.

Interestingly, ionotropic glutamate receptors (iGluRs) seem to share more resemblance with the voltage-gated potassium channel than other LGIC, suggesting that voltage and ligand-gated ion-channels evolved from a common ancestral protein (Chen *et al.*, 1999a;Panchenko *et al.*, 2001;Brejc *et al.*, 2001;Miyazawa *et al.*, 2003). Both receptor families are tetramers, share critical residues involved in gating and access multiple subconductance levels before

reaching the fully open state (Zheng & Sigworth, 1998;Zheng & Sigworth, 1997;Jahr & Stevens, 1987). Interestingly, the identification of new homologs of bacterial GluRs, such as GluR0 bacterial glutamate-gated potassium channels, supports a potential evolutionary link between these two receptor families (Chen *et al.*, 1999a;Kuner *et al.*, 2003).

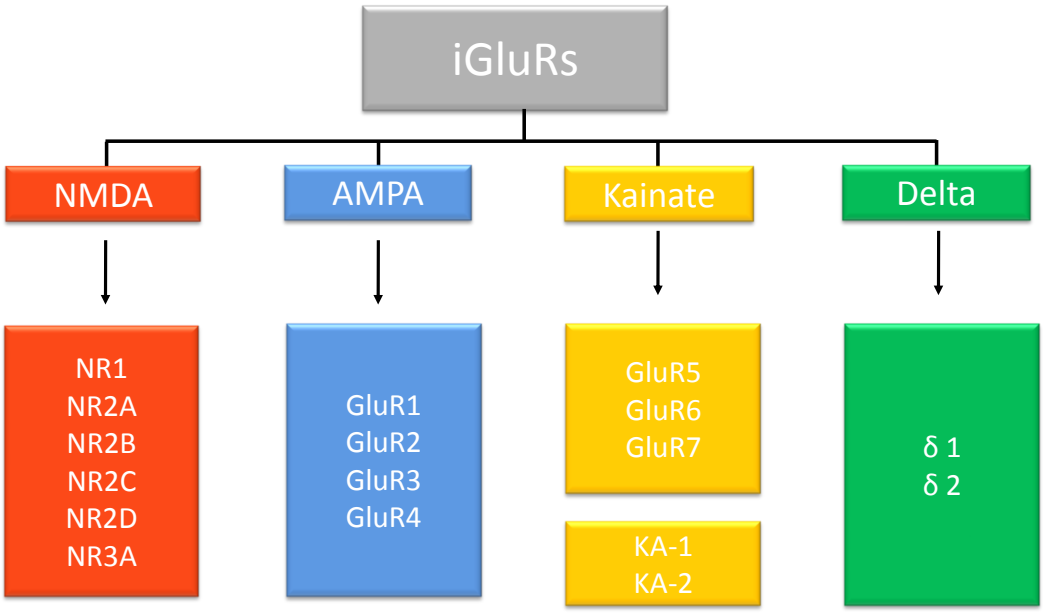


Figure 1. Schematic of the ionotropic glutamate receptor subfamilies

Though this work has focused on transmitter-gated ion-channels, it is important to note that insight into other classes of ligand-gated ion-channels such as the trimeric peptide-gated sodium channels and the intracellular ligand-gated ion-channels such as the tetrameric cyclic nucleotide gated (CNG) channels or the transient receptor potential channels may be useful to understand the strutcture and function of iGluRs. Given that these ion-channels are out of the scope of this thesis, these will not be adressed in this thesis (or only briefly to relate relevant findings).

1.2. Ionotropic Glutamate Receptors Fulfill Distinct Roles in the CNS

Although it is now well established that almost all excitatory neurotransmission in the mammalian central nervous system (CNS) is mediated via the action of L-glutamate (Dingledine et al., 1999;Erreger et al., 2004;Madden, 2002;Watkins, 2000), this recognition was very slowly accepted within the scientific community (Bowie, 2008a). This amino acid was isolated in 1866 by the German chemist, Karl Ritthausen (1866), but it was not until more than 90 years later that its putative function as a neurotransmitter was investigated. The fact that L-glutamate was already known as a protein component and key metabolite of the citric acid cycle represented major barriers for recognizing its role as a neurotransmitter (Palmada & Centelles, 1998).

After years of research and controversy, it is now widely recognized that L-glutamate is the main excitatory neurotransmitter in the CNS where it binds to both ionotropic and metabotropic GluRs (mGluRs) located on the postsynaptic neuron (Watkins, 2000). While mGluRs mediate the slower biochemical component of synaptic transmission, our work focuses on a subfamily of iGluRs where the free energy of agonist binding has been conventionally thought to drive channel pore opening and/or intracellular calcium elevation (Dingledine et al., 1999). Synaptic transmission may be subsequently halted by a combination of glutamate re-uptake, diffusion out of the synaptic cleft, and receptor desensitization (Clements *et al.*, 1992;Glavinovic & Rabie, 1998;Kidd & Isaac, 2001). This simplistic description, however, is complicated by recent evidence showing that L-glutamate also acts on presynaptic receptors that regulate the strength of synaptic transmission (Pinheiro & Mulle, 2008).

Together, iGluRs are responsible for basal excitatory synaptic transmission in the CNS and many forms of synaptic plasticity, such as long-term potentiation (LTP) and long-term depression (LTD), which are thought to underlie learning and memory (Asztely & Gustafsson, 1996;Cull-Candy *et al.*, 2006;Bortolotto *et al.*, 1999;Whitlock *et al.*, 2006). Pharmacological tools and engineered knock-out mice have helped dissect the specific roles of each iGluR family. For example, NMDA receptors have been shown to function primarily as coincidence detectors

since their voltage-dependent $Mg2^{+}$ block allows them to simultaneously monitor changes in the membrane potential and the presence of L-glutamate in the synaptic cleft (Collingridge & Bliss, 1995). Additionally, their high calcium permeability, slow kinetics and little receptor desensitization allows for calcium influx to drive intracellular $Ca2^{+}$ dependent signaling (Debski *et al.*, 1990;Constantine-Paton, 1990;Nicoll & Malenka, 1999). NMDAR activation also requires binding of the co-agonist, glycine to the NR1 subunit (Johnson & Ascher, 1987).

In contrast to NMDA, AMPA and KA receptors have faster kinetics (Stern et al., 1994;Rosenmund et al., 1998), smaller conductance, exhibit different calcium permeability and are strongly desensitizing in the presence of glutamate (Sprengel & Seeburg, 1993;Dingledine *et al.*, 1999). Despite these similarities, AMPA and KA receptors fulfill both overlapping and divergent roles in the CNS. It is well recognized that AMPA receptors are the main mediators of the fast component of excitatory synaptic currents (Dingledine et al., 1999;Erreger et al., 2004). Moreover, cellular models of synaptic plastiticity have implicated the trafficking of postsynaptic AMPA receptors where synaptic strenghetening (i.e. LTP) and weakening (i.e. LTD) may be achieved via incorporation of specific AMPA receptor subunits (reviewed in Kessels & Malinow, 2009). On the other hand, since KA receptors are thought to be expressed at fewer synapses in the CNS and because of the lack of selective pharmacological tools, our understanding of the KA receptor subfamily function has trailed behind other iGluR subfamilies (reviewed in Jane et al., 2009). Consequently, our research work has focused on elucidating the basic properties of KA receptors, which will be the major purpose of the remainder of the introduction.

2. EMERGENCE OF KAINATE RECEPTORS AS PART OF A MAJOR NEUROTRANSMITTER SYSTEM

2.1. The Neurotoxin Kainate: A Tool Distinguishing iGluR Subfamilies

Kainic acid was originally isolated from the red marine algae *Digenea simplex* in 1953 by a Japanese group (Murakami et al., 1953). In the early 1960s, this marine toxin was originally administered for its anthelminitic properties to treat large populations of children against intestinal worms (reviewed in Olney et al., 1974). A few years later, L-glutamate and short chain dicarboxylic amino acids were shown to be a strong excitant of neurons in the mammalian CNS (Curtis & Crawford, 1969). Given that many anthelmintics also contained a L-glutamate backbone, it was hypothesized that these molecules might also have a comparable effect on neurons (Shinozaki & Konishi, 1970). Specifically, the effect of KA on the CNS by electrophoretic application with current pulses on rat cortical neurons revealed that the toxin induced a spike discharge response similar to that previously observed with L-glutamate, but with a higher frequency (Shinozaki & Konishi, 1970;Johnston *et al.*, 1974). This observation provided the first evidence for the potent excitatory action of KA.

Given that KA was identified as a conformationally restricted analog of L-glutamate, a potent excitant and neurotoxic amino acid, researchers suspected that KA may also exhibit similar properties. Consistent with this idea, studies showed that subcutaneous, systemic, intracerebral injection or oral administration of KA in rodents induced convulsions and brain damage (Olney et al., 1974;Nadler, 1981;Nadler et al., 1978). As a result of the work by Olney and colleagues, the value of KA as a potent lesioning tool exhibiting specificity for neuronal somata and dendrites was quickly recognized (Coyle et al., 1978). This neurotoxin has now become the most investigated model of temporal lobe epilepsy (Nadler, 1981;Sperk, 1994;Pinheiro & Mulle, 2006).

Likewise, in the early characterization of iGluRs, KA proved to be an important pharmacological tool in discriminating between receptor subtypes. The notion that L-glutamate was acting through multiple receptors emerged in 1974 from work by Arthur Duggan, in which he showed differential sensitivities of Renshaw cells and spinal interneurons to L-aspartate and L-glutamate, respectively (Duggan, 1974). This observation was further supported by the finding that KA and NMDA also exhibited large potency differences on the same groups of cells (McCulloch et al., 1974). Moreover, the effect of KA was insensitive to conventional NMDA antagonists such as external magnesium (Evans et al., 1979) and APV, but sensitive to known non-NMDA receptor blockers such as L-glutamic acid diethyl ester (GDEE), γ-D-glutamylglycine (γ-GG) and kynurenic acid (reviewed in Bowie, 2008a).

Experiments on pain conducting C-fibers in dorsal root fibers warranted further refinement of the non-NMDA classification because these fibers responded to KA, but not NMDA or quisqualate (Davies *et al.*, 1979;Agrawal & Evans, 1986;Huettner, 1990). In line with this, radioligand binding assays showed a distinct pattern of high-affinity binding sites for [^{3}H] KA in the rat and human brain suggesting that KA was acting via a distinct subset of glutamate receptors (London & Coyle, 1979b;London & Coyle, 1979a). As described below, it was the subsequent cloning of various iGluR subunits that permitted the definite classification of KA receptors as a distinct iGluR subfamily.

2.2. Elucidating the Molecular Identity of Kainate Receptors

The application of molecular biology techniques to study iGluRs in the 1980s confirmed the existence of three main glutamate receptor families: NMDA, AMPA and KA receptors (Figure 1) (Hollmann & Heinemann, 1994;Hollmann *et al.*, 1989;Sommer & Seeburg, 1992;Paschen *et al.*, 1994). At the same time the delta orphan subunits (GluRδ) which do not form functional glutamate gated-channels or seem to assemble with other iGluR families were identified (Figure 1) (Lomeli *et al.*, 1993;Schmid & Hollmann, 2008).

AMPA and KA receptor subfamilies were found to exhibit cross-activation by their defining agonists and rapid drug application techniques revealed a more complex pharmacology than initially anticipated. For instance, KA was found to activate large sustained currents at AMPA receptors (Patneau & Mayer, 1990a) (but see (Patneau et al., 1993)) while evoking a rapidly-desensitizing response at KA receptors (Huettner, 1990). Additionally, AMPA was also found to activate some heteromeric KA receptors (Herb et al., 1992).

At the beginning of the 1990s, five KA receptor subunits were cloned using screening with low stringency hybridization probes to AMPA receptor subunits (Hollmann & Heinemann, 1994). Examination of amino acid identity and agonist-binding properties revealed that KA receptors could be further divided into two different subclasses. The first subclass includes GluR5, GluR6 and GluR7, which are approximately 75%-80% identical, exhibit a low affinity for glutamate and can form homomeric channels. The diversity of KA receptors is further increased with the existence of splice variants for these three subunits, denoted as GluR5a, GluR5b, GluR5c, GluR6a, GluR6b, GluR7a and GluR7c (Bettler & Mulle, 1995;Jaskolski *et al.*, 2005;Lerma, 2003).

In contrast, the high-affinity subunits KA-1 and KA-2 display approximately 70% sequence homology and must be expressed as heteromers in combination with either GluR5, GluR6 or GluR7 to form functional receptors with altered pharmacological and biophysical properties (Dingledine *et al.*, 1999;Pinheiro & Mulle, 2006). These KA subclasses share about 45% sequence identity with each other, but only 30-40% with AMPA receptors and 10-20% with NMDA receptor subunits, setting them apart as a distinct receptor subfamily (Hollmann & Heinemann, 1994). Cloning of iGluR receptors eventually permitted crystallization of their agonist-binding domain (see section 3.2.4) and the design of engineered knock-out mice, which played a key role in clarifying the structure and role of specific subfamilies and receptor subunits at the synaptic level (Mulle et al., 1998;Jia et al., 1996;Meng et al., 2003;Zamanillo et al., 1999;Sakimura et al., 1995;Strutz-Seebohm et al., 2005;Armstrong et al., 1998).

Curiously, in addition to the identification of mammalian KA subunits, several other homologous proteins, which also bind KA were cloned around the same time. Although their role has yet to be defined, these include KA-binding proteins from other species including goldfish (Ziegra et al., 1992), frog (Hampson & Wenthold, 1988;Wada *et al.*, 1989), toad (Henley et al., 1992;Henley et al., 1989a;Kerry et al., 1993) and chick (Henley et al., 1989b;Klein et al., 1988;Gregor et al., 1989;Henley, 1994). Despite the fact that these proteins are thought to share the same subunit topology as iGluR subunits, they lack apparent intrinsic ion-channel function and do not seem to form functional channels when expressed alone or in combination with subunits (reviewed in Henley, 1994). Interestingly, however, engineered chimeras of the ion-channel domain of the different KA-binding proteins into GluR1 and GluR6 subunits generated functional channels suggesting that these proteins are unable to convert the energy of binding to gate the channel or have the ability to operate as ion-channels but lack some key property (Villmann et al., 1997).

Finally, consistent with the observation that the different KA receptor subunits display distinct pharmacological and electrophysiological profiles (Wilding & Huettner, 2001), the next section discusses the various approaches that have been used to examine the localization and distribution of KA receptor subunits in the CNS (Wisden & Seeburg, 1993;Mulle *et al.*, 2000).

2.3. Distribution of Kainate Receptors in the CNS

Despite the fact that KA receptors are found in the embryo, most of the distinct subunit expression patterns emerge in the postnatal period (Bahn et al., 1994). Interestingly, the initial suggestion that KA receptors played an important physiological role followed the observation that high affinity binding sites to KA in striatal membranes dramatically increased during the development of animals from birth (Campochiaro & Coyle, 1978).

KA receptor subunits are widely expressed throughout the nervous system, including the dorsal root ganglia, cerebellum, hippocampus, and amygdala (Huettner, 2003). Of particular relevance to this work, the GluR6 subunit is

abundantly expressed in brain structures implicated in learning and memory (such as the hippocampus) and in regions serving motor and motivational aspects of behavior (such as the basal ganglia and the cerebellum) (Mulle et al., 1998). *In situ* hybridization, single cell reverse transcription-polymerase chain reaction (RT-PCR) and northern blot analysis have been utilized to map out the distribution of cells expressing KA receptor mRNA, revealing heterogeneous, yet overlapping expression patterns in the CNS (Huettner, 2003).

More specifically, northern blot analysis of mRNA extracted from dorsal root ganglia (DRG) neurons revealed that GluR5 is the most prominent subunit expressed in this cell type, while GluR7 and KA-1 were also detected, but at much lower levels (Partin et al., 1993). In the cerebellum, *in situ* hybridization histochemistry showed that the GluR5 mRNA is mainly found in the Purkinje cell layer and in the Golgi cells of the granule cell layer, whereas GluR6 is abundantly expressed in the granule cells and GluR7 is expressed at lower levels in the inhibitory neurons of the molecular layer. In the hippocampus, the GluR5 transcript is primarily expressed in *stratum radiatum* interneurons while GluR6 is mainly expressed in pyramidal cells but is also found in both *stratum oriens* and *radiatum* interneurons (Paternain et al., 2000).

Additionally, electrophysiological recordings demonstrated that the GluR5 and GluR6 subunits were co-expressed to form functional channels in *stratum radiatum* interneurons (Paternain et al., 2000). On the other hand, the KA-1 subunit is almost exclusively found in hippocampal CA3 and dentate granule neurons (Huettner, 2003). In the amygdala, GluR5 mRNA expression levels are higher than in the hippocampal formation, whereas the opposite applies for GluR6 and KA-2 mRNA (Li et al., 2001;Bureau et al., 1999). KA receptors have also been identified in trigeminal ganglion neurons, where single-cell PCR revealed high levels of the GluR5 and KA-2 transcripts (Sahara et al., 1997). Functional recordings of native KA receptors in the hippocampus, DRG and spinal cord has clearly shown that this molecular diversity also gives rise to KA receptors with specific physiological and pharmacological profiles (Wilding & Huettner, 2001).

Even though these studies provide information pertaining to the general localization of KA receptors, they do not offer insight into their specific localization and compartmentalization within neurons. Immunocytochemistry studies have yielded limited information because of the lack of subunit-specific KA receptor antibodies. The available anti-GluR5/6/7, anti-GluR6/7, anti-KA-2 and the recently developed anti-KA-1 antibodies have placed KA receptors both presynaptically in a subpopulation of glutamatergic terminals as well as postsynaptically on dendrites and spines (Huntley *et al.*, 1993;Good & Morrison, 1995;Good *et al.*, 1993;Siegel *et al.*, 1995;Petralia *et al.*, 1994;Charara *et al.*, 1999;Darstein *et al.*, 2003). Interestingly, a detailed analysis of the subcellular and subsynaptic localization of the KA receptor subunits GluR6/7 and KA-2 have placed more than two thirds of the subunits intracellularly and found that amongst the plasma-membrane bound KA receptor subunits, almost two-thirds were expressed extrasynaptically (Kieval et al., 2001). This pattern of expression mimics that of G-protein-coupled metabotropic receptors, which are mainly found intracellularly or at non-synaptic sites (Rodriguez-Moreno & Sihra, 2007), an observation consistent with the metabotropic action of KA receptors (see subheading *Metabotropic Action of Kainate Receptors & Presynaptic Kainate Receptors* under section 3.4). As discussed below, the development of specific antibodies should help us to delineate the composition of KA receptors responsible for postsynaptic and presynaptic mechanisms.

2.4. Kainate Receptors and Neurotransmission

The observations that KA and AMPA receptors were co-expressed in the same cell (Lerma et al., 1993;Frerking et al., 1998) and have overlapping pharmacological properties (Patneau & Mayer, 1990a;Patneau *et al.*, 1993;Huettner, 1990;Herb *et al.*, 1992) posed two major hurdles for the functional study of native KA receptors. Functional differentiation between these two iGluR subfamilies proved difficult until the development of the noncompetitive AMPA receptor antagonists, the 2,3-benzodiazapine compounds, amongst which GYKI 53655 seemed to be the most selective (Wilding & Huettner, 1995;Paternain *et*

al., 1995). To date, the most commonly used test for the presence of functional KA receptors is a combination of resistance to GYKI 53655 and sensitivity to the AMPA/KA receptor antagonist CNQX (cyano-7-nitroquinoxaline-2,3-dione) (Frerking & Nicoll, 2000).

Using these pharmacological tools, neuroscientists have described two major contributions of KA receptor to synaptic transmission. First, KA receptors have been shown to contribute to the excitatory postsynaptic current (EPSC) (Castillo *et al.*, 1997;Vignes & Collingridge, 1997) and more recently, to metabotropic functions (Ruiz et al., 2005;Rozas et al., 2003) following L-glutamate release. Secondly, it has been suggested that KA receptors modulate neurotransmission through a presynaptic mechanism that is still fairly controversial (Cossart et al., 1998;Rodriguez-Moreno et al., 1997;Clarke et al., 1997).

Postsynaptic Kainate Receptors. Although immunostaining experiments had placed KA receptors postsynaptically (Petralia et al., 1994), functional evidence supporting this localization remained elusive for quite some time (Bettler & Mulle, 1995;Mayer, 1997;Lerma, 1997). Two research groups simultaneously demonstrated that KA receptors contribute to the EPSC at mossy fibers synapses into CA3 pyramidal cells in the hippocampus (Castillo *et al.*, 1997;Vignes & Collingridge, 1997). Since then, KA receptor-mediated EPSCs have been observed at a number of other synapses including GABAergic interneurons of the CA1 region, cerebellar Golgi cells, both pyramidal and interneurons of the neocortex, the superficial dorsal horn of the spinal cord, "Off" bipolar cells of the retina and in the basolateral amygdala (reviewed in Pinheiro & Mulle, 2006). In contrast with AMPA, most KA receptor-mediated EPSCs ($EPSC_{KA}$) exhibit a much smaller amplitude and slower decay kinetics ((Kidd & Isaac, 1999), but see (DeVries & Schwartz, 1999;Epsztein *et al.*, 2005)). The small amplitude of $EPSCs_{KA,}$ makes these currents difficult to measure using electrophysiological techniques and may thus be easily missed. It is therefore likely that with time the list of synapses containing KA receptor-mediated EPSCs will become considerably longer (Frerking & Nicoll, 2000).

An attractive possibility proposed for the functional role of $EPSCs_{KA}$ (i.e., small peak amplitude and slow decay kinetics) is that they can lead to temporal summation at frequencies as low as 1 Hz and thus provide information about the global firing rate (Frerking & Nicoll, 2000). This role is distinct, but complementary to AMPA receptor-mediated EPSCs that display faster kinetics and are therefore better suited to transmit information encoded by afferent synchronization (Konig *et al.*, 1996;Frerking & Nicoll, 2000). Interestingly, the number of each type of synapse is developmentally regulated through an activity-dependent mechanism (Kidd & Isaac, 1999).

The kinetic profile of synaptic KA receptor responses (Kidd & Isaac, 2001) was quite surprising given that activation of recombinant KA receptors by L-glutamate evokes larger response amplitude and exhibit faster channel kinetics (Schiffer *et al.*, 1997;Swanson & Heinemann, 1998;Bowie & Lange, 2002). Several hypotheses have been set forth to explain these paradoxical observations. In principle, this phenomenon could be explained by the presence of extrasynaptic KA receptors since it would predict a significantly slower glutamate-evoked current because of diffusion.

Although this proposal is consistent with the finding that repetitive stimulation of the mossy fiber synapse is necessary to facilitate $EPSC_{KA}$ (Castillo *et al.*, 1997;Vignes & Collingridge, 1997), there are several lines of evidence in the literature that do not support this hypothesis. For example, examination of L-glutamate's time course in the synaptic cleft proposed that it is highly implausible that the neurotransmitter concentrations remain high for such an extended period (Clements, 1996). Consistent with this, electron microscopic data localizing GluR6/7 and KA-2 also indicated that this explanation is unlikely, since at least some KA receptors were found at postsynaptic densities and therefore in direct apposition to presynaptic release sites (Petralia et al., 1994). This finding is also in agreement with a study demonstrating that various manipulations to increase L-glutamate concentration in the synaptic cleft, including inhibition of L-glutamate re-uptake, did not increase $EPSCs_{KA}$ (Kidd & Isaac, 2001;Castillo *et al.*, 1997;Vignes & Collingridge, 1997).

A second explanation for this phenomenon is that intermediate desensitized states contribute to membrane conductance (Bowie & Lange, 2002), reconciling anatomical, synaptic and biophysical studies mentioned above. According to this explanation, activating postsynaptic GluR6 receptors at 1 Hz would decrease their response by more than 80% because of their slow recovery from desensitization (Bowie & Lange, 2002), a prediction that is consistent with the smaller events recorded *in vitro* (Cossart *et al.*, 1998;Frerking *et al.*, 1998;Kidd & Isaac, 1999). In addition, given that most KA receptors reside in the intermediate desensitized state, decay would be slower, a phenomenon independently observed in these synaptic studies.

Finally, the association of KA receptors with novel accessory proteins may offer a likely explanation for this observation. For example, recent work has shown that the decay kinetics of $EPSCs_{KA}$ were significantly slower in granule cells from *stargazer* that were co-transfected with GluR6 (K696R mutant) and neuropilin tolloid-like 2 protein (NETO-2), compared to the GluR6 mutant alone (Zhang et al., 2009). Moreover, intracellular signals may also provide a potential explaination the slow time course of $EPSCs_{KA}$ and/or the expression of heteromeric KA receptors with distinct biophysical properties (Swanson et al., 2002).

"Pure" Kainate Receptor Synapses. Although postsynaptic KA receptors are thought to be co-expressed with AMPA receptors (Lerma et al., 1993;Frerking et al., 1998) at most central synapses, the existence of synapses exclusively expressing postsynaptic KA receptors (i.e., 'KA synapse') has been identified in two studies. In the first case, DeVries & Schwartz demonstrated that in the retina, depolarization of an individual cone evoked a fast EPSC in off-bipolar cells that was insensitive to GYKI 53566 and APV, but blocked by CNQX, an observation clearly indicating its dependence upon KA receptors (DeVries & Schwartz, 1999). Curiously, the fast kinetics of the $EPSC_{KA}$ at this synapse are unlike all other slower $EPSCs_{KA}$ recorded elsewhere, but similar to the kinetics of recombinant

KA receptors displaying fast activation, as well as rapid and pronounced desensitization (Schiffer *et al.*, 1997;Swanson & Heinemann, 1998).

The developing thalamocortical synapse is a second example of a KA synapse. It was found to elicit either a slow $EPSCs_{KA}$ or a typical faster AMPAR-mediated EPSC (i.e., 'AMPA synapse') (Kidd & Isaac, 1999). Although cortical cells can express both KA and AMPA receptors, these two subfamilies never seem to co-localize at the same synapses, which could be defined as either KA- or AMPA-type, depending on their kinetic properties (Kidd & Isaac, 1999).

Metabotropic Action of Kainate Receptors & Presynaptic Kainate Receptors. At the molecular level, electrophysiological recordings and electron microscopy showed that KA receptors are also expressed presynaptically. Consistent with this, KA receptors were shown to modulate the release of neurotransmitters (GABA and L-glutamate) through a presynaptic mechanism (Mulle et al., 2000;Contractor et al., 2001).

Interestingly, although the action of KA receptors has been classically thought to occur via its ionotropic properties, recent evidence has shown that some of its pre- and postsynaptic activity must be attributed to a metabotropic mechanism (Rodriguez-Moreno et al., 2000). Namely, endogenous release of L-glutamate to activate KA receptors reversibly inhibited the slow $Ca2^{+}$-activated K^{+} current (sAHP) at mossy fiber synapses through a metabotropic effect (Ruiz et al., 2005). Moreover, the activation of GluR5-containing KA receptors by KA in cultured DRG neurons have been shown to elicit, in the absence of apparent permeation, an increase in intracellular Ca^{2+} while blocking the activation of voltage-dependent Ca^{2+} channels (Rozas et al., 2003). These effects were inhibited by conventional antagonists of phospholipase C (PLC) and protein kinase C (PKC) (Rodriguez-Moreno & Sihra, 2007). Specifically, subunit knock-out studies report that this G-protein-coupled mechanism is abolished in the KA-2 mutant while KA receptor-mediated EPSC are conserved (Ruiz et al., 2005). This provides one example of the metabotropic actions of postsynaptic kainate receptors.

Taken together, these studies have demonstrated the critical role of KA receptors in regulating various synaptic processes such as maturation, plasticity as well as neurotransmitter release (Pinheiro & Mulle, 2006;Bortolotto *et al.*, 1999;Hartmann *et al.*, 2004). The functional evidence indicating that KA receptors primarily play a modulatory role in neurotransmission rather than being the main postsynaptic target for synaptically released glutamate, as is the case for AMPA and NMDA receptors, makes them an appealing target for drug action. As described below, this finding has important implications for a considerable number of neuropathological conditions (Dingledine et al., 1999;Jane et al., 2009).

2.5. Kainate Receptors and Disease States

Compared to other iGluR subfamilies, the role of KA receptors in pathology is only beginning to emerge (Bowie, 2008a). Human molecular genetic screening, KA receptor subunit knock-out mice and animal disease models combined with the use of "selective" antagonists have been used to show the potential involvement of these receptors in a number of developmental, neurodegenerative as well as mental disorders (Figure 2) (Bowie, 2008a).

KA receptor subunit knock-out mice engineered by the Heinemann group (Mulle et al., 1998) revealed distinct physiological roles in synaptic transmission and plasticity (Bureau et al., 1999;Contractor et al., 2001;Contractor et al., 2000;Huettner, 2001), but here we have focused almost exclusively on pathologies involving GluR6-containing KA receptors, since the work presented in this thesis has primarily dealt with homomeric GluR6 receptors. As an example of the different roles for KA receptor subunits, knock-out animals have shown that the GluR6 subunit plays an important role in fear memory, while GluR5-expressing KA receptors have been implicated in plasticity mechanisms of the amygdala (Ko et al., 2005). Here, I have highlight some key findings linking KA receptors to specific neurological disease states.

Developmental	Autism	—	$GluR6_{M867I}$	—	—	—
	Schizophrenia	GluR5	GluR6	GluR7	KA1	—
Mood Disorder	Bipolar	GluR5	GluR6	GluR7	KA1	—
	Depression	—	—	GluR7	KA1	—
Anxiety	Obsessive / Compulsive	—	GluR6	—	—	—
	Fear Memory	GluR5	GluR6	—	—	—
Neuropathy / Neurodegenerative	Neuropathic Pain	GluR5	GluR6	—	—	—
	Epilepsy	GluR5	GluR6	—	KA1	KA2
	Huntington's		GluR6	—	—	—

Figure 2. Kainate receptors have been implicated in a number of disease states. Modified from Bowie (2008) CNS & Neurological Disorders-Drug Targets 7: 129-143.

The implication of GluR6 in epilepsy is perhaps one of the most well-established roles of a KA receptor subunit in a neurological disorder (Bowie, 2008a). The first suggestion that KA receptors may be involved in the pathology of epilepsy followed the observation that systemic or intracerebral injections of KA in rodents elicited seizures and epileptiform discharges in the hippocampus and amygdala (Nadler, 1981). Since then, the kainic acid injection has become the most commonly used animal mode of temporal lobe epilepsy (Pinheiro & Mulle, 2006). The later observation that submicromolar concentrations of KA elicited epileptogenic effects in the CA3 region of a slice preparation (Berger et al., 1986), a region highly enriched in high affinity KA-binding sites, provided stronger evidence that this effect was mediated via KA (and not AMPA) receptors (Monaghan & Cotman, 1982;Tremblay *et al.*, 1985).

Subsequently, mutant GluR6 -/- mice were shown to have an increased threshold for epileptic activity induced by KA (Mulle et al., 1998). Moreover, high affinity binding sites in the hippocampus and the activation of currents by low concentration of KA were both abolished in the knock-out animals (Mulle et

al., 1998). At the circuit level, both KA-induced gamma oscillations as well as epileptiform bursts in slice recordings were prevented in the GluR6, but not the GluR5 knockout mice, thereby further confirming the critical role of GluR6-containing KA receptors in this pathology (Fisahn et al., 2004).

Additionally, morphological changes such as glutamatergic fiber sprouting and the establishment of novel synapses in the dentate gyrus of epileptic animals, as in the human condition, are thought to contribute to the epileptiform discharge (Ben Ari & Represa, 1990). Moreover, recent evidence from the Ben-Ari group has demonstrated that this enhanced glutamatergic excitatory drive is largely mediated via *de novo* expression of KA receptors at recurrent mossy fiber synapses (Epsztein et al., 2005).

Besides its well-established role in epilepsy, GluR6-containing KA receptors have also been linked to a number of other neurological disorders. For example, recent work tracking receptor changes have found a decrease in GluR5/R6 expression in the orbitofrontal cortex of schizophrenic patients (Garey et al., 2006). An increasing number of human molecular genetic studies have also suggested that alterations in the GluR6 receptor gene (GRIK2) are associated with various neurological conditions. Examples of these include early-onset Huntington's disease ((Rubinsztein et al., 1997), but see (Metzger et al., 2006)), autism (Jamain et al., 2002), schizophrenia (Bah et al., 2004;Shibata et al., 2002), mania (Shaltiel et al., 2008), mental retardation (Motazacker et al., 2007) and obsessive-compulsive disorder (Delorme et al., 2004). As discussed in more detail elsewhere, it is most likely, however, that these neurological disease conditions do not represent an exclusive dysfunction of the glutamatergic synapse, but rather a combination of other signaling anomalies (Bowie, 2008a).

In order to gain a better understanding of the implication of KA receptors in CNS disorders, the basic structural and gating properties of the KA receptor need to be elucidated. In light of this, the next chapter discusses specifically the structure of the KA receptor subfamily of iGluRs with a focus on their subunit topology and receptor stoichiometry and assembly.

3. THE ARCHITECTURE OF KAINATE RECEPTORS

Since the identification of the first primary amino acid sequence of an iGluR at the end of the 1980s (Hollmann et al., 1989), a colossal number of experiments have been carried out in order to examine the different domains and amino acid residues that are important to receptor function. As discussed in the next chapter, more recent structural analysis has also provided important clues in relating binding to gating at KA receptors. Before reviewing the structure and stoichiometry of KA receptors, however, it is important to consider how ideas about iGluR receptor architecture evolved.

3.1. Historical Considerations

Following cloning of the first glutamate receptor in 1989 (Gregor et al., 1989;Hollmann et al., 1989), many other subtypes were cloned and confirmed the existence of three main iGluR subfamilies (Bettler et al., 1990;Moriyoshi et al., 1991;Keinanen et al., 1990;Boulter et al., 1990). Both posttranscriptional and posttranslational modifications such as RNA splicing (Sommer et al., 1990), RNA editing (Sommer et al., 1991;Lomeli et al., 1994;Seeburg, 1996;Swanson et al., 1997;Swanson et al., 1996;Teague, 2003) and phosphorylation (Wang et al., 1991) were found to confer even more functional diversity to these receptors. Notably, editing of a glutamine residue (Q) in the genomic sequence to encode for an arginine (R) in the mature polypeptide (i.e. Q/R site) was shown to affect channel permeability (Sommer et al., 1991;Swanson et al., 1996), polyamine block (Bowie & Mayer, 1995), single-channel conductance (Howe, 1996) and calcium permeability of KA receptor subunits (Egebjerg & Heinemann, 1993;Kohler *et al.*, 1993).

Subsequently, structural homology between the hydropathy plot of the first glutamate receptors and nAChRs, known to have a four transmembrane domains and an extracellular N- and C-termini, led researchers to propose that glutamate receptors also displayed a comparable transmembrane topology (Gasic & Hollmann, 1992). A first key breakthrough following sequencing of iGluRs was

the recognition that portions of its amino acid sequence were related to bacterial amino acid-binding proteins. In fact, Nakanishi et al. first recognized the sequence similarities between iGluR sequences and the glutamine binding protein (QBP) (Nakanishi et al., 1990). Subsequently, the N-terminal part of the amino acid sequence of both iGluRs and mGluRs were found to share strong similarities with the folding of the leucine-isoleucine-valine binding protein (LIVBP) (O'Hara et al., 1993).

Secondly, two sequence segments showed sequence homology with the lysine-arginine-ornithine binding protein (LAOBP) (Oh et al., 1993). Curiously, these two segments were found in regions of the proteins which were at the time predicted to be on opposite sides of the membrane (Kohler et al., 1993). Like the LAOBP, which was known to form a "clamshell" or "venus-flytrap" with two lobes that together form an amino acid-binding pocket, L-glutamate was predicted to be partially buried in a cleft between the globular S1S2 domain (Stern-Bach *et al.*, 1994a). This localization was in sharp contrast with the model proposed for nAChRs, where ligand binding is thought to occur at the interface between adjacent subunits (Kubalek et al., 1987;Unwin, 1993;Brejc et al., 2001).

At the same time, increasing evidence questioning the proposed four-transmembrane topology for iGluRs emerged and served to further differentiate them from the nAChRs. For instance, antibodies against the C-terminal peptide of rat brain AMPA/KA receptor were also found to stain the cytoplasmic side of the plasma membrane (Petralia & Wenthold, 1992), an observation inconsistent with the nAChR model placing the C-terminal on the extracellular side of the membrane. In agreement with this finding, the C-terminal of NMDA receptors was also shown to be phosphorylated (Tingley et al., 1993). Together with the identification of N-glycosylation sites previously thought to be located on the intracellular side of KA receptors (Wo & Oswald, 1994), these studies provided strong evidence for a revised three-transmembrane domain topology with a re-entrant loop.

The transmembrane topology of iGluR subunits was further confirmed by two main technical approaches. The first involved genetic engineering of

glycosylation and proteolytic enzyme recognition sites and subsequent expression in eukaryotic cells and analysis of shifts in molecular weight following incubation with glycosidases and proteases. Combined with hydropathy plots which determined the boundaries of the extracellular domains, inferences on the intracellular and transmembrane segments were made (Hollmann *et al.*, 1994;Bennett & Dingledine, 1995;Wo & Oswald, 1995a;Wo & Oswald, 1994)

The second approach took advantage of the revised topology and the similarities between the agonist-binding domain of iGluRs and periplasmic-binding proteins to perform homology modeling and subsequently site-directed mutagenesis and domain swapping to confirm the correct topology (Stern-Bach *et al.*, 1994a;Quiocho & Ledvina, 1996). Taken together, these studies defined the correct three-transmembrane domain topology of iGluRs with an extracellular N-terminal, an intracellular C-terminal and the putative second transmembrane domain forming a re-entrant pore (Figure 3).

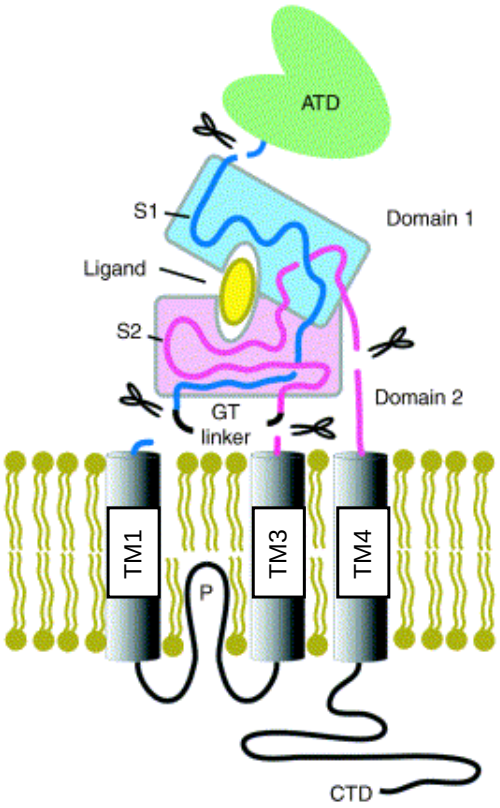


Figure 3. iGluR building blocks. Schematic representation of a typical iGluR subunit. Notice the extracellular N-terminal and the intracellular C-terminal. Together the S1 and S2 domain make the clamshell-like agonist binding domain. The transmembrane domains (TM) 1, 3 and 4 as well as the P-loop that forms the channel pore are also illustrated.

A second important breakthrough following sequencing of iGluR genes in 1989 was the construction of clones expressing the soluble agonist-binding domain of GluR4 (Kuusinen et al., 1995). This provided an adequate substrate for subsequent crystallographic studies (see sections 3.2.4 and 4.2.2). As detailed below, these discoveries were pivotal in shaping our current understanding of the details of KA receptor architecture and stoichiometry.

3.2. Topology of Individual Kainate Receptor Subunits: Modular Building Blocks

Though the complete three-dimensional structure of an intact KA receptor has yet to be solved, the proposed modular nature of individual iGluR subunits has permitted a reductionist approach to investigate their architectural properties. Each subunit that makes up a functional iGluR measures approximately 11 x 14 x 17 nm for GluR2 (McFeeters & Oswald, 2004) and has a molecular mass of ~119 kDa (for GluR6) (Strutz-Seebohm et al., 2005). As illustrated in Figure 3, the typical iGluR subunit is composed of four modular domains:

(1) Extracellular amino-terminal domain
(2) Intracellular C-terminal domain
(3) Three transmembrane domains (TM1, TM3 and TM4) and a reentrant loop which forms the pore of the channel (M2 or P region)
(4) Agonist-binding domain (S1S2) that is built from two separate amino acid sequences. The S1 domain is drawn from the sequence adjacent to the TM1 domain and the S2 domain is drawn from the TM3-TM4 loop (Dingledine et al., 1999)

3.2.1. Amino-Terminal Domain

The amino-terminal domain is a ~400 amino acid polypeptide chain that is an important determinant of subunit assembly at both KA (Ren et al., 2003b) and AMPA receptors (Leuschner & Hoch, 2003). Consistent with this, the amino-terminal domain is absent from the obscure KA-binding protein found in frog, chick and goldfish, which do not form functional channels. This domain shows

strong similarities with the folding of the LIVBP and the agonist-binding domain of mGluRs (Sack et al., 1989;Nakanishi et al., 1990;O'Hara et al., 1993). An important difference, however, is that unlike mGluRs or LIVBP, which have two globular domains connected via 3 β strands, iGluRs only have a pair of β-strands (Sack et al., 1989;Kunishima et al., 2000). Although this may suggest that the amino-terminal domain of iGluRs may also encode a ligand binding domain, only binding sites for allosteric modulators such as Zn^{2+} and ifenprodil at NMDA receptors have been identified so far (Choi & Lipton, 1999;Masuko *et al.*, 1999;Paoletti *et al.*, 1997;Rachline *et al.*, 2005;Perin-Dureau *et al.*, 2002).

On the other hand, no endogenous ligand has yet to be found to bind to the N-terminal of KA (or AMPA) receptors. As described in more detail in the next chapter, however, allosteric modulators such as lectins have been shown to alter KA receptor responses (Kehoe, 1978;Huettner, 1990). In fact, carbohydrate side chains have been involved in many diverse receptor functions including: subunit assembly, formation of agonist binding sites, receptor targeting to the cell surface, and recognition by extracellular modulators (Lis & Sharon, 1993). Interestingly, all iGluR subfamilies possess N-glycosylation sites that can influence channel activity, but which are not essential for receptor function (Everts et al., 1997;Everts et al., 1999).

Of particular relevance to our work, the GluR6 KA receptor subunit has nine potential N-glycosylation sites that conform to the universal consensus sequence, N-X-S/T, with $X \neq P$, which are located both on the N-terminal domain as well as in and around the agonist-binding domain (Figure 4) (Everts et al., 1999). As discussed later, these sites are thought to bind plant lectins, such as concanavalin-A (Con-A) to alter channel responses (Everts *et al.*, 1997;Bowie *et al.*, 2003a).

Lastly, even though more work is required to unravel the physiological role of the N-terminal domain in KA receptors, at least for NMDA receptors, this modular domain is involved in regulating desensitization (Villarroel et al., 1998;Krupp et al., 1998). Recent findings also reveal that this region, which acts as a clamshell-like domain that bind allosteric modulators, also governs the subunit-specfici gating of NMDARs through differences in the spontaneous

equilibrium between open- and closed-cleft conformations of the N-terminal domain of the NR2 subunit (Gielen et al., 2009). Interestingly, the crystal structure of the amino-acid terminal of the GluR1, GluR2 and GluR6 subunits have been recently resolved and shown to form dimers in solution, a finding which is significantly different that the conformation adopted by mGluRs (Kumar et al., 2009;Jin et al., 2009). This domain structure may begin to shed some light on the precise role in subunit assembly and their potential association with allosteric ligands or modulators.

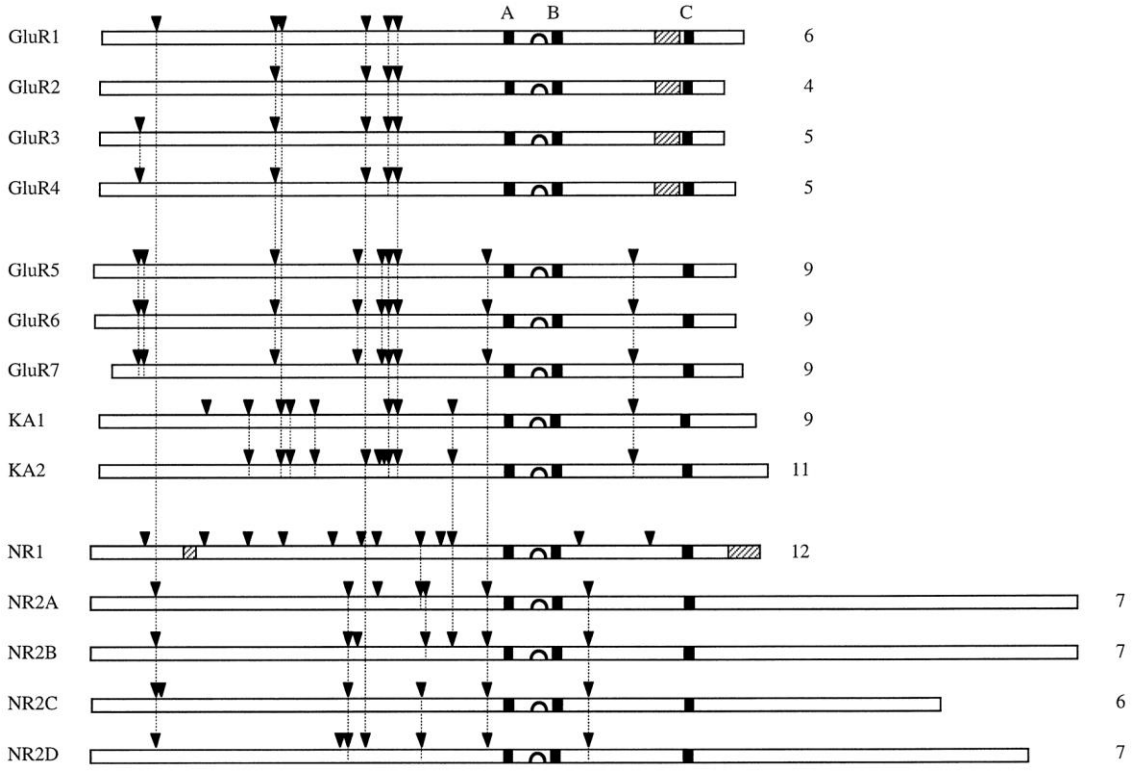


Figure 4. Extracellular N-glycosylation sites of all iGluR subunits (arrowheads). Each bar structure represents the subunit listed on the left. The black squares represent the location of transmembrane domains 1, 2, and 3 while the hairpin loop structure (positioned between domains 1 and 2) shows the location of the pore-forming domain. Stippled vertical lines connect conserved consensus sites at homologous positions in different receptors. At the right of the bar structures, the total number of sites is indicated. Reproduced from Molecular Pharmacology, Volume 52 (5), 861-873.

3.2.2. Carboxyl-Terminal Domain

The carboxyl-terminal sequence of iGluRs exhibits the largest sequence diversity in the entire protein, ranging from 20 to 500 amino acids in length (Mayer & Armstrong, 2004). In contrast to the agonist-binding domain and N-terminal domain, the structure of the C-terminal domain has yet to be solved. In fact, the short C-terminal domain of KA receptors does not form a stable structure when expressed as a soluble protein (Oswald, 2004). Nonetheless, several studies have shown that this domain is critical in the regulation of KA receptor function and surface localization (Oswald, 2004;Sheng & Kim, 2002).

For example, the C-terminal domain has been shown to interact both *in vitro* and *in vivo* with cytoskeletal protein containing a PDZ domain in the postsynaptic density to control trafficking, targeting, clustering and localization of iGluRs (Bolton et al., 2000;Yan et al., 2004). PDZ-domain containing proteins that interact with KA receptors include: PSD-95, SAP102 (102-kDa synapse-associated protein), SAP97 (Garcia et al., 1998), CASK (calmodulin-associated serine/threonine kinase) (Coussen et al., 2002), GRIP (glutamate receptor-interacting protein), PICK1 (protein interacting with C kinase 1) and syntenin (Hirbec et al., 2003). Interestingly, PDZ domain interaction sites are required for trafficking of NMDA receptors (Standley et al., 2000), but not KA receptors (Coussen et al., 2002;Ren et al., 2003b;Ren et al., 2003c). Additionally, PSD-95 was found to accelerate recovery from desensitization by binding to the cytoplasmic tail region of KA subunits with an N-terminal PDZ domain (Bowie *et al.*, 2003a). This interaction is thought to be important in maintaining the shape, the position and the aggregation of ion-channel clusters (Garcia *et al.*, 1998;Bowie *et al.*, 2003a).

Additionally, both *in vitro* and *in vivo* work has demonstrated that GluR6a-containing KA receptors interact with cadherin–catenin adhesion complexes (Coussen et al., 2002). At least in heterologous expression systems, this interaction was shown to result in KA receptor recruitment in regions where cadherins were highly concentrated. Given that adhesion proteins such as cadherins are found in synaptic junctions, specifically in perisynaptic domains,

interaction of these proteins with KA receptors might be important in localizing these receptors at synapses (Jaskolski et al., 2005).

More recently, the complement C1r/C1s, Uegf, Bmp1(CUB)-domain-containing proteins, NETO1 and NETO2 have been identified as the first members of the kainate receptor regulatory proteins (KARPs) (Zhang et al., 2009). In addition to modulating KA receptor properties in both recombinant and native systems, the NETO2 protein was shown to increase the frequency of KA receptor-mediated EPSCs (Zhang et al., 2009). Interestingly, although this transmembrane protein did not affect KA receptor trafficking, surface expression of the NETO2 protein was decreased in the GluR6 knock-out mice suggesting that KA receptors may modulate the surface expression of NETO2 (Zhang et al., 2009).

Several intracellular enzymes have also been shown to modulate KA receptors. Namely, protein kinase A (PKA) potentiates GluR6 KA receptor responses (Wang et al., 1991;Wang et al., 1993) by increasing open channel probability while the phosphatase calcineurin decreases this probability (Traynelis & Wahl, 1997). Though the molecular basis for this effect remains controversial since it placed the amino acid residue on the extracellular side of the membrane (Traynelis & Wahl, 1997;Wang *et al.*, 1993), recent evidence showed that the double mutation of S825A/S837A in the C-terminal abolished the effect of PKA on KA receptor responses (Kornreich et al., 2007). Further, protein kinase C-dependent phosphorylation of the C-terminal domain of GluR5 and GluR6 have been reported to stabilize binding of GRIP (Hirbec et al., 2003), suggesting that interaction with GRIP might anchor KA receptors at synapses. As exemplified here, interactions of KA receptors with cytosolic proteins adds yet an additional dimension to synaptic signaling.

3.2.3. Pore Region and Transmembrane Domains

The S1 and S2 amino acid sequences that make up the agonist binding domain are interrupted by the inclusion of two transmembrane domains, which in combination with the p-loop helix and pore loop, constitute the narrowest portion

of the pore (Chen *et al.*, 1999a;Panchenko *et al.*, 2001;Kuner *et al.*, 1996;Kuner *et al.*, 2001). Soon after the three transmembrane domain topology of iGluRs was identified, scientists recognized that their p-loop exhibited sequence homology with the p-loop of K^+ channels (Wo & Oswald, 1995b;Wood *et al.*, 1995). Similar to potassium channels, the p-loop sequence of iGluRs partly lines the channel and has been shown to regulate many electrophysiological properties such as calcium permeability and polyamine block (Panchenko et al., 2001;Verdoorn et al., 1991). Despite this homology, the ion selectivity of eukarytotic iGluRs and K^+ channels is very different. Permeability experiments have shown that the more promiscuous iGluR pore allows passage of both Na^+ and K^+ and sometimes Ca^{2+}, suggesting that it may either be wider or more flexible (Mayer & Armstrong, 2004) than the K^+ channel pore, which allows passage of dehydrated K^+ ions exclusively (Doyle et al., 1998;Zhou et al., 2001). Interestingly, in contrast with K^+ channels, the iGluR pore is thought to permit the passage of ions in a hydrated state (Zhou et al., 2001).

Yet, compared to potassium channels, the pore orientation in the membrane of iGluRs is proposed to be inverted, with a hairpin P-loop dipping into the structure from the cytoplasmic side of the membrane (Panchenko et al., 2001) and not from the extracellular side, as observed in K^+ channels (Doyle et al., 1998). The model that emerged from these studies suggested that two transmembrane α-helices (TM1 and TM3) support a pore-loop motif which is partly helical and forms the closest point of contact with ions near the cytoplasmic entrance to the channel (Wollmuth & Sobolevsky, 2004). As observed in K^+ channels, the narrow constriction or selectivity filter in the glutamate receptor channel is positioned near the tip of the loop (Panchenko et al., 2001). Furthermore, scanning mutagenesis was used to reveal the location of the negatively charged amino acid binding site for polyamines at the cytoplasmic entrance of the pore (Panchenko et al., 2001).

Although the channel pore is primarily lined by residues from the TM3 segment, increasing evidence in the literature suggests that the M4 domain might also play an important role in glutamate receptor gating. For instance, the Lurcher

mutation in the M4 domain of the various iGluR subunits was shown to convert inactive receptors to constitutively active channels, thereby acting as a molecular switch governing its gating properties (Zuo et al., 1997;Kohda et al., 2000). Interestingly, the M4 domain is the most highly conserved transmembrane across glutamate receptors and exhibits sequence homology with the M3 region and the helix downstream of the p-loop in K^+ channels (Kuner et al., 2003). Moreover, mutations in the fourth transmembrane domain of NMDA receptors were also shown to influence desensitization and channel open time properties, supporting the proposal that this transmembrane domain is also important in governing receptor gating kinetics (Ren et al., 2003a). Further determining the precise details of the channel pore awaits additional structural and functional analysis.

3.2.4. Agonist-Binding Domain

As mentioned previously, an important breakthrough following sequencing of iGluRs in 1989 was the recognition that its S1S2 domain shared sequence homology with the bacterial periplasmic protein, LAOBP from *Salmonella typhimurium*, which was known to form a "clamshell" or "venus-flytrap" structure (Oh *et al.*, 1993;Stern-Bach *et al.*, 1994a). In this design, the agonist was predicted to be partially buried in a cleft between the globular S1S2 domains. Shortly after, the GluR2 S1S2 domain was excitingly found to be expressed as a monomeric, soluble protein (Kuusinen et al., 1995), which proved appropriate and relevant for additional structural investigations. In fact, the S1S2 domain was later overexpressed in *Escherichia coli*, denatured and refolded into a fully functional, monomeric water-soluble protein (Chen & Gouaux, 1997). This permitted resolution of the crystal structure of the S1S2 construct in the absence of and in complex with various ligands (Mayer & Armstrong, 2004;Armstrong *et al.*, 1998;Armstrong & Gouaux, 2000a;Jin & Gouaux, 2003;Hogner *et al.*, 2002;Madden, 2002), as well as the characterization of backbone nuclear magnetic resonance (NMR) dynamics (McFeeters & Oswald, 2002).

The first crystallized S1S2 construct was the KA-bound GluR2 AMPA receptor (Armstrong et al., 1998), which confirmed the suspected bi-lobed

structure (Stern-Bach *et al.*, 1994a). Surprisingly, the two lobes of the agonist-binding domain were not made exclusively from the amino-terminal segment before the TM1 domain and the second one from the sequence between TM3 and TM4 as expected from homology modeling (Sutcliffe et al., 1996), but the two lobes were found to intercross with each other so that both segments contribute to both lobes.

In the absence of an agonist, the clamshell is thought to be in an open conformation and the gate of the intramembrane ion channel pore closed. Binding of the agonist closes the clamshell structure and the energy of binding permits opening of the pore to allow ion flow. Accordingly, this concept will be discussed in more detail in the section on the gating properties of KA receptors (see section 4.2.2), along with the assumptions underlying interpretations of iGluR crystal structures (see section 4.2.3). Armed with a basic knowledge of individual iGluR subunits begs a number of obvious questions such as: "How many subunits come together to make a mature receptor?" and "What are the molecular mechanisms governing the assembly process?"

3.3. Kainate Receptor Stoichiometry

It is now well-established that iGluRs are composed of four subunits (Dingledine et al., 1999). In fact, biochemical, crystallographic and functional studies suggest that AMPA receptors assemble as a dimer of dimers (Sobolevsky *et al.*, 2004;Bowie & Lange, 2002;Tichelaar *et al.*, 2004;Horning & Mayer, 2004;Sobolevsky *et al.*, 2004;Armstrong & Gouaux, 2000a;Sun *et al.*, 2002;Armstrong *et al.*, 2006a;Robert & Howe, 2003). In support of this, two independent detailed studies of AMPA receptor desensitization demonstrated that they operate via a cooperative dimer model (Bowie & Lange, 2002;Robert & Howe, 2003). Interestingly, the assembly of AMPA iGluRs is quite unique in that the assumed 4-fold symmetry of the ion channel pore does not correspond to the putative 2-fold symmetry of its ligand-binding core dimers (Horning & Mayer, 2004).

It is reasonable to assume that KA receptors are tetramers, since immunoprecipitation protocols with a carboxyl-terminal antibody demonstrated that GluR6 KA receptors formed tetramers (Mah et al., 2005). Although this idea has yet to be directly demonstrated, KA receptors have generally been assumed to adopt a similar dimer of dimer model (Plested et al., 2008;Priel et al., 2006). Surprisingly, KA receptors were also shown to form homomers, dimers and trimers (Mah et al., 2005). Consistent with this, detailed functional analysis of KA receptor desensitization was most consistent with a tetrameric model and not a dimer of dimer arrangement (Bowie & Lange, 2002). This critical structural difference raises an important issue as to whether the basic gating mechanisms of these two iGluRs are comparable, as it has been previously thought (Weston *et al.*, 2006a;Lerma *et al.*, 1997).

As with most ion-channels, iGluRs can assemble as homo- and hetero-oligomers (Dingledine et al., 1999). Although the three main subfamilies can be expressed in the same cell (Bettler & Mulle, 1995), only subunits within a subfamily can co-assemble (with some exceptions at KA receptors as described previously). Although the precise mechanism of assembly are beginning to emerge, both the N- and C-terminal have been implicated in the specificity of subunit assembly (Leuschner & Hoch, 2003;Ren *et al.*, 2003b;Yan *et al.*, 2004;Ayalon *et al.*, 2005). Moreover, the large divergence in their C-terminal sequence opens the possibility that trafficking of the different receptor subtypes might be differentially regulated by proteins that interact with this region (Jaskolski et al., 2005).

Compared to NMDA and AMPA receptors, much less is known about the assembly process of KA receptors. Although mutations of residues in the N-terminal domain decreased surface expression of homomeric GluR6a receptors (Fleck et al., 2003), future work is required to determine whether these mutations affect a trafficking signal or protein folding. One important issue to examine is which subunits co-assemble. It was initially proposed that AMPA receptor heterodimerization was solely governed by the extracellular N-terminal domain (Leuschner & Hoch, 2003). As discussed below, however, more recent evidence

has suggested that assembly of KA and AMPA receptors also involves the C-terminal.

The sorting mechanisms in the endoplasmic reticulum (ER) and membrane delivery of KA receptors was shown to depend on the subunit composition and splice variants (Jaskolski et al., 2004;Ren et al., 2003b). For example, the KA-1, KA-2 and GluR5c subunits do not traffic to the membrane when expressed alone, but are confined to the ER (Jaskolski et al., 2004;Ren et al., 2003b;Gallyas, Jr. et al., 2003;Hayes et al., 2003). The presence of a retention motif (RXR sequence) in the C-terminal has been identified on both GluR5c and KA-2 to account for this behavior (Ma & Jan, 2002). Additionally, a dileucine motif in KA-2 has been suggested to act as a intracellular signal for clathrin-dependent endocytosis (Ren et al., 2003b).

In contrast, the GluR6a splice variant is highly expressed at the plasma membrane when assembled as homomeric or heteromeric channels. Interestingly, co-expression of GluR6a was shown to promote trafficking of subunit splice variants that were normally retained in the ER through a forward trafficking domain in its C-terminal, which occludes the ER retention signal (Yan et al., 2004;Jaskolski et al., 2004). Receptor assembly in the endoplasmic reticulum (ER) is thought to involve the formation of intermediates of subtype-specific subunit dimers that are subsequently assembled into tetramers (Ayalon & Stern-Bach, 2001).

Taken together, these studies illustrate how KA receptor subunit composition and splice variants are thought to regulate membrane delivery of these receptors and explain their differential cell-surface expression (Jaskolski et al., 2005). Furthermore, it is the multi-subunit nature of KA receptors that provides the necessary basis for profound structural rearrangement. As discussed in more detail in the next section, these ion-channels possess multiple agonist binding sites that are located outside the membrane pore, intuitively suggesting the presence of a gating mechanism.

4. THE GATING PROPERTIES OF KAINATE RECEPTORS

In order to better appreciate how ligand binding leads to KA receptor activation and desensitization, it is first necessary to introduce the concept of gating. To do this, a section on the historical emergence of ideas about gating mechanisms is included here, followed by a section discussing the two main models that have been developed to explain agonist behavior at ligand-gated ion-channels.

4.1. Development of Ideas About Gating Mechanisms

Until the 1950s, it was conveniently assumed that receptor occupancy was synonymous with receptor activation (reviewed in Steinbach, 2008). In simple terms, a submaximal agonist response was explained by the fact that not all binding sites were occupied. In the mid-1950s, however, agonists eliciting submaximal responses even at saturating concentrations at motor end-plate acetylcholine 'receptors' were discovered (Del Castillo & Katz, 1957). This observation could not be explained by a model solely based on receptor occupancy (Colquhoun, 1998).

To explain this discrepancy, del Castillo & Katz proposed a two-step model to describe agonist behavior (Katz & Thesleff, 1957). The kinetic model proposed for binding of the agonist molecule (A) to receptor in the closed state (R) followed by channel opening (AR*) is illustrated in Figure 5. The first step in this gating scheme involves ligand binding and the second step (with different kinetics and affinity) implicates a conformational change that switches the inactive ligand-bound receptor into an active conformation (Katz & Thesleff, 1957).

This simplistic model assumes that the initial binding reaction is so rapid that the AR complex is always in equilibrium with free A and R. Upon removal of the agonist, the response decays with an exponential closing of open receptors (AR*) with a rate constant α. This value represents the burst length at the single-channel level. Upon closing of the channel, the complex AR quickly dissociates, leaving

unoccupied receptors and free agonist molecules (which may then bind again, or be subsequently removed or degraded). This mechanism has important implications since it recognizes that an agonist molecule csn form a complex with its receptor, which is still inactive (i.e., shut, AR), and that activation of the channel requires an additional conformational step beyond binding: a process termed gating (Hille, 1992).

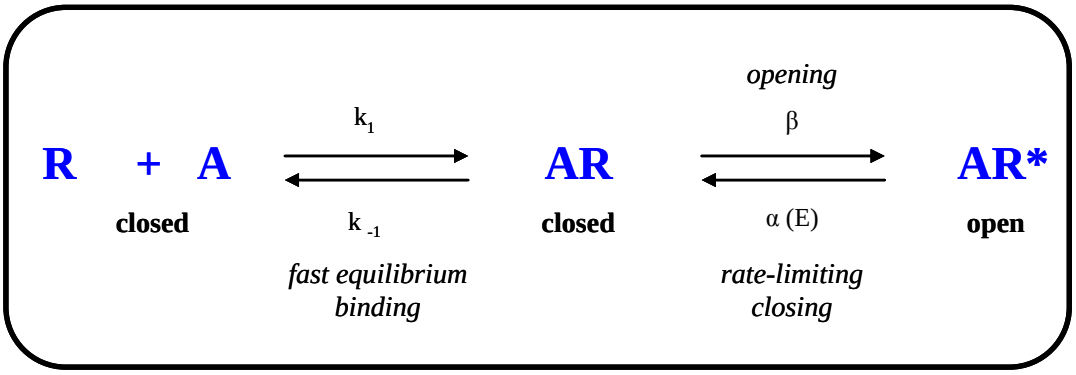


Figure 5. The del Castillo-Katz mechanism. The agonist (A) binds to a vacant receptor (R), forming a complex (AR) that is still closed (inactive state). The AR complex can then undergo a conformational change to open (AR*) (i.e.: active state). The rates of agonist binding and dissociation are defined by K1 and K-1, respectively. The rates of channel opening and closing are correspondingly described by β and α. The ability of the agonist to bind is quantitatively defined by the equilibrium constant KA (KA = k-1/k1). The ability of the agonist to activate the receptor, once bound, is described by the equilibrium constant E (E = β/α). Adapted from Hille B (1992) Ionic channels of excitable membranes. Sunderland: Sinauer Associates.

Defining Receptor Affinity & Efficacy

Although the ability of a molecule to activate a receptor (i.e.: efficacy) is an all-or-none property, these abilities may be graded. Classically, the efficacy of an agonist is measured by its capacity to initiate a response once it occupies all the receptor sites. Hence, at saturating concentration, a full agonist, by definition, produces a maximal effect whereas a partial agonist induces a submaximal effect. A competitive antagonist competes for the same binding site as an agonist, but does not cause the channel activation.

Defining affinity and efficacy and experimentally deciphering between these two concepts has been an exceptionally challenging task. Colquhoun carefully defines these two terms:

> Affinity…is simply the microscopic equilibrium (or rate) constant(s) for binding to the inactive state(s). Efficacy is everything else. So efficacy is simply the set of all the other microscopic equilibrium (or rate) constants, which describe all the transduction events that follow the initial binding reaction. Note that the efficacy constants must include those for binding of agonist to the active states, those for binding G proteins, as well as those for conformational changes. In addition, they must include quantities such as single channel conductance, and other such later parts of the transduction pathway. The definition is simple. It is measuring the values that is often hard (1998).

4.1.1. Models of Ion-Channel Gating

Any model attempting to describe gating behavior must consider several factors such as the strength of ligand binding, the geometrical arrangements of the subunits, the nature and degree of interaction between subunits, the energy associated with conformational change and the effect of non-identical subunits (Rang et al., 2003). In the mid-1960s, two models were developed to explain cooperativity (i.e., the functional interactions between distinct sites) in proteins and have greatly influenced and directed research in the last 35 years. These models, known as the concerted and multi-state models, are also often named after the scientists who initially described them: the Monod, Wyman and Changeux model and the Koshland-Nemethy-Filmer model, respectively (see Figure 6).

4.1.1.1. Monod-Wyman-Changeux Model

Monod, Wyman and Changeux (MWC) first suggested the concerted model to describe allosteric transitions of proteins made up of identical subunits. (Monod et al., 1965). Also referred to as the two-state model, it postulated that the protein can only occur in two states: inactive (shut) and active (open). The entire protein thus oscillates between these two conformations. In the absence of ligand, there is a conformational equilibrium that strongly favors the inactive state. Given that ligands bind with higher affinity to the activated state, ligand binding then shifts the equilibrium towards the activated conformation (Monod et al., 1965). Given that symmetry of the quaternary structure is retained, only two parameters are

required to explain behavior of the model at equilibrium: the dissociation constant of the ligand for each state, as well as the conformational equilibrium constant in the absence of ligand.

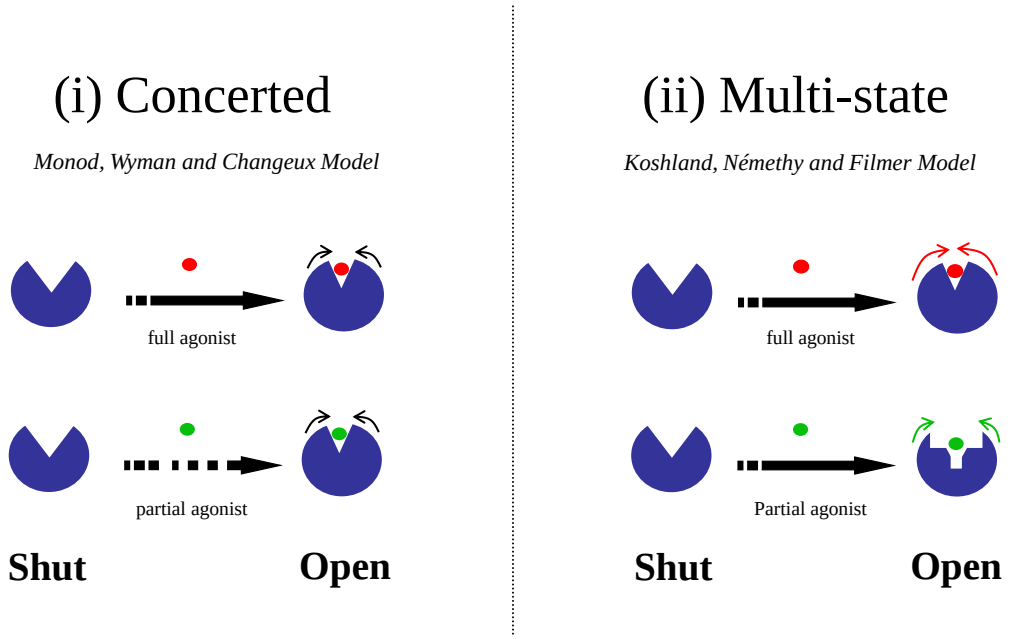


Figure 6. Schematic of the Monod, Wyman and Changeux (MWC, left) and Koshland-Nemethy-Filmer (KNF, right) models describing agonist behavior. In the MWC model, Binding of both full and partial agonist induces the same conformation of the receptor. The probability of entering the open state, once the agonist is bound, is higher for a full agonist compared to a partial agonist. In contrast, according to the KNF model, full and partial agonists elicit distinct conformations in the receptor.

In this case, the difference between a full agonist and a partial agonist is the probability of entering the open state once the agonist is bound (Monod et al., 1965). Proteins that gate in accordance to the MWC scheme have identical and non-interacting binding sites (Colquhoun & Sivilotti, 2004). In this paradigm, a partial agonist has a small equilibrium constant ($E = \beta/\alpha$, see Figure 5), which, in turn, elicits a response that is much smaller than the maximum possible response ($E/(1+E)$) of 1 (Colquhoun & Sivilotti, 2004).

Interestingly, most ligand-gated ion channels, with the exception of AMPA receptors and CNG channels, are thought to operate via the concerted model (Colquhoun & Sivilotti, 2004;Ruiz & Karpen, 1997). Although KA receptors have been thought to behave like AMPA receptors, their behavior has yet to be characterized. Given this, elucidating the gating mechanism of KA receptors has

been one of the major themes addressed in the work presented in this thesis. A particularly appealing part of the MWC model is that it seems to capture the most obvious simple transitions between closed and fully open conformations in single-channel recordings. This model, for example, can explain spontaneous channel opening observed in the absence of agonist at nAChRs (Jackson, 1986). Nonetheless, such oversimplification may be misleading. For instance, an unrealistic assumption of this model is that ligand binding to one subunit does not alter the conformation of that subunit more than the others. However, crystal structures of several proteins reveal that quaternary structural changes are associated with ligand binding. In fact, occupancy of only one of six available binding sites has been shown to induce quaternary rearrangements in some proteins (Macol et al., 2001).

4.1.1.2. Koshland-Nemethy-Filmer Model

The second model describing agonist behavior is the Koshland-Nemethy-Filmer (KNF) model, also referred to as the sequential, multi-state or induced-fit model (Figure 6). It proposes that each ligand has the ability to induce distinct conformational changes in the receptor (Koshland, 1958;Koshland, Jr. et al., 1966). According to this model, ligand binding to a subunit elicits a conformational change in that particular subunit alone. Consequently, binding of different number of ligands allows for various protein conformations. In this case, cooperativity arises from altered contacts between subunits. Despite the fact that the sequential model has fewer states than the concerted model, more parameters are necessary to specify the functional effects of a conformational change on one subunit to its neighbors (Karpen & Ruiz, 2002). In this model, the energy resulting from the binding of a full agonist will be more efficaciously translated into gating (i.e.: activation) than when a partial agonist is bound. In other words, it is not the probability of entering the open state that accounts for distinct agonist behavior as is the case in the MWC model, but rather the specific changes in the protein structure upon ligand binding (Koshland, 1958;Koshland, Jr. et al., 1966). An important drawback of this model is that it cannot explain spontaneous

activation in the absence of ligand or the apparent synchronous changes in quaternary structure (Karpen & Ruiz, 2002).

4.1.2. Limitations of the Classical Models of Ion-Channel Gating

It is important to bear in mind that for most ion-channels, limited structure and/or functional resolution has impeded our ability to delineate between these two classical models. The main obstacle in interpreting single-channel investigation is that ligands constantly bind and dissociate from each site, rendering measurements of the direct functional consequences of a single binding event very difficult. This challenge arises because there is no technique available which allows us to measure these parameters simultaneously. In fact, with the exception of homomeric $GABA_C$ receptors (Chang & Weiss, 1999) and CNG channels (Ruiz & Karpen, 1997), direct links between gating and the binding step for ion-channels remain purely speculative.

The main reason why such resolution has been possible for CNG channels is that unlike most neurotransmitter systems, they do not desensitize (Liu et al., 1996). CNG receptors are non-selective tetrameric cation channels found in photoreceptors and olfactory sensory neurons that are activated in response to intracellular cyclic adenosine monophosphate (cAMP) and cyclic guanosine monophosphate (cGMP) (Varnum et al., 1995). In an elegant study by Ruiz & Karpen, high resolution single-channel recordings were combined with the use of a photoaffinity analogue of cGMP to covalently tether cGMP moieties to the binding site of a single CNG receptor, thus allowing the isolation of channels with one, two, three or four ligands permanently attached (Ruiz & Karpen, 1997). Thorough kinetic analysis revealed that each liganded state can induce various open states and that the equilibrium constant for channel opening does not increase by a constant amount for each bound ligand. This complex behavior is not consistent with the MWC model, as previously proposed from these receptors (Ruiz & Karpen, 1997).

Moreover, most functional data available represents an ensemble average of various liganded states that prevents any direct correlation between the number of

bound ligands and a particular behavior (Karpen & Ruiz, 2002). Nonetheless, as the resolution of structural proteins and kinetic events continues to evolve, there are an increasing number of observations which cannot be explained by the two classical models of ion-channel gating. To illustrate this, I provide here four examples of such cases.

Subconductance States. A first observation, which cannot be reconciled with conventional models, is that many receptor systems display openings that are less than the maximal conductance, a phenomenon termed subconductance state. Moreover, the subconductance states of many ion-channels (Taylor & Baylor, 1995;Ruiz & Karpen, 1997;Chapman *et al.*, 1997;Root & MacKinnon, 1994), including iGluRs (Rosenmund *et al.*, 1998;Smith & Howe, 2000) have been associated with distinct activation levels. According to this scheme, smaller subconductance levels are more prevalent in partially activated receptors, but lead to the main conductance observed in maximally activated channels. Nevertheless, the concerted model cannot explain how the binding of a different number of ligands is linked to multiple conformational states.

More specifically, single-channel recordings from a GluR3/GluR6 chimera showed that the open channel conductance of a single iGluR depends on the number of subunits occupied by the agonist (Rosenmund et al., 1998). Taken in isolation, this observation seems to support subunit-based conformational changes and thus the KNF model. However, an additional complication is that single-channel analysis coupled with x-ray crystallographic snapshots of the GluR2 S1S2 construct proposed that full and partial agonists access the same subconductance states, but with different relative frequencies (Jin *et al.*, 2003a).

Experimental Design. Another element of complexity arises when considering that some technical parameters unexpectedly alter ion-channel structure and kinetics (Kash et al., 2003;Virginio et al., 1999). In fact, altering the agonist concentration and the length of the agonist application has important functional and structural repercussions (Kash et al., 2003). Specifically, the

$GABA_A$ receptor has been shown to adopt a conformation that was upon the agonist concentration-dependent. In fact, only high GABA concentrations were associated with disulphide bond formation in 2 loops critical for gating (Virginio et al., 1999).

Additionally, brief adenosine triphosphate (ATP) application (<1 second) caused permeation of small cations at three P_2X receptor subtypes (P2X2, 4 and 7) while prolonged agonist application (10-60 seconds) allowed passage of larger cations such as N-methyl-D-glucamine (NMDG) presumably by causing the channel to dilate (Virginio et al., 1999). Together, with work by Zheng & Sigworth (1998) on *Shaker* potassium channels, these studies have provided the only direct demonstrations in an ion-channel that different open states have distinct permeation properties. Though there is currently no data demonstrating that this specifically applies to iGluRs, these findings have critical implications in approaching experimental design to investigate agonist behavior.

Modal Gating. Modal gating represents a third issue which cannot be explained by classical gating models of ion-channels. Given that single-channel behavior is a stochastic process, kinetics can abruptly change while experimental conditions are held constant. Other than the obvious reasons of patch drift or instability, there are two main explanations for this behavior (Blatz & Magleby, 1986). One possibility is that many ion-channels have multiple shut states. Another possibility is that the sudden shift in kinetic activity is due to a shift between different apparent modes of activity, a phenomenon called modal gating (Magleby, 2004;McManus & Magleby, 1988). Modal gating can arise from changes in multiple parameters such as open times, closed times or both, and is usually associated with changes in the open channel probability (Blatz & Magleby, 1986). For instance, Popescu & Auerbach (2003) identified three different modes of NMDA receptor gating: high, medium and low, with mean open times of ~25 ms, 8 ms and 1 ms. Switching between modes was revealed in the absence of extracellular Mg^{2+} which normally blocks the channel at negative

membrane currents and occurred on a timescale ranging from tens of seconds to minutes (Popescu & Auerbach, 2003).

Although conventional models of ion-channel gating cannot explain such complex behavior, Popescu & Auerbach developed a kinetic model based on linear free analysis suggesting that the different modes utilize a common gating scheme but with distinct free energy of the open and closed states for each mode. Given that these experiments were performed on recombinant receptors, it remains to be established whether modal gating occurs at functional synapse and what the physiological significance of this phenomenon is. Though no information is available on the actual mechanism causing receptors to switch between gating modes, it has been suggested that alterations in the ion-channel structure at the intracellular level may account for this phenomenon (Popescu & Auerbach, 2003;Popescu, 2005). Notably, many intracellular partners of NMDA receptors have been shown to alter average channel open time (Wang & Salter, 1994;Yu *et al.*, 1997;Rycroft & Gibb, 2002). Although modal gating has yet to be examined for KA receptors, specific cellular components such as PKA or calcineurin and the recently identified NETO2 proteins which have also been shown to increase GluR6 open channel probability (Traynelis & Wahl, 1997;Zhang *et al.*, 2009), may potentially provide clues into the basis of such mechanism.

A flipping receptor. Lastly, a recent study by the Colquhoun group has overturned the conventional understanding of agonist behavior for the cys-loop receptor family, where partial agonists were thought to elicit smaller responses than full agonists because they were less efficient at converting the shut state into the active state of the receptor (Lape et al., 2008). Using two members of the cys-loop receptor family (nAChR and GlyRs) they reported that the open-shut reaction was surprisingly comparable across agonist efficacy. Instead, according to this new paradigm, partial agonists are thought to often fail to trigger an earlier conformational change in the receptor, a state that actually precedes the actual channel opening - hence, the term for this conformational change – flipping (Lape

et al., 2008). This observation cannot be explained by a conventional model; illustrating the complexity of dissecting the molecular events leading to channel activation and desensitization. Taken together, these studies illustrate the complexity of ion-channel gating which cannot be readily explained by conventional models of ion-channel behavior.

4.2. Activation of the Kainate Receptor

Proteins are dynamic systems, exhibiting motion on multiple time scales (Hille, 1992). This is especially true for iGluRs, which display movement in regions thought to be important for function in the orders of microseconds to milliseconds (Valentine & Palmer, 2005;McFeeters & Oswald, 2002). Binding of the neurotransmitter L-glutamate to the agonist-binding domain of KA receptors induces short-lived and rapid opening at multiple conductance levels with varying levels of peak open probability (Swanson *et al.*, 1996;Zhang *et al.*, 2008a;Swanson *et al.*, 1997;Howe, 1996). Though the precise mechanism by which agonist binding leads to channel activation remains elusive, it is well-known that following activation by L-glutamate, KA receptors undergo desensitization, a process whereby the agonist is still bound but the receptor becomes refractory or insensitive to further stimulation (Huettner, 1990;Erreger et al., 2004).

This obviously raises the question as to whether the agonist-binding domain adopts the same conformation in the activated and desensitized states. Moreover, although the desensitized state has been assumed for some time to be non-conducting (Raman & Trussell, 1992;Patneau *et al.*, 1993;Heckmann *et al.*, 1996;Bowie *et al.*, 1998;Smith & Howe, 2000;Mulle *et al.*, 2000;Robert *et al.*, 2001), detailed characterization and modeling of the desensitization properties of KA receptors suggest that this state may be conducting (Bowie & Lange, 2002). This concept will be addressed in a later section on the plant lectin, Con-A (see section 4.2.5.4)

In order to fully understand the context in which this work on the basic properties of KA receptor activation and desensitization was performed, the

remainder of this **REVIEW OF THE LITERATURE** provides an overview of what was known about the gating process of KA receptors ***before* the beginning of my thesis (*i.e.: before 2004*)**. This is to allow the reader to fully appreciate the scientific context which motivated our research work. Relevant information published during the course of my thesis will be provided in the **RESULT CHAPTER PREFACES** and addressed in the **DISCUSSION AND CONCLUSIONS** in the context of the findings presented here.

4.2.1. Conceptual Model of Kainate Receptor Activation

From a structural point of view, ligand binding occurs within four extracellular S1S2 agonist-binding domains. From a conceptual perspective, the first necessary step to determine the opening and closing kinetics of a channel is to be able to distinctly record agonist binding and channel opening. This can only be resolved by performing single-channel recordings since macroscopic patch recordings cannot distinguish between these two phenomenon (Colquhoun, 1998). Specifically, the kinetic properties of KA receptors are important to understand since their channel properties participate in shaping and modulating synaptic transmission (Lerma, 2003). Since there is much less information about the KA receptor, valuable information may also be gained from studying the closely-related AMPAR.

The first quantitative measurements of AMPA receptor activation kinetics were extracted from analysis of macroscopic currents and suggested that (at least) two binding steps were required for agonist activation (Hausser & Roth, 1997;Raman & Trussell, 1995;Clements *et al.*, 1998;Jonas *et al.*, 1993). In 1998, single-channel recordings of a recombinant non-desensitizing GluR3/GluR6 chimera clearly demonstrated that the channel can open to three distinct conductance levels (5, 15 and 23 pS) (Rosenmund et al., 1998). As shown in Figure 7, Rosenmund et al. cleverly equilibrated a single GluR3/GluR6 receptor with a slowly dissociating competitive AMPA receptor antagonist, NBQX (6-nitro-7-sulphamoylbenzo[f]quinoxaline-2,3-dione), before replacing the bathing solution with a high affinity agonist, quisqualate.

In this paradigm, antagonist dissociation from each binding site was immediately accompanied by binding of an agonist molecule and kinetic analysis showed that two agonist molecules are required to activate the lowest resolvable conducting state. As the number of agonist molecules progressively increased to three and four, a clear stairway opening to three distinct conductance levels was observed (Rosenmund et al., 1998).

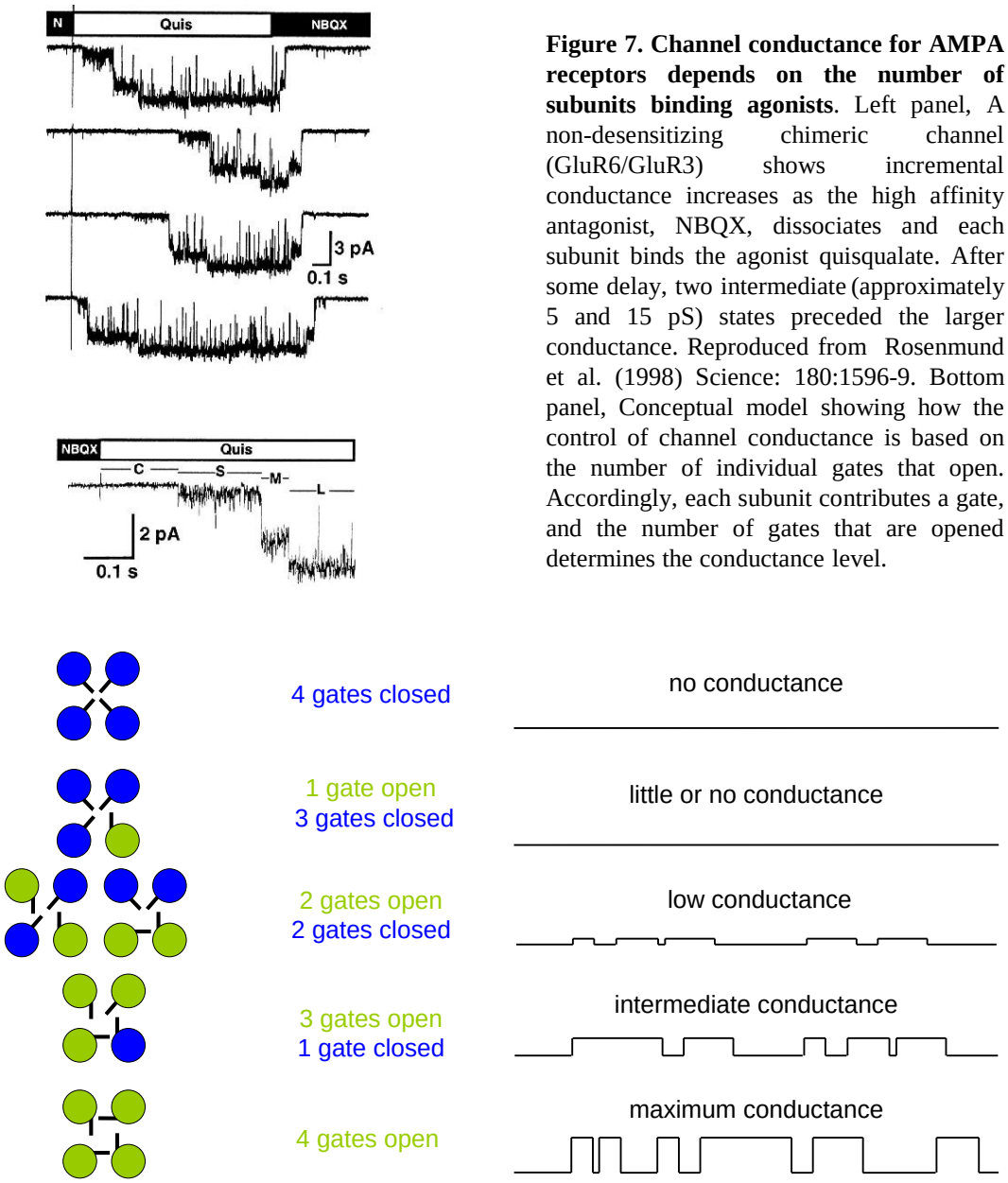


Figure 7. Channel conductance for AMPA receptors depends on the number of subunits binding agonists. Left panel, A non-desensitizing chimeric channel (GluR6/GluR3) shows incremental conductance increases as the high affinity antagonist, NBQX, dissociates and each subunit binds the agonist quisqualate. After some delay, two intermediate (approximately 5 and 15 pS) states preceded the larger conductance. Reproduced from Rosenmund et al. (1998) Science: 180:1596-9. Bottom panel, Conceptual model showing how the control of channel conductance is based on the number of individual gates that open. Accordingly, each subunit contributes a gate, and the number of gates that are opened determines the conductance level.

Besides suggesting that AMPA receptors have four binding sites, the most parsimonious explanation for these observations is that the receptor opens to a larger conductance level as each subunit binds an agonist molecule (Rosenmund *et al.*, 1998;Smith & Howe, 2000) (Figure 7). The largest conductance level (i.e., 23 pS) is therefore thought to represent the fully liganded state (i.e., four agonists) while the singly liganded channel is either non-conducting or has a conductance that is too small to resolve (Rosenmund et al., 1998). Moreover, the subconductance state occupancy was shown to be agonist-concentration dependent at AMPA receptors (Smith & Howe, 2000). Comparable results were obtained in wildtype GluR3 in the presence of a desensitization blocker (cyclothiazide, CTZ), therefore strengthening the significance of their findings (Rosenmund et al., 1998). Although a similar subunit-dependent gating scheme had also been proposed for K^+ and CNG channels (Zheng & Sigworth, 1997;Ruiz & Karpen, 1997), this was the first evidence for the direct relationship between the number of agonist bound and the conductance levels in non-NMDA receptors (Rosenmund et al., 1998).

Subsequently, a series of experiments by the Howe laboratory on native AMPA and KA receptors further confirmed the presence of multiple single-channel subconductance states (Smith & Howe, 2000;Smith *et al.*, 1999;Smith *et al.*, 2000). In contrast to the study by Rosenmund and colleagues (Rosenmund et al., 1998), however, they found that approximately half of the patches exhibited, not only three, but four distinct levels of channel activation (Smith & Howe, 2000). Importantly, single-channel analysis revealed fundamental differences between the gating mechanism of AMPA and KA receptors. For example, Smith & Howe (2000) reported that the relative occupancy of subconductance states at AMPA receptors is agonist concentration-dependent, consistent with the experiments performed by Rosenmund et al. On the other hand, occupancy of KA receptors was independent of the agonist concentration used, as only KA receptors seem to occupy multiple subconductance levels even at high agonist concentration (Smith & Howe, 2000). One important drawback of the experiments performed by the Howe laboratory is that long application of the high

affinity and desensitizing agonist domoate implies that the authors were studying the desensitized state of the receptor and not the activated state.

The observation that KA (like NMDA, but unlike AMPA) receptors display single-channel conductances independent of the agonist concentration (Smith & Howe, 2000) seems consistent with a concerted model of KA receptor activation. An independent gating scheme would predict a concentration-dependent behavior. Although this might be due to the potential problem associated with domoate, an agonist that exhibits strong level of desensitization, further studies are clearly required to elucidate this issue.

Many questions in regards to the mechanism of KA receptor activation still remain. For example, why are some agonists more efficacious than others? What is the molecular basis for the action of an agonist versus an antagonist? Although an important limiting factor has been the lack of available ligands acting at KA receptors, resolution of the agonist-binding domain of AMPA receptors has began to shed light of some of these issues.

4.2.2. Structural Features of Kainate Receptor Activation

4.2.2.1. Structure and Dynamics of the Ligand Binding Site

Even though the structure of an intact, membrane-bound iGluR has yet to be resolved, the modular nature of its agonist-binding domain provided key structural insight. Over a decade after the first AMPA S1S2 crystal (Armstrong et al., 1998), over fifty crystal structures for different subunits from AMPA and NMDA receptors have been resolved in the absence or presence of various agonists, antagonists and modulators (for example see: (Armstrong *et al.*, 1998;Armstrong & Gouaux, 2000a;Jin *et al.*, 2002;Hogner *et al.*, 2002;Sun *et al.*, 2002;Armstrong *et al.*, 2003;Jin & Gouaux, 2003;Hogner *et al.*, 2003;Lunn *et al.*, 2003;Mayer *et al.*, 2001;Jin *et al.*, 2003a;Furukawa & Gouaux, 2003)). At the beginning of this work in 2004, however, the agonist-binding core of KA receptors had yet to be resolved.

Importantly, these crystals have provided pivotal insights into the specifics of agonist behavior at iGluRs. Here, I highlight four main findings from these studies:

(1) First, except for the NR1 subunit (Furukawa & Gouaux, 2003), the binding cavity of the available iGluR crystals is substantially larger than is necessary to accommodate the neurotransmitter L-glutamate (Armstrong & Gouaux, 2000a). The different sizes and chemistry of the iGluR binding pockets may thus help to confer receptor subtype selectivity for ligands (Armstrong *et al.*, 1998;Armstrong & Gouaux, 2000a).

(2) Secondly, the extra space in the binding cleft can accommodate a number of surrogate water molecules which provide surrogate ligand atoms and additional hydrogen bond interactions (Armstrong & Gouaux, 2000a). GluR2 AMPA receptors can accommodate three to four such water molecules (Armstrong & Gouaux, 2000a). Displacement of these water molecules permits binding of bulkier glutamate analogs such as AMPA and the willardiines (Armstrong & Gouaux, 2000a;Jin *et al.*, 2003a).

(3) Thirdly, the differences in amino acid residues located in the cleft and in the ligand structure allows for receptor subtype specificity. Since no KA receptor crystal was available, comparisons of amino acid sequences combined with homology modeling make important predictions. For instance, while all AMPA and KA receptors have a serine residue at position 654, GluR6 KA receptors possess an alanine at this position. Modeling studies have predicted that H-bonding between L-glutamate and serine via a water molecule is probably disrupted in GluR6 (De Luca et al., 2003).

Another example is the Leu650 residue in GluR2, which accepts an H-bond from the α-amino group of L-glutamate while the equivalent residue at GluR5, GluR6 and GluR7, is a valine. This residue difference explains the selectivity of the agonist SYM 2081 ((2S,4R)-4-methylglutamate) for KA versus AMPA receptors (Zhou et al., 1997). In fact, the more rigid valine favorably interacts with SYM 2081`s methyl group, while the bulkier leucine would be in the same

location as the optimally positioned methyl group of SYM 2081 (Pentikainen et al., 2003).

(4) Lastly, receptor subtypes with identical residues at specific positions have also been shown to exhibit agonist-dependent differences in the strength of interaction between specific residues and the ligand. For example, infrared spectroscopy measurements at GluR2 AMPA receptors indicate that the partial agonist, KA, interacts more strongly with Arg485 and more weakly with Pro478 than the full agonist, L-glutamate (Jayaraman et al., 2000). As detailed below, evaluation of the AMPA receptor ligand binding core in complex with full and partial agonists as well as antagonists revealed varying degrees of domain closure.

4.2.2.2. Partial Agonists, Antagonists and Domain Closure

Although at the beginning of this work, the binding mode of L-glutamate to KA receptors was still unknown, useful information could be drawn from AMPA receptors, where the two lobes of the clamshell structure (D1 and D2) bind distinct portions of the agonist molecule (Armstrong *et al.*, 1998;Armstrong & Gouaux, 2000a). The L-glutamate backbone (the α-substituents) binds to a relatively fixed portion of the binding site (D1) whereas the equivalent side chain of glutamate (γ-substituents) binds to a more dynamic portion (D2) (Armstrong & Gouaux, 2000a;McFeeters & Oswald, 2002).

Interestingly, the separation between the two *lobes* of the S1S2 construct is proposed to correspond positively with the extent of receptor activation at AMPA receptors. The energy associated with agonist binding is thought to be transduced across the interface that connects adjacent subunits (dimer interface). As discussed further in the following section (4.2.4), receptor desensitization has been shown to include dissociation of the dimer interface between subunits, thus abolishing the transduction of agonist binding energy to the ion-channel pore (Sun et al., 2002).

The first evidence supporting a correlation between agonist efficacy and domain closure came from work on GluR2 AMPA receptors where binding of the partial agonist, KA induced a smaller amount of closure in the binding core than

the full agonists, glutamate and AMPA (12^{o} vs. 20^{o}, respectively) (Armstrong & Gouaux, 2000a). Furthermore, comparison of the GluR2 S1S2 domain in complex with a series of willardiine molecules, which exhibit different efficacies at GluR2 channels, revealed a correlation between the degree of domain closure and agonist efficacy (Patneau *et al.*, 1992;Jin *et al.*, 2003a). In addition, crystal structures of GluR2 in complex with the competitive iGluR antagonists (CNQX and DNQX) also appear to support a correlation between closure and agonist efficacy. Compared to the ligand-free state (i.e: apo state), the competitive antagonist DNQX elicited a modest degree of domain closure (2.5^{o}-6.0^{o}), a change which was not considered sufficient to cause channel activation of the wildtype receptor (Armstrong & Gouaux, 2000a).

Taken together, this behavior is consistent with the KNF model of ion-channel behavior (Jin *et al.*, 2003a;Armstrong *et al.*, 2003). This finding is in contrast with functional studies on nAChR and NMDA receptors where the chemical nature of the agonist is not thought to influence the (average) channel conductance, but rather the probability of entering the open state (Colquhoun & Sivilotti, 2004).

Based on this model, one may predict that the extent of domain closure to be directly correlated with the single-channel conductance of AMPA receptors, given that the degree of gate opening should regulate ion flow through the channel. As discussed above, however, Gouaux and colleagues observed that both full and partial agonists accessed similar subconductance levels, but with different probabilities (Jin *et al.*, 2003a). It remains to be determined, then, how closure may govern open channel probabilities. Moreover, as described in the subsequent section, interpretation of these findings is confounded by several factors.

4.2.3. Does the Isolated Binding Core Behave Like the Intact receptor?

In assessing the implications of the structural data obtained through x-ray crystallography, there are three main issues to consider. First, the D1 and D2 domain are artificially linked by a short GT linker and lack transmembrane domains as well as the N-and C-terminals (Armstrong et al., 1998). Consequently,

the conformational changes observed may not be representative of the strain present in the intact receptor.

Secondly, while the crystallized construct is generally taken to represent the open state (Jin *et al.*, 2003a;Madden, 2002) its actual state remains uncertain and some have argued it may represent a pre-opening conformation, the desensitized state or the transition to the desensitized state (Madden, 2002;Naur *et al.*, 2005;Colquhoun & Sivilotti, 2004).

Thirdly, the data obtained through x-ray crystallography only provides low temperature static images of the receptor that is constrained by crystal-packing forces and thus cannot fully account for the dynamics of the receptor (reviewed in Erreger et al., 2004). For instance, motility of the S1S2 domain was shown to occur on a very fast time scale (micro- to milliseconds) using NMR spectroscopy (McFeeters & Oswald, 2002). This finding generates many obvious questions including: "Does the interaction with auxiliary proteins affect the domain closure of glutamate receptor?" and "What is the mechanistic basis for this observation?" Answers to these questions remain unknown as crystallization of the entire complex has yet to be acheived.

The static information provided through crystallography combined with other functional and structural analyses, nonetheless, has significantly advanced our understanding of the molecular basis of agonist, antagonist and modulator binding at iGluRs. Although the pharmacological profile of the S1S2 construct and the wildtype receptor display strong similarities and are often regarded as identical, it is imperative to consider these issues.

4.2.4. Receptor Desensitization and the Dimer Interface

Following activation of most ligand-gated ion channels, they enter an inactive state despite the continued presence of bound agonist, a process known as desensitization. First described by Katz and Thesleff in nAChRs, this process has now become a hallmark of almost all ion-channels involved in rapid synaptic transmission (Katz & Thesleff, 1957;Jones & Westbrook, 1996). Glutamate receptor desensitization is critical in shaping the postsynaptic response at

synapses in the brain by limiting ion flow to a few milliseconds following binding of the endogenous neurotransmitters (Rosenmund & Mansour, 2002). Interestingly, a hallmark of KA (and AMPA) receptors is their ability to desensitize at agonist concentrations much lower than those needed to elicit currents (Chittajallu et al., 1999).

Although our understanding of KA receptor desensitization is still emerging, insights into its mechanism can be drawn from its more studied counterpart, AMPA receptors. Recent data from x-ray crystallography, electrophysiology, sedimentation equilibrium and site-directed mutagenesis studies have demonstrated the crucial role of the stability of the dimer complex for desensitization. In support of this, an unstable dimer interface may be generated by either mutagenesis of an amino acid located at the subunit interface of the GluR1 (Sun et al., 2002) or by binding of allosteric modulators such as CTZ to the dimer interface (Sun et al., 2002;Leever et al., 2003). In both cases, the receptor response showed a reduced rate and degree of desensitization. Together, these pointed to the role of a salt bridge, hydrogen bond network and intermolecular van der Waals contacts in maintaining the stability of the dimer and, in turn, desensitization.

To examine if AMPA receptor desensitization depends on the number of subunits occupied by glutamate, Robert and Howe (2003) cleverly used a combination of fast glutamate application protocols and kinetic simulations. From their data, they developed a kinetic scheme which includes desensitization and incorporates four binding sites for glutamate and multiple concentration-dependent open levels. In this model, each of the four binding steps is capable of opening to a distinct subconductance level or desensitizing the channel (Robert & Howe, 2003). This model explained many properties of AMPA receptor behavior including the Hill slope of dose response curves, the faster recovery from desensitization for singly occupied channels compared to receptors found with two to four glutamates and the concentration dependence of single-channel properties observed (Robert & Howe, 2003). Moreover, this gating scheme clarified the relationship between affinity of glutamate for the closed and

desensitized states by explaining the similar affinity of glutamate for both these receptor states (Robert & Howe, 2003).

In agreement with this, detailed characterization of the functional stoichiometry of glutamate receptor desensitization demonstrate that KA, but not AMPA, receptors operate via an independent gating scheme (Bowie & Lange, 2002). In addition, using subunit-selective agonists, Swanson et al. (2002) showed that individual subunits comprising heteromeric KA receptors can independently contribute a distinct channel conductance. One important question raised by these studies is whether the desensitized state is truly non-conducting. Although it is generally hypothesized to represent a non-conducting state, detailed analyses have suggested an alternate explanation in which subunits in the desensitized state of non-NMDA receptors (particularly KA receptors) contribute to membrane conductance (Bowie & Lange, 2002). Compared to NMDA and AMPA receptors, our understanding of the basic gating features of KA receptors has lagged behind due primarily to the relative absence of specific ligands (Lerma et al., 2001). Nonetheless, as detailed below, several pharmacological tools such as agonists, antagonists and allosteric modulators including external ions and lectins have proved useful in elucidating the properties of KA receptors.

4.2.5. Pharmacological Profile of Kainate Receptors

Compared to AMPARs, which display relatively uniform pharmacological sensitivity, KA receptors are assembled from subunits with strikingly different pharmacological properties (Huettner, 2003;Swanson *et al.*, 2002;Herb *et al.*, 1992;Swanson *et al.*, 1998;Hollmann & Heinemann, 1994). In fact, the high affinity KA-1 and KA-2 subunits, which do not form functional homomeric receptors, assemble as a heteromeric complex with lower affinity subunits to alter their pharmacological and physiological properties. As described here, relevant pharmacological tools are important to unravel the basic kinetic properties of receptor activity. Yet, at the beginning of the work presented here, there was an urgent need to identify more KAR ligands and modulators.

4.2.5.1. Agonists

The only known endogenous ligand at KA receptors is the main excitatory neurotransmitter, L-Glutamate (Brauner-Osborne et al., 2000). This amino acid is thought to act as a full agonist at all KA receptors and displays low affinity in the high micromolar EC_{50} range (Traynelis & Wahl, 1997;Sommer *et al.*, 1992). Natural and synthetic agonists often used to elicit KA receptor responses include KA, domoate and SYM 2081 ((2S,4R)-4-methylglutamate) (Jones et al., 1997;Brauner-Osborne et al., 2000).

Although KA is the prototypic agonist for KA receptors, it is also activates AMPA receptors (Stensbol et al., 2002). Given this, a concentration window at which this marine toxin is thought to act exclusively on KA receptor needs to be carefully considered. For example, in hippocampal pyramidal cells, low KA concentrations (i.e., less than 1 μM) exclusively activate GluR6-containing KA receptors, while higher concentrations have been shown to activate both KA and AMPA receptors (Mulle et al., 1998;Bureau et al., 1999). Domoate also activates KA receptors but exhibits even less selectivity as it also activates AMPA receptors (Bleakman et al., 2002). Similarly, SYM2081 is not selective for iGluRs, but is also an agonist at both NMDA and metabotropic GluRs (mGluRs).

Other KA receptor agonists include (S)-5-Iodowillariine (IW) and (RS)-2-amino-3-(3-hydroxy-5-terbutylisoxazol-4-uyl) propanoic acid (ATPA). IW was isolated from the seeds of *Acacia willardiiana* and exhibits subunit selectivity since it activates GluR5 subunit-containing receptors, but not homomeric GluR6 or GluR7-containing receptors (Brauner-Osborne et al., 2000). Interestingly, IW weakly activates GluR6 and GluR7 in heteromeric assembly with KA-2 (GluR6-7), a sensitivity conferred because IW is thought to bind to KA-2 to elicit a current (Swanson et al., 1998;Swanson et al., 2002). ATPA also shows strong selectivity for GluR5 over GluR6 containing receptors (Wilding & Huettner, 2001) (Brauner-Osborne et al., 2000).

The naturally-occurring glutamate analog, dysiherbaine, was more recently isolated from the marine sponge *Dysidea hebacea* (Swanson et al., 2002). While this toxin activates KA and AMPA receptors, the wide range of affinities for the

different subunits has been cleverly used to demonstrate that individual subunits in a GluR5/KA-2 receptor could be differentially activated (Swanson et al., 2002).

4.2.5.2. Antagonists

In addition to agonists, the orthosteric site of KA receptors has also been shown to bind different classes of antagonists. As previously described, the physiological role of KA receptors has lagged behind other iGluRs, in great part because of the absence of exclusively selective antagonists (reviewed in Jane et al., 2009).

For example, the quinoxalinediones (such as CNQX and NBQX) also block AMPA receptors (and NMDA receptors at higher concentrations) (Brauner-Osborne et al., 2000;Bleakman et al., 2002). Similarly, the first identification of a putative KA-selective antagonist, NS-102 (5-nitro-6,7,8,9-tetrahydrobenzo[g]-2-3-dione-3-oxime) was later questioned by another group, which showed that it also could also bind to AMPA receptors (Chittajallu et al., 1999). The identification of the decahydroisoquinolines as iGluRs ligands with high selectivity for GluR5 over other KA and AMPA receptor subunits also marked an important achievement, though some of these compounds also exhibit cross-activation (Bleakman et al., 2002). Although there is clearly a need for the design of selective antagonist at KA receptors, as presented below, allosteric modulators have also been cleverly utilized to unravel the properties of these receptors.

4.2.5.3. External Ions

It has been known for many years that ion-channels do not gate normally under various ionic conditions, such as changes in ionic species or ionic strength (Yellen, 1997). Perhaps the best example of this is the “foot-in-the-door” effect in which high ionic concentrations of specific ions have been shown to slow channel closing presumably because ions restrict channel closure, suggesting that the gating process and permeation may be coupled (Ascher *et al.*, 1978;Marchais & Marty, 1979). For example, reducing the concentration of permeant ions for both

Na^+ and K^+ channels significantly decreased the channel opening probabilities (Townsend et al., 1997;Lopez-Barneo et al., 1993;Pardo et al., 1992).

iGluRs are no exception to this, as different ions in the extracellular milieu have been known for some time to modulate all three main subfamilies (Dingledine et al., 1999). KA (and AMPA) receptors are regulated by a number of exogenous anions (e.g., thiocynate, F^-, Cl^-, Br^-, I^-, NO_3^-) (Bowie & Smart, 1993;Arai *et al.*, 1995;Partin *et al.*, 1996;Bowie, 2002a) and cations (H^+, Zn^{2+}, Ca^{2+}, Mg^{2+}, Hg^{2+}, K^+, Li^+, Rb^+, Cs^+) alike (Mott & Dingledine, 1999;Ihle & Patneau, 2000;Rassendren *et al.*, 1990;Perouansky & Grantyn, 1989;Bowie & Mayer, 1996;Kiskin *et al.*, 1986;Umbach & Gundersen, 1989;Bowie, 2002a). Importantly, external ions first revealed that KA and AMPA receptors may operate via distinct gating mechanisms (Bowie, 2002a). In fact, both the response amplitude, deactivation rate and desensitization kinetics of KA, but not AMPA receptors, were strongly regulated by the ionic concentration and species of the extracellular solution (Bowie, 2002a;Bowie & Lange, 2002).

Similarly, both cations and anions regulate the gating behavior of voltage-dependent Na^+ and K^+ channels through surface charge screening in or around the voltage sensor (Hille, 1992;Dani *et al.*, 1983;Kao & Stanfield, 1968;Hille *et al.*, 1975). In accordance with electrostatic principles, the effects of cations and anions on Na^+ and K^+ channels were distinct, suggesting that non-identical, local surface charges are present on each protein structure (Dani et al., 1983;Hille, 1992).

At KA receptors, however, this observation cannot be explained by surface charge screening or ion-agonist competition at the orthosteric site because deactivation rates decreased in high ionic strength solutions (Bowie, 2002a). A channel block mechanism is also very unlikely since this effect was voltage-independent. One possibility is that the differential effect of cations at KA receptors may be explained by assuming a single anion-binding site (Eisenman, 1962;Hille, 1992), where cations stabilize the open state of the channel. Consistent with this, the rank order of cation potency $Na^+ > Li^+ > K^+ > Rb^+ > Cs^+$

matches the sequence X of the Eisenman series, an observation consistent with a mechanism favoring binding of smaller rather than bulkier cations.

Despite this interesting correlation amongst external cations, it is difficult to also explain the effect of anions on the receptor kinetics. In contrast with a charge-screening mechanism as observed at Na^+ and K^+ channels, both cations and anions evoked apparently identical effects, suggesting a common site of action for ions of opposite charge. Though this may, at first, seem to violate electrostatic principles, there are two possible explanations to account for this observation. One possibility is that cations bind to a single anion-binding site and that anions bind to a cation-binding site that both merge into a common pathway.

Alternatively, external ions may modulate KA receptors through a dipole mechanism as described for the permeation of cations through amphotericin B channels (Borisova et al., 1986). Borisova and colleagues have shown that under specific conditions, the channels strongly select for anions over cations and in the absence of anions, they are practically impermeable to any cation. However, in the presence of a permeant anion, the contribution of monovalent cations to channel conductance grows with an increase in the anion concentration. This suggests that a cation may only enter an anion-bound channel to form a dipole at the center of the channel and subsequently slip past the ions to exit the channel to the intracellular milieu (Borisova et al., 1986).

In this case, an external anion binds the positive binding site to attract and allow passage of external cations (Borisova et al., 1986). Since the cation-anion interaction is dependent on the ion species (Khutorsky, 1996), this mechanism for cation permeation may also explain the effect of ions at KA receptors. In line with the idea that this phenomenon may also take place at other receptors, the reverse scenario at anionic voltage-dependent chloride channels has also been observed (Franciolini & Nonner, 1987).

Although synaptic activity induces important changes in the pH concentration in the brain, the physiological implication of ion regulation remains unknown. (Mott et al., 2003). Finally, a fundamental consideration to address is whether ions regulate the basal gating properties or are absolute requirement for the gating

of KA receptors. Given this, examining the molecular mechanism for this effect constitutes one of the main objectives of the present thesis.

4.2.5.4. The Plant Lectin Concanavalin-A

Historical Significance

The plant lectin Con-A was extracted from the jack bean (*Canavalia ensiformis)* by Nobel Prize winner James B. Sumner (Sumner & Howell, 1936). This discovery bears considerable historical significance since Con-A was the first agglutinin to be isolated, purified and crystallized. Importantly, this opened up the notion of carbohydrate recognition by lectins, which subsequently allowed researchers to elucidate the biochemical nature of the ABH-blood group system (Morgan & Watkins, 1953;Morgan *et al.*, 2000).

While Con-A is found as a dimer (M.W.: 53KDa) at low pH (5.6-5.6) (McKenzie et al., 1972), the lectin predominantly exhibits a tetrameric form with four saccharide binding sites at physiological pH levels (Wang et al., 1971;Edelman et al., 1972). Subsequently, chemical derivation of Con-A was shown to yield stable dimeric molecules with altered properties, yielding syccinyl Con-A and acetyl Con-A at pH 7 (Gunther et al., 1973). These findings were consistent with the proposition that at least some of Con-A's effect appear to be valence-dependent and involved in cross-linking.

Before the cloning of iGluRs, Con-A was used in the purification of glutamate-binding glycoproteins, which were isolated from rat brain synaptosomes (Michaelis, 1975). The effect of Con-A on receptor kinetics was first noticed on invertebrate muscle fibers (Mathers & Usherwood, 1976) and neurons (Kehoe, 1978) but it was not until later that its effect on vertebrate neurons was recognized (Mayer & Vyklicky, 1989). Interestingly, the effects of Con-A was state-dependent at insect muscle fibers (Mathers & Usherwood, 1976). In contrast with its initial use in agglutination, Con-A's effect seemed unrelated to its ability to produce redistribution of receptor sites in cell membranes (Kehoe, 1978).

Quite unexpectedly, Con-A was also the first tool used to partly distinguish between AMPA and KA receptors (Partin et al., 1993). Later sequencing and mutagenesis studies revealed that the preferential effect of Con-A on KA receptors over AMPA receptors may be mediated by the fact that AMPA receptors have 4-6 N-glycosylation sites while KA receptors contain 8-10 (Dingledine et al., 1999;Partin et al., 1993). Curiously, Con-A potentiated the peak response of GluR7b, a splice variant that is also interestingly antagonized by domoate, but had no effect on its equilibrium response (Schiffer et al., 1997).

How Does Concanavalin-A Regulate Kainate Receptors?

The effect of Con-A on KA receptors may be explained by one of two mechanisms. The majority of studies have inferred that the lectin blocks or significantly decreases the onset of receptor desensitization (Mayer & Vyklicky, 1989;O'Dell & Christensen, 1989;Huettner, 1990;Partin *et al.*, 1993;Wong & Mayer, 1993;Yue *et al.*, 1995;Wilding & Huettner, 1997;Everts *et al.*, 1997;Everts *et al.*, 1999;Mathers & Usherwood, 1976) by sterically interfering with protein conformational changes associated with the desensitization process. The most compelling argument supporting this notion is that the rapid onset of KA receptor desensitization before Con-A treatment is completely or almost completely absent following lectin treatment (Huettner, 1990;Everts *et al.*, 1999;Partin *et al.*, 1993;Patneau *et al.*, 1994;Wilding & Huettner, 1997). Furthermore, Lerma's research group observed a leftward shift of the agonist dose-response relationship after Con-A treatment, which led them to propose that the lectin converts the high-affinity, non-conducting state of the receptor into a conducting state (Paternain et al., 1998).

However, careful examination of KA receptor kinetics recorded from outside-out excised patches using a fast-perfusion system revealed that Con-A treated receptors exhibit a larger agonist-evoked steady-state response, but dose-response relationships, peak responses as well as desensitization remained unchanged compared to control (Bowie *et al.*, 2003a). This finding was inconsistent with the proposal that Con-A blocked receptor desensitization since this mechanism would

have predicted a change in the rates into and out of desensitization which was not observed at a faster kinetic resolution (Bowie *et al*., 2003). Importantly, the claim that this lectin inhibited receptor desensitization emerged from observations from whole-cell recordings (Paternain et al., 1998), which undoubtedly underestimate peak responses. Thus, this difference most likely represents the slow perfusion conditions utilized in the former studies, which could not reveal detailed kinetic properties.

Bowie & Lange developed an alternative mechanism which reconciles this data by proposing that Con-A targets specific open states, a model that assumes that desensitized GluR6 KA receptors are ion-conducting (Bowie *et al.*, 2003a). In fact, fitting the recovery kinetics before and after lectin treatment using a gating scheme for KA receptors in which they recover from desensitization in multiple conformational steps (Bowie & Lange, 2002), suggests that Con-A shifts the relative contribution of the specific open state of the receptor (i.e., including the desensitized state) (Bowie *et al.*, 2003a). This mechanism fully explains the selective effect of Con-A on the steady-state response and the absence of effect on glutamate evoked peak responses, dose-response relationships as well as rates into and out of desensitization.

From a structural viewpoint, modulation of KA receptor responses by Con-A may be attributed to lectin binding to native (or ectopic) N-glycosylation sites located on the extracellular surface of the protein (Everts et al., 1997;Everts et al., 1999). Although the precise mechanism by which Con-A shifts the contribution of distinct open states has yet to be elucidated, the number of carbohydrate binding sites (Everts et al., 1997) and the lectin's valence (Gunther et al., 1973) are important factors in mediating this effect, suggesting that Con-A may also act by cross-linking distinct N-glycosylation sites.

Clearly, there is a real gap in KA receptor pharmacology. The identification of novel agonists is obviously required and further characterization of the allosteric modulators will play an important role in elucidating the gating properties of KA receptors. In light of the critical role of KA receptor in numerous physiological

processes and pathophysiological conditions, additional structure-activity studies will be pivotal in determining key parts of these ligands that are involved in interactions, which, in turn, will be central in designing clinically relevant compounds.

RATIONALE & OBJECTIVES

The aim of this thesis is to examine whether the basic properties of the KA-type iGluR are consistent with the closely related AMPA-type iGluR, as proposed from previous work (Weston *et al.*, 2006a;Lerma *et al.*, 1997). From the literature review, we derive three main issues motivating this field at the beginning of my PhD thesis. Briefly, the first major issue pertains to examining the conformational changes that occur in the agonist of an *intact* KA receptor. Accordingly, it is critical to develop a method to assess conformational changes in the intact KA receptor (**CHAPTER 1**). A second pressing issue relates to the lack of known agonists at KA receptors which has greatly hampered our understanding of KA receptors compared to other iGluR subfamilies. Hence, the identification of new ligands is pivotal to further advance this field (**CHAPTER 2**). In turn, this will allow us to examine the relationship (if any) between the conformation adopted by the ligand-binding domain of intact KA receptor structures and their relative agonist efficacy (**CHAPTER 2**), as performed for the AMPAR. The third outstanding issue to consider is the structural basis underlying the effects of external ions on KA receptor kinetics (**CHAPTER 3**). In order to address these issues, we have asked the following inter-related questions:

CHAPTER 1

DOES THE AGONIST-BINDING DOMAIN OF INTACT KA RECEPTORS ADOPT LIGAND-DEPENDENT CONFORMATIONS?

Crystal structures of the isolated ligand binding core of AMPA receptors confirmed that it operates as a clamshell structure which closes upon agonist binding (Stern-Bach *et al.*, 1994a;Jin *et al.*, 2003a). Comparison of the different crystal structures in complex with full and partial agonists led to the proposal that the extent of domain closure correlates with agonist efficacy (Jin *et al.*, 2003a). At the beginning of this work, in 2004, the structure of the agonist-binding domain of a KA receptor had not been crystallized, but its similarities to AMPA receptors suggest that they might also operate via a similar scheme. Given this and the

limitations of interpreting functional states from crystal structures (Madden, 2002), we examine whether the intact KA receptor also adopts agonist-dependent changes in its ligand-binding domain. To address this question, it appears crucial to develop a methodology to assess conformational changes in the agonist-binding in the full and intact KA receptor.

Here we propose to use the state-dependent properties observed with the plant lectin Con-A (Everts *et al.*, 1999;Fay & Bowie, 2003) to investigate if the conformational changes in the agonist-binding domain are dependent on the nature of the bound agonist. Interestingly, the effects of this lectin were state-dependent at insect muscle fibers (Mathers & Usherwood, 1976) and in oocytes injected with GluR6 cRNA (Everts et al., 1999), whereby Con-A preferentially modulated resting versus desensitized receptors suggesting that the state of the receptor determines the extent of Con-A binding (and modulation). Consistent with this, desensitization at AMPA receptors has been shown to induce important structural rearrangements (Sun et al., 2002) which may alter the accessibility of Con-A to its binding site and hence receptor activity at KA receptors. In light of this, we have used the state-dependence of Con-A to test if this property may be used to investigate conformational changes upon agonist binding. Moreover, another important question raised by these studies is if and how Con-A modulates the kinetics of other KA receptor agonists. Part of the work presented here aims to shed light on the mechanism and specificity of lectin modulation at KA receptors. To further examine the molecular explanation for this phenomenon, we also perform mutagenesis of key binding sites for the plant lectin.

For this results chapter and subsequent ones, we have expressed cloned KA or AMPA receptors in recombinant HEK 293cells, allowing us to study homomeric receptors of known composition in isolation. Moreover, to capture the rapid kinetics of KA receptors we have performed outside-out patch recordings using an ultra-fast drug application system.

CHAPTER 2:

DOES CLOSURE OF THE AGONIST-BINDING DOMAIN CORRELATE WITH AGONIST EFFICACY AT KA RECEPTORS?

While the extent of domain closure of the agonist-binding domain of closely-related AMPA receptors is thought to correlate with agonist efficacy, no such extensive study had been performed for KA receptors, although the expectation is that they will behave similarly. The paucity of KA receptor agonists has greatly hampered any attempt to investigate the relationship between the degree of closure and agonist efficacy. In light of this, the aim of this study is to identify and characterize the kinetic profile of novel amino acids acting at KA receptors which can then allow us to examine the correlation, if any, between these two parameters.

To do this, we use an electrophysiological assay as described above combined with the computational ligand docking program, FITTED (Corbeil *et al.*, 2007a). Importantly, this docking tool treats the ligand/protein as a realistically dynamic system while also accommodating for displaceable bridging water molecules (Corbeil *et al.*, 2007a;Moitessier *et al.*, 2006). Since the agonist-binding domain of KA receptors is known to adopt ligand-dependent conformations (**CHAPTER 1**) (Mayer, 2005a;Nanao *et al.*, 2005a) and to accommodate a number of surrogate water molecules, FITTED therefore provides the most appropriate docking approach to investigate iGluRs. With this in mind, we took advantage of the five KA receptor co-crystals resolved after the beginning of this work to validate the use of a ligand docking software to predict the preferred conformation adopted by the agonist-binding domain with the novel KA receptor agonists identified here. Finally, we use the methodology developed in **CHAPTER 1** to further assess the extent of conformational change elicited by the novel KA receptor agonists.

CHAPTER 3:

ARE EXTERNAL IONS AN ABSOLUTE REQUIREMENT FOR KA RECEPTOR ACTIVATION?

At the beginning of this work, an important line of evidence differentiating AMPA and KA receptors was that only the decay kinetics and response amplitude of the latter are regulated by external cations and anions (Bowie, 2002a). The underlying mechanism for this effect was unknown, but suggested that these two iGluR subfamilies may operate through distinct gating mechanisms as proposed from detailed kinetic characterization of their desensitization profiles (Bowie & Lange, 2002). Moreover, the methionine-770 (M770) residue in the S2 domain of GluR6 KA receptor has been shown to be important in conferring ion-sensitivity (Paternain et al., 2003). To examine the molecular basis of the effect of ions on KA receptors, we design experiments to determine if (1) ions regulate the basal gating properties of these receptors or (2) if they are an absolute requirement for KA receptor gating. To do so, we perform electrophysiological recordings in the absence and presence of different ions. Additionally, we test the effect of substituting the nonpolar M770 (Paternain et al., 2003) residue in GluR6 with its equivalent positively charged lysine residue, K752, in GluR1 AMPA receptors.

PART TWO

RESULTS CHAPTERS

CHAPTER 1:

DOES THE AGONIST-BINDING DOMAIN OF INTACT KAINATE RECEPTORS ADOPT LIGAND-DEPENDENT CONFORMATIONS?

PREFACE TO CHAPTER 1

At the beginning of my thesis, much of what we knew about KA receptor structure came from its more thoroughly studied counterpart, the AMPAR, since the crystal structure of its agonist binding domain had been available for some time (Armstrong et al., 1998). While writing the manuscript presented here, however, two separate studies reported co-crystals of the GluR6 KAR subunit with five different agonists (Mayer, 2005a;Nanao *et al.*, 2005a). These crystals have provided pivotal insights into the specific amino acid residues involved in ligand binding and reveal important similarities and differences with AMPARs. Importantly, the separation between the two lobes of the isolated S1S2 construct was also proposed to correspond positively with the extent of receptor activation at KARs (Mayer, 2005a;Nanao *et al.*, 2005a), as previously suggested for AMPARs (Jin *et al.*, 2003a).

Given this, an outstanding issue to resolve is whether the intact receptor also adopts agonist-dependent conformations consistent with the KNF model of allosteric proteins – thus raising the question: "What state of the receptor do these constructs represent?" In order to address these questions, we must be able to visualize or infer by comparison the conformation adopted by the agonist-binding domain of the receptors in different states, in the absence and presence of ligands with varying efficacies. Here, we develop a methodological approach **to investigate conformational changes in the agonist-binding domain of intact kainate receptors**. Using the plant lectin Concanavalin-A as a reporter molecule, we assess differences in the agonist-induced conformational changes in the agonist-binding domain of these receptors. Our study provides the first evidence of **ligand-specific conformations adopted by the receptor during desensitization at KA receptors**. This chapter was published as a research article in the Journal of Physiology (Fay & Bowie, 2006).

Article title:

Concanavalin-A reports agonist-induced conformational changes in the intact GluR6 kainate receptor

ABSTRACT

The agonist-binding domain of ionotropic glutamate receptors (iGluRs) has recently been crystallized as two polypeptide chains with a linker region. Although work on the structure of this isolated ligand-binding core has been invaluable, there is debate over how it relates to conformations adopted by intact receptors. iGluR crystals are proposed to represent the activated state as their degree of domain closure correlates well with agonist efficacy. However, iGluR crystals exhibit high agonist affinity that more closely matches that of desensitized receptors. Consequently, conformations adopted by iGluR crystals may represent this state. To test this, we have employed the plant lectin, concanavalin-A (Con-A) to report conformational changes elicited by kainate (KA) iGluR agonists during desensitization. When GluR6 KA receptors (KARs) were pre-incubated with Con-A, equilibrium responses evoked by the full agonist, L-glutamate (L-Glu), increased almost 30-fold. However, in the continued presence of L-Glu, Con-A exerted no effect suggesting that it has restricted access to its binding sites when the agonist is bound. However, Con-A does not discriminate well between agonist-bound or -unbound states with the weak partial agonist, domoate. Accessibility experiments with KA were intermediate in nature consistent with its equilibrium efficacy at GluR6 KARs. Our results suggest that full and partial agonists elicit distinct conformational changes in KARs during desensitization. This finding can be reconciled with crystallographic data if the agonist-binding domain adopts the same conformation in the activated and desensitized states. However, other interpretations are possible suggesting future work is required if this issue is to be resolved.

INTRODUCTION

The concept that agonist molecules act on allosteric proteins such as ligand-gated ion channels with different efficacy was first recognized almost 50 years ago (Ariens, 1954; Stephenson, 1956; del Castillo & Katz, 1957). At fully occupied receptors, agonists that elicit the maximum response are referred to as full agonists whereas partial agonists evoke submaximal responses. Two distinct models have been developed to account for agonist behaviour: the concerted (Monod et al. 1965) and multi-state (Koshland et al. 1958, 1966) models. In the concerted model, full and partial agonists evoke identical conformational changes in protein structure, but differ in their ability to activate channel openings. Nicotinic acetylcholine receptors (nAChRs) exemplify this behaviour since membrane currents elicited by full and partial nAChR agonists have identical single-channel conductance but differ in open-channel probability (Gardner et al. 1984). Moreover, this gating behaviour is widespread amongst other signalling proteins such as glycine, $GABA_A$ and NMDA receptors, as well as cyclic-nucleotide-gated channels (Zagotta & Siegelbaum, 1996; Colquhoun & Sivilotti, 2004; Lynch, 2004; Auerbach & Zhou, 2005). In the multi-state model, full and partial agonists elicit distinct conformational changes in protein structure. Contrary to the concerted model, single-channel recordings reveal that conformations in protein structure are governed by agonist concentration (Rosenmund et al. 1998; Smith & Howe, 2000) as well as agonist type (Swanson et al. 1997; Jin et al. 2003). Although few ligand-gated ion channels operate by this mechanism, recent work on the agonist-binding domain of AMPA and KA iGluRs has suggested that their agonist behaviour is best described by this model.

Detailed X-ray analysis of iGluR subtypes has been possible since their agonist-binding domains can be reconstituted as two polypeptide chains using a linker peptide to replace transmembrane regions (Armstrong et al. 1998; Furukawa & Gouaux, 2003; Mayer, 2005). From work on AMPA iGluRs, it is proposed that agonist binding promotes closure of the isolated ligand-binding core which in the intact receptor would lead to channel opening (Armstrong et al. 1998; Armstrong & Gouaux, 2000). Therefore, conformations adopted by the

isolated ligand-binding core are understood to represent the activated state. In support of this, full and partial AMPAR agonists elicit complete and partial cleft closure, respectively, correlating well with agonist efficacy (Armstrong et al. 2003; Jin et al. 2003). Ligand-binding constructs of KAR iGluRs apparently behave similarly since full and partial agonists also promote distinct conformations (Mayer, 2005) consistent with the multi-state model already proposed from functional analysis of intact KARs (Bowie & Lange, 2002; Swanson et al. 2002). However, a potential caveat is that unitary current measurements indicate that single AMPA and KAR activations are short-lived, lasting only a few milliseconds (Swanson et al. 1996, 1997; Howe, 1996). Consequently, X-ray crystal structures may represent another protein conformation that is more thermodynamically stable, such as the desensitized state(s).

Here we have characterized the state-dependent modulation of GluR6 KARs by Con-A. Previous work from our laboratory has established that this plant lectin selectively regulates desensitized GluR6 receptors (Bowie et al. 2003). We have used this property of Con-A to test if full and partial agonists elicit distinct conformations in the extracellular domain of intact GluR6 KARs during desensitization. In agreement with recent work on GluR6 crystal structures, we show that different agonists evoke distinct conformations in intact receptors. This finding further establishes that agonist efficacy at KARs is best explained by a multi-state model. Our observations on desensitized channels can be reconciled with crystallographical data if the activated and desensitized states adopt comparable conformations. However, as discussed below, alternative interpretations are possible suggesting that future structure–function analysis of KA iGluRs must address this issue.

MATERIALS & METHODS

Cell culture and transfection

Techniques used to culture and transfect mammalian cells to express GluR6 KARs have already been described in detail elsewhere (Bowie, 2002, 2003; Bowie & Lange, 2002). Briefly, tsA201 cells, a transformed human kidney (HEK 293) cell line stably expressing on SV40 temperature sensitive T antigen (provided by R. Horn, Jefferson Medical College, PA, USA) were maintained at a confluency of 70–80% in minimal essential medium with Earle's salts, 2 mM glutamine and 10% fetal bovine serum supplemented with penicillin (100 units ml^{-1}) and streptomycin (100 μg ml^{-1}). After plating at low density (2×10^4 cells ml^{-1}) on plastic dishes, cells were transfected with cDNA encoding unedited wild-type glutamate receptor subunit 6 (GluR6Q) or mutant GluR6Q receptor subunits using the calcium phosphate technique as previously described (Bowie et al. 1998). The cDNA for enhanced green fluorescent protein (EGFP S65T mutant) was routinely cotransfected to help identify transfected cells.

Site-directed mutagenesis

Mutation of N-glycosylated residues located in close proximity to the agonist-binding domain of GluR6 KARs was performed to disrupt lectin modulation (Fig. 1.4). To generate mutants, three of the N-glycosylated consensus sites (N-X-S/T, where $X \neq P$) in the GluR6 sequence were changed from an S/T to an A and will be referred to as GluR6(Q)ΔNG5,6,7 according to the nomenclature of Everts et al. (1999) (Fig. 1.4A). Alanine substitutions of T414 (NG5), T425 (NG6) and S432 (NG7) were performed in two steps using the Quickchange II XL site-directed mutagenesis kit (Stratagene, La Jolla, CA, USA) using PfuUltra DNA polymerase and custom primers (Alpha DNA, Montreal, Quebec, Canada). Mutant cDNAs were amplified using XL10-Gold ultra-competent cells (Stratagene), purified with the QIAprep Spin Miniprep kit (Qiagen Inc., Mississauga, Ontario, Canada), initially identified by restriction digest using BamH I or Sac I (New England Biolabs, Beverly, MA, USA) and later confirmed by automated sequencing (McGill University and Genome Quebec Innovation

Center, Montreal, Quebec, Canada). To obtain larger quantities of mutant cDNA, GluR6 mutants were amplified in bacterial cultures (Top10 cells, Invitrogen) and the cDNA purified using QIAfilter Maxiprep kits (Qiagen Inc.).

Electrophysiological solutions and techniques

Excitatory amino acid agonists were dissolved in external solutions containing 150 mM NaCl, 5 mM Hepes and 0.1 mM each of $CaCl_2$ and $MgCl_2$. All concentrated agonist stocks were adjusted to pH 7.3 with NaOH before being stored at −20°C. Saturating agonist concentrations chosen for L-glutamate (10 mM), KA (1 mM) and domoate (50 μM) were at least 5-fold larger than published EC_{50} values at GluR6 receptors (Köhler et al. 1993; Tygesen et al. 1994; Jones et al. 1997; Donevan et al. 1998; Bowie, 2002; Alt et al. 2004). We empirically confirmed that these concentrations were saturating by doubling the agonist concentration in each case and observing that peak response amplitudes were unchanged. The internal solution was composed of 115 mM NaCl, 10 mM NaF, 5 mM Hepes, 5 mM Na_4BAPTA, 0.5 mM $CaCl_2$, 1 mM $MgCl_2$ and 10 mM Na_2ATP to chelate endogenous polyamines (Bähring et al. 1997; Bowie et al. 1998). The pH and osmolarity of internal and external solutions were adjusted to 7.3 and 295 mosmol l−1, respectively. Con-A and succinyl Con-A (Sigma, St Louis, MO, USA) were prepared in glucose-free saline solution and filtered (0.2 μm filter, Corning) immediately before use as previously described (Bowie et al. 2003). All recordings were performed with an Axopatch 200B amplifier (Axon Instruments Inc., CA, USA) using thin-walled borosilicate glass pipettes (2–5 MΩ) coated with dental wax to reduce electrical noise. Control and agonist solutions were rapidly applied to outside-out patches excised from transfected tsA201 cells as previously described (Bowie et al. 1998, 2002; Bowie & Lange, 2002). Solution exchange (10–90% rise time = 25–50 μs) was determined routinely at the end of the experiment by measuring the liquid junction current (or exchange current) between the control and agonist-containing solution in which total Na^+ content was reduced by 5%. Current records were filtered at 5 kHz, digitized at 25–50 kHz and series resistances (3–10 MΩ) compensated by 95%. Recordings were performed at −20 mV membrane potential to ensure adequate

voltage clamp control of peak currents. Data acquisition was performed using pCLAMP9 software (Axon Instruments Inc.). All experiments were performed at room temperature.

RESULTS

iGluR molecular rearrangements and structural information has been inferred from the state-dependent behaviour of a number of pharmacological agents including channel blockers (Benveniste & Mayer, 1995; Bähring & Mayer, 1998; Bowie et al. 1998) and the accessibility of substituted cysteine residues (Kuner et al. 1996, 2001). At KARs, the binding and modulatory effect of Con-A is also state dependent (Everts et al. 1999). We speculated that this property may be useful in probing gating conformations elicited by full and partial KAR agonists. Therefore, our initial experiments were designed to further characterize the nature of state-dependent modulation of KARs by Con-A.

Con-A modulation of GluR6 KARs is state dependent

Previous work on invertebrate iGluRs has suggested that Con-A binding sites are masked during desensitization (Evans & Usherwood, 1985) whereas more recent studies on mammalian GluR6 receptors has proposed that binding can occur (Everts et al. 1999). However, in the latter study the authors did not exclude the possibility that incubation with desensitizing concentrations of agonists still permit Con-A to bind to GluR6 receptors recycling through the open state (Everts et al. 1999). In such conditions, recycling through the open state would occur with low probability and the onset of Con-A's effects would develop slowly. Since the authors did not determine the time course of modulation (Everts et al. 1999), it is possible that their observations reflect binding to open rather than desensitized channels.

To determine if Con-A is able to bind to desensitized states, GluR6 receptors were stimulated at two frequencies, 0.067 (every 15 s) and 0.33 (every 3 s) Hz, to vary the fraction of desensitized receptors. The time course of Con-A modulation was then compared at each frequency. Figure 1.1A and B shows typical patch recordings where the development of Con-A effects was compared using multiple applications of 10 mM L-glutamate (L-Glu, 250 ms duration, holding potential (V_h) =−20 mV) every 15 s or 3 s, respectively. In each case, L-Glu evoked a rapidly rising inward membrane current that desensitized in the continued presence of the agonist to reach a steady-state level. At 0.067 Hz, GluR6 channels

recover fully from desensitization between agonist applications (Bowie & Lange, 2002) whereas at 0.33 Hz, 50–60% of the peak response is desensitized. Consequently, the peak agonist response at 0.067 Hz was unchanged (Fig. 1.1A), whereas at 0.33 Hz, the peak amplitude initially declined by almost 60% before a new peak level was established (Fig. 1.1B, see arrow). When peak amplitudes stabilized during a recording, the outside-out patch was treated with 10 μM Con-A as previously described (Bowie et al. 2003).

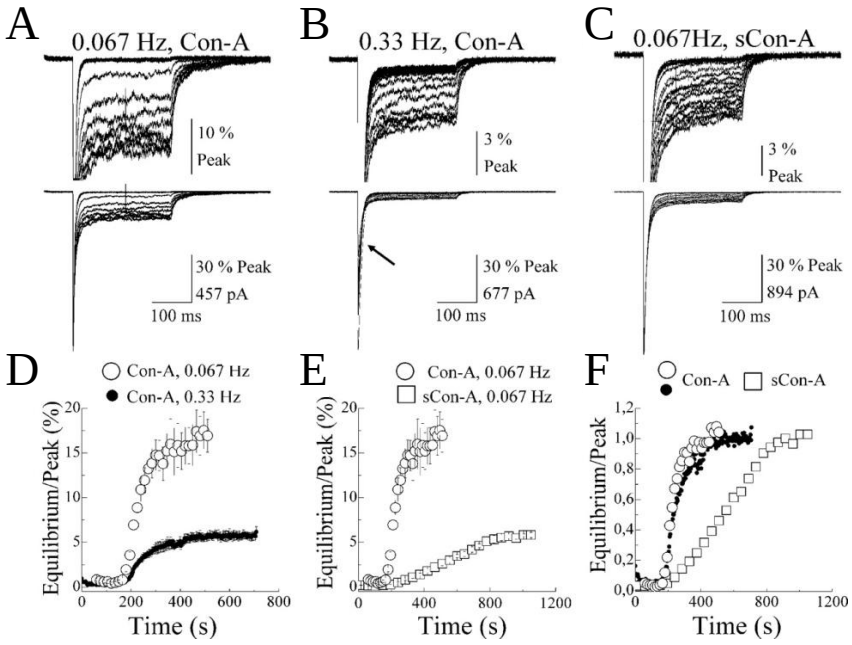


Figure 1.1 Determining the time course for lectin modulation of GluR6 receptors. A–C, time course for the onset of lectin modulation was determined by stimulating GluR6 receptors with 10 mM Glu (250 ms, V_h=−20 mV) every 15 s (A and C, 0.067 Hz patch numbers, 010327p2 and 010712p1) or 3 s (B, 0.33 Hz patch number, 010816p6) in the continuous presence of Con-A or succinyl Con-A (sCon-A). In each case, baseline control responses were first established before each patch was treated until a maximal effect on the equilibrium response was observed. Note, peak responses shown in B initially declined in amplitude when GluR6 receptors were stimulated at 0.33 Hz (see arrow). This effect, due to the onset of desensitization, was permitted to reach equilibrium before the patch was treated with Con-A. D, summary plot showing the development of modulation by Con-A of GluR6 receptors activated every 15 s (○, n= 8) or 3 s (•, n= 10). In each case, the rate of onset was similar, but the degree of modulation differed by more than 3-fold. E, plot comparing the time course for the onset of modulation by sCon-A (□, n= 4) and Con-A (○, n= 10) on GluR6 responses stimulated at 0.067 Hz. F, plot summarizing the data shown in D and E. In each case, the data were normalized to allow comparison between the onset of modulation at different stimulation frequencies and between different lectins. All data are expressed as mean ± S.E.M.

At both stimulation frequencies, Con-A did not significantly affect the peak amplitude but irreversibly potentiated the level of the equilibrium response (Fig. 1.1A and B). Since Con-A binding is irreversible, binding sites are saturated at any concentration where the total number of Con-A molecules is greater than or equal to the number of binding sites. In view of this, Con-A treatment modifies all GluR6 channels in each patch recording. At both stimulation frequencies, the time course for the onset of Con-A modulation reached a maximal effect after approximately 2–3 min of treatment (Fig. 1.1D–F). However, the effectiveness of Con-A on the equilibrium response was dependent on the stimulation frequency (Fig. 1.1D). The equilibrium responses observed at 0.067 and 0.33 Hz were 17.2 ± 2.1% (○, n= 8) and 5.7 ± 0.5% (●, n= 10) of the peak, respectively, representing a 3-fold difference in the effectiveness of Con-A (Fig. 1.1D). Taken together, these observations are not consistent with Con-A binding sites being masked by desensitization (Evans & Usherwood, 1985) since this mechanism would predict equi-effectiveness of Con-A at both stimulation rates but with a slower time course at 0.33 Hz. To account for the different degree of modulation, we propose that the number of glycosylated residues available for Con-A binding is restricted by desensitization.

Consistent with this, when we compared the rate and degree of modulation of GluR6 receptors with the lectin dimer, succinyl Con-A (sCon-A) (Gunther et al. 1973), the rate of onset was slower and the degree of modulation was less (□, 5.7 ± 0.5% Peak, n= 5) (Fig 1.1C, E and F). Since Con-A and sCon-A possess a different number of carbohydrate binding sites, it is likely that differences in stoichiometry sterically hinder binding and/or cross-linking events essential for modulating GluR6 receptors. However, these initial experiments do not exclude the possibility that different modulatory effects of Con-A at 0.067 and 0.33 Hz reflect binding to the open state rather than the desensitized state. Experiments described below and illustrated in Fig. 1.2 resolve this issue.

Con-A modulation of GluR6 KARs is a multi-step process

Figure 1.2 shows the experimental protocol used to determine if Con-A binds to desensitized GluR6 receptors. In each experiment, control responses to 10 mM

L-Glu (250 ms duration) were measured to establish the baseline amplitude of the equilibrium response (Fig. 1.2A, left panel). During the second, longer application of L-Glu (2–3 min), Con-A was continuously co-applied to the equilibrium response for a period previously shown to fully modulate GluR6 receptors (Bowie et al. 2003) (Fig. 1.2A, middle panel). The effect of Con-A was then determined by comparing the amplitude of the equilibrium responses at the beginning (Fig. 1.2A, △) and end of the treatment period (Fig. 1.2A, ▲).

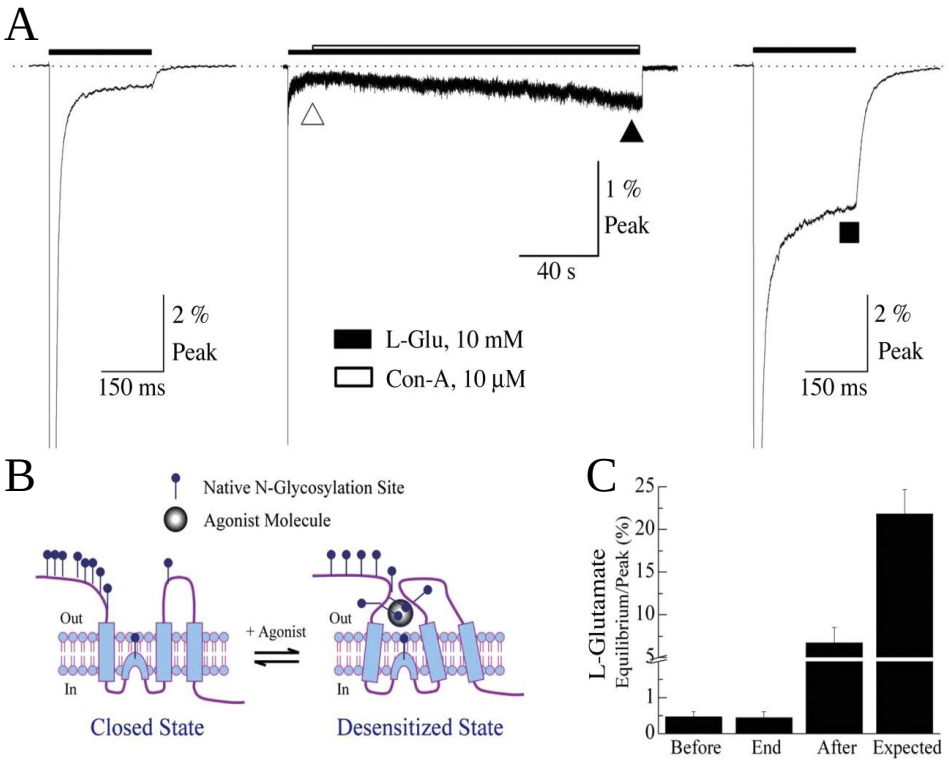


Figure 1.2 Con-A modulation of GluR6 receptors is a multi-step process. A, typical experiment showing the effect of Con-A when applied to predominantly desensitized channels. l-Glu (10 mM) was applied before (▽), during (▲) and after (▪) extensive Con-A treatment (200 s, V_h=−20 mV) to monitor changes in the equilibrium response amplitude (patch number, 010817p6). The filled and open bars indicate the application period of 10 mM Glu and 10 µM Con-A, respectively. The dotted line denotes the zero current level. The first and third applications of 10 mm Glu had a duration of 250 ms. B, schematic diagram illustrating how agonist-binding may prevent access of Con-A to a subset of N-glycosylated residues in the vicinity of the agonist-binding domain. C, summary plot of data from several patches (n= 6) where the amplitude of the equilibrium response was compared at various time points as exemplified by the experiment shown in A. The values for 'Expected' were taken from data shown in Fig. 1.3C. All data are expressed as the mean ± S.E.M.

Interestingly, measurement of the equilibrium response at these two time points revealed that Con-A had almost no effect on equilibrium desensitization (End (▲): 0.44 ± 0.14% Peak, n= 5) when compared to control levels (Before (△): 0.47 ± 0.17% Peak, n= 6) (Fig. 1.2C). Similar results were also observed when patches were co-treated with L-Glu and Con-A for longer periods (e.g. > 5 min).

The lack of effect of Con-A on desensitized GluR6 receptors suggests one of two possibilities. Firstly, lectin binding sites are masked by conformational events that occur during desensitization as suggested from work on invertebrate iGluRs (Evans & Usherwood, 1985). Alternatively, Con-A binding may have occurred but modulation requires an additional conformational step not permissible whilst receptors are desensitized (Fig. 1.2B). To distinguish between these two possibilities, co-treatment of the patch with 10 μM Con-A and agonist was terminated. The receptors were then allowed to fully recover from desensitization and a third 250 ms application of only 10 mM L-Glu was applied (Fig. 1.2A, right panel). Surprisingly, without subsequent Con-A treatment, the equilibrium response increased 14- to 15-fold to 6.74 ± 1.76% of the peak (n= 4) (Fig. 1.2A,■). The increase in the equilibrium response represents only 30% of the modulation observed when GluR6 receptors were treated in the absence of agonist (Fig. 1.2C, Expected: 21.8 ± 2.9% Peak, n= 29). This experiment suggests that Con-A binds to desensitized channels but requires an additional step, involving agonist dissociation, before modulation is observed. It is unlikely that Con-A binds to GluR6 receptors recycling through the open state since the degree of modulation observed with the third L-Glu application is too large. Having established that Con-A can report agonist-induced conformations, we hypothesized that this behaviour may be useful in comparing structural changes evoked by full and partial agonists.

Con-A modulation of GluR6 KARs is agonist dependent

We initially compared the response profile of three structurally related agonist molecules recently crystallized in complex with the GluR6 KAR ligand-binding core (Mayer, 2005; Nanao et al. 2005). Figure 1.3A and B shows typical membrane currents evoked by rapid application of saturating concentrations of L-

Glu (10 mm), KA (1 mm) and domoate (Dom, 50 μm) in the same patch recording before and after treatment with Con-A. Prior to Con-A treatment, peak responses to KA and Dom were 44.9 ± 2.2% (n= 13) and 12.6 ± 3.8% (n= 8), respectively, of the L-Glu response (n= 13) (Fig. 1.3A) confirming that KA and Dom are partial agonists at GluR6 KARs. Although, Con-A increased the amplitude of the equilibrium response for all three agonists, the degree of modulation was agonist specific (Fig. 1.3A–C).

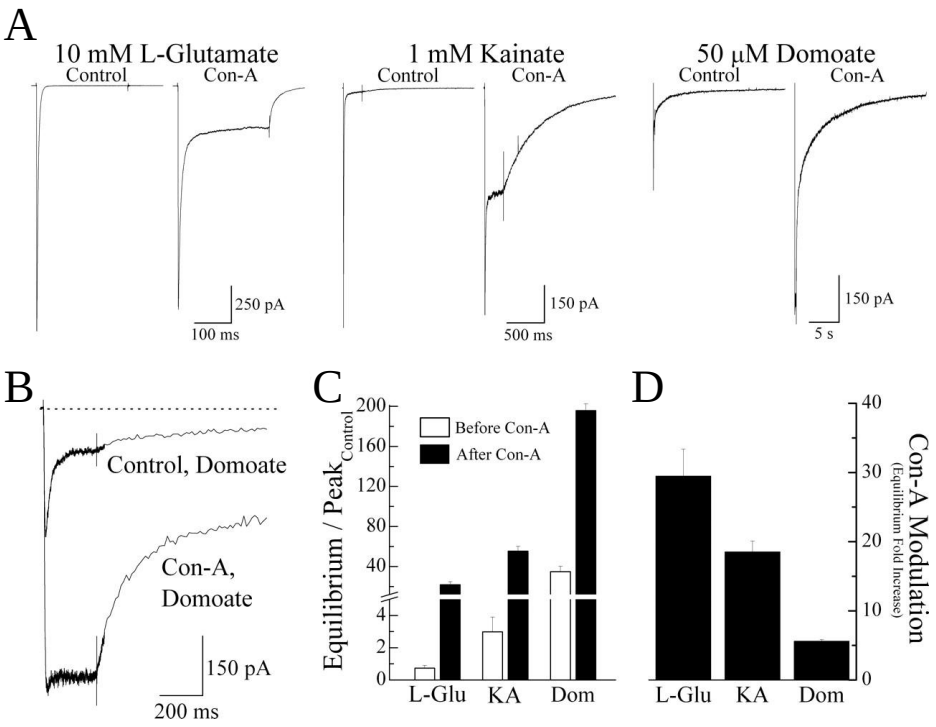


Figure 1.3 Modulation of GluR6 receptors by Con-A is agonist dependent. A, typical membrane currents (250 ms duration, V_h=−20 mV) elicited in the same patch by 10 mM l-Glu, 1 mM KA and 50 μM Dom before and after treatment with 10 μM Con-A (patch number, 030724p2). B, to show the early phase of the Dom response in more detail, agonist-evoked membrane currents prior to and after incubation with Con-A were superimposed. C and D, bar graphs summarizing the effect of Con-A treatment on equilibrium responses (C) and comparing its effects on different KA receptor agonists (D). All data are expressed as the mean ± S.E.M.

For example, the equilibrium response elicited by L-Glu increased 30-fold from an equilibrium/peak ratio of 0.74 ± 0.16% in control conditions to 21.8 ± 2.9% (n= 29) following Con-A treatment (Fig. 1.3C). In contrast, equilibrium/peak ratio

for Dom was 34.8 ± 5.4% (n= 15) in the control response compared to 195.7 ± 6.8% (n= 3) after Con-A treatment, representing a 5-fold change. Finally, consistent with the rank order of agonist efficacy observed in control conditions, modulation by Con-A of equilibrium KA responses was intermediate (Fig. 1.3D).

GluR6 equilibrium responses depend on the summed contribution of several subconductance states (Swanson et al. 1996; Howe, 1996) whose relative proportions may vary with full and partial agonists as recently proposed for AMPA receptors (Jin et al. 2003). We have shown that Con-A's effect on L-Glu responses is due to the up-regulation of a subset of conductance states (Bowie et al. 2003). Consequently, it is likely that Con-A affects KA and Dom equilibrium responses by modulating a different combination of subconductance levels. From a structural standpoint, irreversible binding of Con-A to N-glycosylated residues (Everts et al. 1997, 1999) may restrict conformational changes to a number of regions in the mature protein including the dimer interface, pore region or agonist-binding domain. Movement of the dimer interface governs the rate at which GluR6 receptors desensitize (Bowie & Lange, 2002; Horning & Mayer, 2004). Since Con-A does not affect GluR6 desensitization kinetics (Bowie et al. 2003) it is unlikely that lectin binding influences dimer–dimer interactions. Furthermore, Everts et al. (1999) have shown that N-glycosylated residues important for lectin binding are distant from the pore region (see Discussion) but located in and around the agonist-binding domain. Consequently, Con-A is unlikely to influence the pore region directly but may restrict conformations within the agonist-binding domain. To provide further experimental support for this, we performed mutational analysis of three N-glycosylated amino acid residues in close proximity to the agonist-binding domain.

Mutation of Con-A binding sites in close proximity to the agonist-binding domain

Alanine substitution of three amino acid residues, T414A, T425A and S432A (Fig. 1.4A) was made since previous work had established that each residue was critical for Con-A modulation (Everts et al. 1999). The triple mutant will be referred to as GluR6(Q)ΔNG5,6,7 according to the nomenclature of Everts et al.

(1999). We hypothesized that if full and partial agonists elicit distinct conformational changes during desensitization, the disruption of Con-A modulation by Con-A would be agonist specific.

Figure 1.4 compares experiments where wild-type and mutant GluR6 receptors were modulated by Con-A. As expected from previous work (Everts et al. 1997), removal of N-glycosylated residues did not significantly affect surface expression or the response profile of GluR6 receptor agonists (Fig. 1.4B). However, we did observe some variation in the Dom response. The majority of patches containing wild-type or GluR6(Q)ΔNG5,6,7 receptors exhibited a sustained response to Dom (Fig. 1.4B, right panel), but in some cases, the onset of desensitization was evident (Fig. 1.4B, left panel).

This observation was labile in nature only appearing during the first but not subsequent applications of Dom making it difficult to study. Here, we have included both response types in our dataset since modulation by Con-A was indistinguishable. Compared to wild-type receptors, Con-A was less effective in modulating responses elicited by agonists acting on GluR6(Q)ΔNG5,6,7 receptors (Fig. 1.4B and C). Moreover, this disruption was agonist dependent. For example, Dom responses were rendered almost insensitive to treatment by Con-A in the triple mutant. The equilibrium/peak ratio observed after lectin treatment was only modestly increased compared to the equilibrium/peak ratio prior to Con-A (102.4 ± 6.8%, n= 5 and 84.2 ± 8.6%, n= 5, respectively). With L-Glu, equilibrium responses elicited by mutant receptors increased 15-fold (Fig. 1.4C) after Con-A treatment compared to the 30-fold increase observed in wild-type GluR6 (Fig. 1.3D). Finally, disruption of the modulation of KA responses was intermediate (Fig. 1.4C) consistent with the hypothesis that Con-A can be used to compare conformations elicited by agonists with different efficacies.

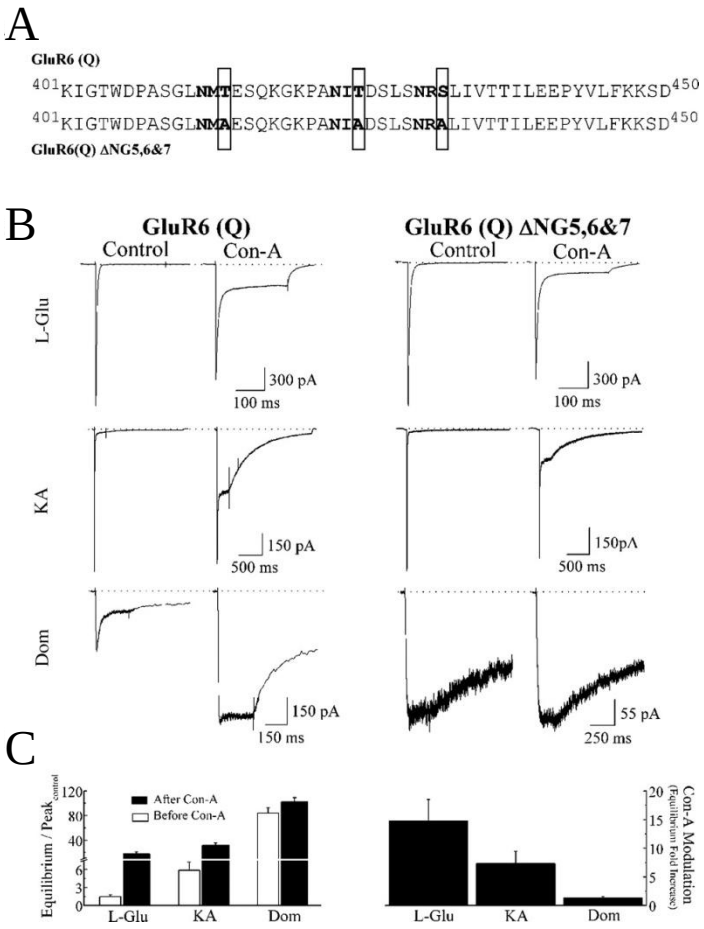


Figure 1.4 Disruption of Con-A binding sites interferes with Con-A modulation. A, amino acid sequence alignment of wild-type GluR6 and GluR6 (Q) ΔNG5,6 & 7 showing three N-glycosylation consensus sites (N-X-S/T, X ≠ P) highlighted in bold. Disruption of Con-A binding was achieved by replacing threonine (T) or serine (S) residues at these sites with alanines (A), as highlighted by grey boxes. B, comparison of the membrane currents evoked by l-Glu, KA and Dom at wild-type (patch number, 030724p2) and mutant (patch numbers, 041015p2 and 041008p2) GluR6 receptors before and after treatment with 10 μM Con-A. Although agonist responses evoked by wild-type and GluR6 ΔNG5,6,7 channels were comparable, the degree of modulation by Con-A was different. C: left panel, summary plot comparing the amplitude of the equilibrium response for GluR6 ΔNG5,6,7 channels with each agonist before and after treatment with Con-A; right panel, bar graph showing that the degree of modulation by Con-A of GluR6 ΔNG5,6,7 is agonist dependent but less than observed with wild-type receptors (cf. Fig. 1.3D).

GluR6 agonists promote distinct conformational changes to intact KARs

To test if agonists cause distinct conformational changes during desensitization, we repeated experiments shown in Fig. 1.2 using prolonged applications of the partial agonists, KA and Dom (Fig. 1.5). Figure 1.5A and C

shows representative experiments where treatment with Con-A was initiated only after responses evoked by 1 mM KA or 50 μM Dom reached equilibrium levels. As previously described (cf. Fig. 1.2), the amplitude of the equilibrium response before and at the end of treatment with Con-A was compared to assess lectin accessibility to the N-glycosylated sites (Fig. 1.5B and D).

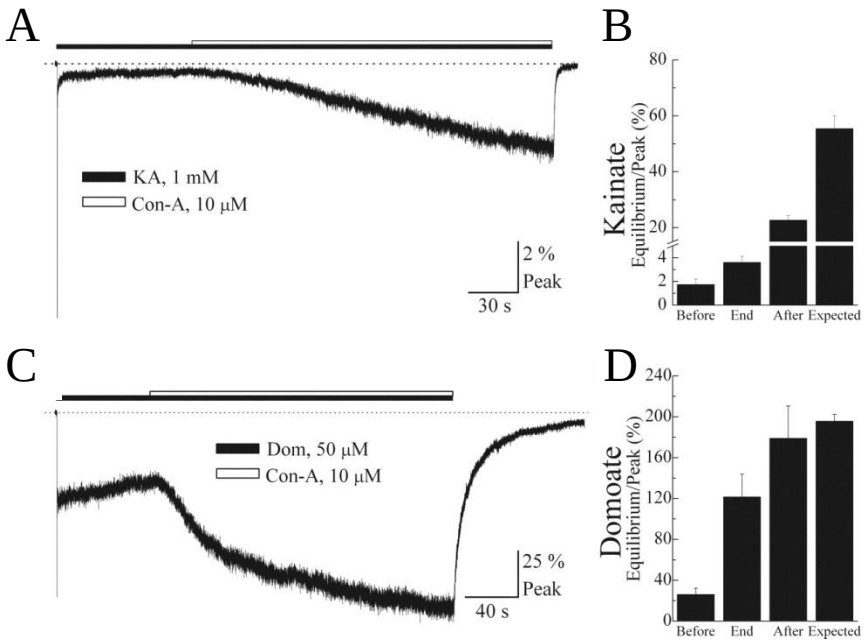


Figure 1.5 Accessibility of Con-A to its binding sites is increased by partial agonists. A and C, typical patch experiments where the effect of Con-A (10 μM) on the equilibrium response evoked by 1 mM KA (patch number, 031118p2) or 50 μM Dom (patch number, 031111p2) was tested. Filled and open bars indicate the application period of agonist and Con-A, respectively, and the dotted line denotes zero current level. Note that, unlike l-Glu, Con-A was able to modulate equilibrium responses elicited by each partial agonist. B and D, summary bar graphs showing the amplitude of the equilibrium response at various time points as described in Fig. 1.2. All data are expressed as the mean ± S.E.M.

Unlike the full agonist L-Glu, Con-A modulated the equilibrium response elicited by partial agonists, KA and Dom (Fig. 1.5A and C). Moreover, it was possible to distinguish between partial agonists since the degree of Con-A modulation observed with Dom was greater than with KA. For example, Con-A treatment increased the equilibrium response (Before: 1.73 ± 0.47% Peak) evoked by KA approximately 2-fold when GluR6 receptors were pre-desensitized with the agonist (End: 3.60 ± 0.51% Peak) compared to an increase of 20- to 30-fold (Expected: 55.4 ± 4.6% Peak) when GluR6 receptors were treated in the absence

of agonist (Figs 1.3D and 1.5B). In comparison, conformational events elicited by Dom only moderately restricted Con-A's accessibility. Here, Con-A increased the equilibrium response 5-fold on Dom-bound GluR6 receptors and approximately 6- to 7-fold when the agonist was absent (cf. Figs 1.3D and 1.5D). It is unlikely that these observations reflect Con-A modulating channels recycling through the open state since this mechanism would predict a greater effect on L-Glu responses than on KA or Dom responses. Indeed, our observations report the opposite effect where Con-A has a greater effect on equilibrium responses elicited by Dom or KA when compared to L-Glu (cf. Figs 1.2 and 1.5). It is also improbable that Con-A binds to resting channels since GluR6 receptors would be fully bound due to the saturating agonist concentrations used in these experiments. Taken together, these observations are in agreement with recent crystallographic data (Mayer, 2005) showing that partial agonists promote less closure of the agonist-binding domain than full agonists.

Figure 1.6 summarizes our results with Con-A in the presence and absence of full and partial agonists. Using the full agonist, L-Glu, Con-A's effect was strictly state dependent since the degree of modulation of the equilibrium response was dependent on whether GluR6 receptors adopted an agonist-bound (Co-Application: (0.92 ± 0.3)-fold increase) or unbound conformation (Pre-Incubation: (29.49 ± 3.9)-fold increase) (Fig. 1.6A). These two measurements were used to calculate an accessibility index ratio of 0.03 for L-Glu (Fig. 1.6B) which is consistent with crystallographic data (Mayer, 2005; Nanao et al. 2005). This finding also demonstrates that the small equilibrium response elicited by L-Glu at equilibrium (Fig. 1.3A) is associated with substantial conformational changes in the agonist-binding domain. In contrast, with the weak partial agonist Dom, the degree of Con-A modulation was similar whether lectin treatment occurred in the presence (Co-Application: (4.2 ± 1.0)-fold increase) or absence of agonist (Pre-Incubation: (5.62 ± 0.2)-fold increase) (Fig. 1.6A). In this case, the accessibility index ratio of 0.75 indicates that partial agonists promote weaker conformational changes upon binding which is associated with larger equilibrium responses (Fig. 1.3A and B).

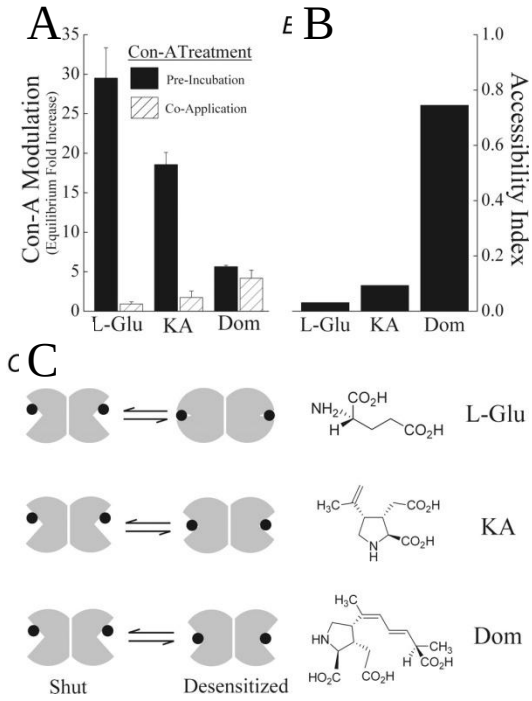


Figure 1.6 Multi-state model accounts for agonist behaviour at GluR6 receptors. A, summary plot comparing the effect of Con-A on the equilibrium response evoked by each agonist, either following a period of treatment with Con-A alone (filled bars) or in the presence of agonist (hatched bars). Note that although modulation by Con-A is state dependent with l-Glu, Con-A discriminates poorly between ligand-bound or ligand-free states with Dom. The plot is constructed using data in Figs 1.2, 1.3 and 1.5. All data are expressed as the mean ± S.E.M. B, data from A were used to determine an accessibility index as described in Results. C, schematic diagram illustrating that full and partial agonists promote distinct conformational changes in the agonist-binding domain of GluR6 receptors. The extended molecular structure of each agonist is shown opposite revealing that L-Glu is the most compact, and domoate is the most bulky in nature.

As expected, the accessibility index for KA (0.09) is consistent with its intermediary behaviour compared to full and weaker partial agonists (Fig. 1.6B). Interestingly, structural comparison revealed that Dom was the most bulky and L-Glu the most compact (Fig. 1.6C) suggesting that the physical nature of the agonist molecule may place constraints on the extent of domain closure. Taken together, these results suggest that the efficacy of full and partial agonists at equilibrium (Fig. 1.3) reflect distinct conformational changes in the agonist-binding domain of intact GluR6 KARs.

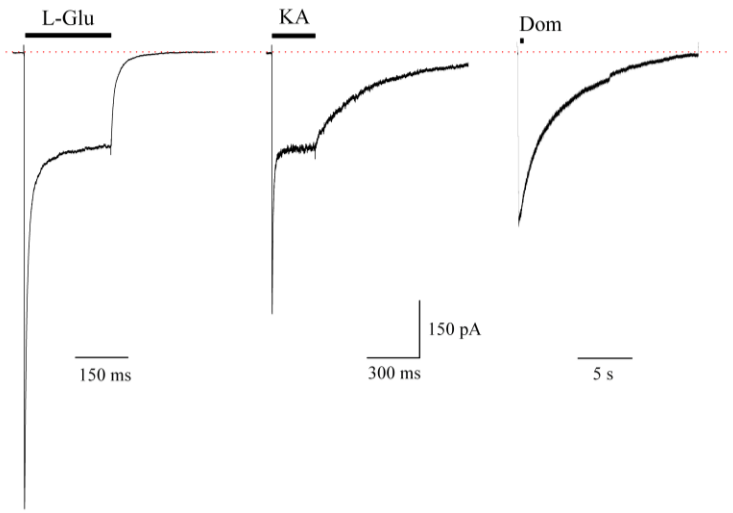
DISCUSSION

We show that Con-A can be employed to report agonist-induced conformational changes in the extracellular portion of intact GluR6 KARs. As discussed below, the most parsimonious explanation for our observations is that Con-A reports structural alterations in the agonist-binding domain. Crystallographical studies have not been able to provide structural information on the entire KAR due to technical considerations. Therefore, agonist behaviour has been examined by reconstituting the agonist-binding domain as two polypeptide chains with a linker domain. In agreement with reports describing KAR crystal structures, we show that GluR6 receptors adopt distinct conformations in the ligand-bound and unbound states. Moreover, the state-dependence of Con-A modulation is agonist-specific suggesting that full and partial agonists elicit distinct conformational changes in the agonist-binding domain during desensitization. Crystal structures of AMPA and KA receptors are thought to represent the activated state of the receptor since the extent of closure in the isolated ligand-binding core correlates with agonist efficacy. However, as addressed below, correlating conformational changes in this structure to functional properties of intact iGluRs remains an unresolved issue.

Comparison with previous studies

Although Con-A has been employed extensively as a pharmacological tool (Mayer & Vyklicky, 1989; Huettner, 1990; Wong & Mayer, 1993; Yue et al. 1995; Everts et al. 1997, 1999; Paternain et al. 1998), the state-dependence of its effects have not been examined in detail. State-dependent binding of Con-A was first observed in invertebrate iGluRs where Con-A-mediated effects (Mathers & Usherwood, 1976) were ineffective on desensitized channels (Evans & Usherwood, 1985). The authors concluded that structural rearrangements during desensitization masked carbohydrate moieties essential for Con-A binding (Evans & Usherwood, 1985). Since then, Con-A effects on mammalian GluR6 KARs have been documented (Yue et al. 1995; Everts et al. 1997; Paternain et al. 1998; Lerma et al. 2001) although state-dependent modulation has been described to a

much lesser extent (Everts et al. 1999). This may reflect the difficulty in comparing observations with Con-A between different laboratories. For example, a significant variability in the potentiation of GluR6 KARs by Con-A has been reported in the literature ranging from 30- to 150-fold (Partin et al. 1993; Yue et al. 1995; Bowie et al. 2003) to 5000- to 6000-fold change (Everts et al. 1997, 1999). The reason for these differences is not clear but it does not reflect the electrophysiological recording techniques used (e.g. whole-cell versus patch) or the surrogate expression system (e.g. oocyte versus mammalian cell) chosen to study recombinant GluR6 receptors. In support of this, in separate experiments where we treated KARs with Con-A before or after excising patches, the degree of Con-A modulation was indistinguishable (Supplementary Figure S1).



S1: Supplementary Figure 1. Whole-cell recording following Con-A treatment. Typical whole-cell membrane currents (250 ms, V_h = -20mV) elicited in the same cell by 10 mM L-Glu, 1 mM KA and 50 μM Dom after Con-A treatment. Note that the, the degree of Con-A modulation was indistinguishable from experiments using excised patches also expressing GluR6 KA receptors (c.f. Figure 1.3).

Based on previous work, there are two possible explanations to account for Con-A's modulatory effect on equilibrium responses evoked by full and partial agonists (cf. Fig. 1.3). The first possibility is that Con-A blocks the onset of desensitization (Huettner, 1990; Partin et al. 1993; Wong & Mayer, 1993; Yue et al. 1995; Everts et al. 1997, 1999; Wilding & Huettner, 1997; Paternain et al.

1998). As a result, the potentiation of equilibrium responses evoked by strongly desensitizing agonists (e.g. L-Glu) would be expected to be greater than weakly desensitizing agonists (e.g. Dom). This explanation is unlikely, however, as there is no direct experimental evidence to support a mechanism whereby Con-A blocks entry into the desensitized state (Bowie et al. 2003). Previous studies had reached the conclusion that Con-A blocked desensitization based on the finding that lectin treatment eliminated the desensitization observed in whole-cell recordings. However, an important caveat in all of this work was that the rate of agonist perfusion used was too slow to accurately resolve the gating properties of GluR6 KARs (Bowie et al. 2003). Consequently, peak agonist responses were significantly underestimated in these studies. When experiments are performed in faster perfusion conditions, rates into and out of the desensitized state are unaffected by lectin binding (Bowie et al. 2003) demonstrating that Con-A does not block desensitization. The second possibility is based on the mechanism proposed by Bowie et al. (2003) whereby ion-conducting, desensitized states (Bowie & Lange, 2002) are up-regulated by lectin treatment. Here, the agonist-dependent nature of Con-A modulation is explained if, as proposed at AMPA receptors (Jin et al. 2003), full and partial KAR agonists activate different relative proportions of subconductance levels. As yet, analysis of single-channel currents activated by different GluR6 agonists has not been performed but would be necessary to delineate between an effect of Con-A on open-channel probability and/or unitary conductance (Bowie & Lange, 2002).

State-dependent modulation of KARs by Con-A

Although GluR6 subunits contain 10 N-glycosylated residues only nine are exposed to the extracellular surface and accessible to Con-A (Everts et al. 1999). The N-linked residue that does not bind Con-A is located in the pore region (Everts et al. 1999). All nine residues are positioned within or in close proximity to the agonist-binding domain of each GluR6 receptor subunit. Everts et al. (1999) have concluded that no single N-linked carbohydrate side chain is an absolute requirement for Con-A's effect, although the degree of modulation is significantly

less with fewer residues present. Moreover, ectopic N-glycosylated sites introduced into the agonist-binding domain also impart sensitivity to Con-A and, as predicted, have a weaker effect compared to the greater number present on wild-type GluR6 receptors (Everts et al. 1999). This observation supports the hypothesis developed here that Con-A binds to different residues in agonist bound or unbound conformations determining the degree of modulation. We further qualify these observations by showing that removal of three amino acid residues (i.e. GluR6(Q)ΔNG5,6,7) is sufficient to abolish the modulation of Dom responses with only a partial effect on L-Glu and KA.

Although used extensively to study invertebrate and mammalian iGluRs, state-dependent binding and modulation by Con-A has been described in only a few studies (Evans & Usherwood, 1985; Everts et al. 1999). As discussed above, Con-A modulates GluR6 receptors by binding to residues in close proximity to the agonist-binding domain (Everts et al. 1997, 1999) and we show here that this property permits inferences to be made about conformations adopted by this structure. We propose that modulation of GluR6 KARs involves two distinct molecular events. Initially, Con-A binds to either agonist-bound, desensitized channels or GluR6 channels in the closed, unbound state. Due to architectural rearrangements that accompany agonist binding (Armstrong et al. 1998; Armstrong & Gouaux, 2000), the number of N-glycosylated residues accessible to Con-A (Everts et al. 1999; Fig. 1.2B) is different for desensitized and unbound channel conformations. At desensitized receptors, bound Con-A molecules do not affect receptor function with full agonists such as L-Glu (Fig. 1.2). However, subsequent agonist dissociation sets off changes in protein structure that promote cross-linking of bound Con-A molecules or adjacent amino acid residues to regulate gating behaviour. This process will be different if Con-A has initially bound to GluR6 receptors in the desensitized or closed, unbound state. We propose that this cross-linking event, in both cases, restricts allosteric movement(s) of the external surface of GluR6 receptors affecting gating behaviour.

Correlating Con-A modulation to conformational changes in GluR6 receptors

In principle, the state-dependence of Con-A modulation may reflect conformational changes to the dimer interface, the pore region or the agonist-binding domain. Although Con-A may affect the dimer–dimer interface, our previously published findings (Bowie et al. 2003) provide experimental evidence that does not support this possibility. Specifically, we have shown that Con-A binding to GluR6 KARs does not affect rates into and out of desensitization. Since Horning & Mayer (2004) have argued that the dimer interface of KARs (and AMPARs) determines desensitization kinetics, by implication, our data demonstrate that Con-A does not affect dimer–dimer interactions. Likewise, Con-A's action is unlikely to reflect binding to the pore since amino acid residues critical for lectin binding and modulation are distant from this region. Instead, our experiments on GluR6(Q)ΔNG5,6,7 receptors and work by Everts et al. (1999) demonstrate that amino acid residues critical for lectin modulation are located in and around the agonist-binding domain. Other mechanisms may emerge as our understanding of KARs progresses. However, given these limitations, the most straightforward explanation of our data is that Con-A modulation reports conformational changes in the agonist-binding domain. In support of this, recent X-ray analysis of the isolated ligand-binding core of GluR6 KARs (Mayer, 2005; Nanao et al. 2005) also reported that full and partial agonists elicit distinct conformational changes in this region of the protein.

A potential caveat amongst these studies is that our experiments have focused on desensitized receptors whereas crystal structures of iGluRs are thought to represent the agonist-binding domain in the activated state of the channel (Jin et al. 2003). Three possible explanations may account for this apparent discrepancy. The first possibility is that published structures of the KA (and AMPA) receptor ligand-binding core represents the conformation adopted during ion channel activation (i.e. channel openings) as already proposed (Armstrong et al. 1998; Hogner et al. 2002; Mayer, 2005) but does not represent the binding cleft during desensitization. However, an important issue is that unitary current measurements indicate that single AMPA or KA receptor activations are very short-lived, lasting

only a few milliseconds (Swanson et al. 1996, 1997; Howe, 1996). Consequently, it is more likely that X-ray crystal structures of the ligand-binding core represent another conformational state that is more thermodynamically stable.

The second possibility, therefore, is that following agonist binding the ligand-binding core adopts a much more stable conformation such as the desensitized state. To date, the possibility that crystal structures of the iGluR ligand-binding core represent the desensitized state has not been examined experimentally though it has been suggested by some authors (Madden, 2002; Colquhoun & Sivilotti, 2004; Naur et al. 2005). In support of this, experimental protocols that require an extended incubation period with the ligand (e.g. radioligand-binding assays) are known to accumulate ligand-gated ion channels into high-affinity desensitized states (Colquhoun, 1998). By analogy, crystallization of the ligand-binding core may also promote formation of the desensitized state. Moreover, estimates of the apparent affinity of L-Glu for desensitized GluR6 receptors (IC_{50}= 0.44–0.5 μM (Paternain et al. 1998; A.Y.C. Wong, A.-M. L. Fay & D. Bowie, unpublished observations) and the isolated ligand-binding core (K_i= 1.4 μM) (Mayer, 2005) are almost identical whereas affinity for the activated state (EC_{50}= 694 μM) (Bowie et al. 2003) is more than 1000-fold lower.

The third and final possibility is that the conformation adopted by the ligand-binding core is identical whether the pore region is in the activated or desensitized state. This latter possibility would explain our observations on desensitized channels whilst agreeing with recent X-ray crystallographic data. However, this model, would have to reconcile with the fact that L-Glu evokes the largest peak response amongst all the agonists (see Fig. 1.3A) whereas the amplitude of its equilibrium response is the smallest (see Fig. 1.3C). In structural terms, the fact that partial agonists elicit responses of larger amplitude at equilibrium appears at odds with the proposed relationship between closure of the agonist-binding domain and agonist efficacy (Armstrong & Gouaux, 2000; Jin et al. 2003; Mayer, 2005). Clearly, further experimentation is required if these issues are to be resolved.

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CHAPTER 2:

DOES CLOSURE OF THE AGONIST-BINDING DOMAIN CORRELATE WITH AGONIST EFFICACY AT KAINATE RECEPTORS?

PREFACE TO CHAPTER 2

In *Results* **CHAPTER 1**, using three KA receptor agonists (L-glutamate, kainate and domoate) we have shown that the conformation adopted by the agonist-binding domain of intact KA receptors is agonist-dependent during desensitization (Fay & Bowie, 2006a). Consistent with this, X-ray crystal structures of the GluR6 KA receptor agonist-binding core in complex with these agonists also revealed that domain closure in the ligand binding core was agonist-dependent. (Nanao *et al.*, 2005a;Mayer, 2005a). The mode of L-glutamate binding was found to be essentially identical for AMPA and KA receptors. As illustrated in Figure P2 the α-carboxyl group of L-glutamate interacts with the conserved Arg523 residue of GluR6 and the α-amino group binds to its conserved Glu738 (Mayer, 2005a). In contrast, the γ-carboxyl group is not bound by a counter charge from a lysine or arginine chain, but rather forms hydrogen-bond interactions with the main chain peptide bond and the hydroxyl group of a conserved Thr690 residue from lobe 2, as seen with GluR2 (Armstrong & Gouaux, 2000a). Together with dynamics studies, the availability of these atomic structures has taken the study of structure-activity relationships at KA iGluRs to a new level of detail.

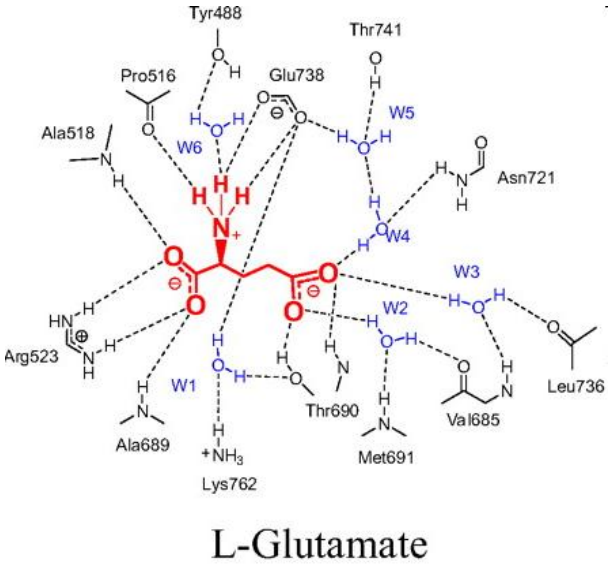


Figure P2: Two-dimensional topographical map of the GluR6 ABD with L-glutamate. The map was deduced from structure complexes obtained with FITTED and shows the number of contact points and the binding orientation of the full agonist, L-Glu. Reproduced, with permission, from (Fay et al, Molecular Pharmacology (2009) May;75(5): 1096-107)

One of the complicating issues in measuring and interpreting lobe closure is that the proposed relationship at KA receptors between agonist efficacy and closure of the agonist-binding domain relies on a limited number agonists (Nanao *et al.*, 2005a;Mayer, 2005a). An additional concern is that the agonist efficacy of two of the five agonists crystallized in complex with GluR6 (i.e., SYM 2081 and quisqualate) has never been systematically examined. As a matter of fact, the lack of available KA receptor agonists has not permitted a detailed structure-activity analysis, as described for AMPA and NMDARs (Patneau & Mayer, 1990; Patneau et al., 1992; Stensbol et al., 2002).

Given this, in the following study we have designed experiments to gain a better understanding of the molecular determinants of agonist behavior at KA receptors. These may, in turn, provide a rationale for the design of more selective pharmacological tools to study physiological and pathological roles of KA receptors. To explore the relationship (if any) between agonist efficacy and the extent of domain closure at KA receptors, we took a two-fold approach.

(1) We first **characterize the profile of known KA receptor agonists and also set out to identify a number of new agonists acting at these receptors**. To define agonist activity at KARs, we have tested a number of L-glutamate analogs using electrophysiological recordings in outside-out patches excised from tsa201 cells transiently expressing GluR6 KARs.

(2) Secondly, we **examine the extent of domain closure predicted for each newly identified agonist using the molecular ligand docking software, FITTED** ((Flexibility Induced Through Targeted Evolutionary Description) (Corbeil *et al.*, 2007a).

FITTED, which uniquely simultaneously allows for flexibility in the protein and ligand whilst allowing the displacement of bridging water molecules using a genetic algorithm, is particularly appealing to model iGluRs. In fact, its agonist binding core is thought to adopt a graded extent of domain closure (Jin *et al.*, 2003a;Nanao *et al.*, 2005a;Mayer, 2005a) and exhibit peptide flipping in the binding pocket (Armstrong & Gouaux, 2000a;Jin & Gouaux, 2003). Furthermore, the consideration of water molecules is particularly relevant for KA receptors,

since the size of the cavity of GluR6 (volume 255 ± 15 Å) is markedly larger than that of GluR2 (218 ± 4 $Å^3$)(Mayer, 2005a). As might be expected from a larger space in the binding cleft, the larger binding cavity of KA receptors accommodates up to six water molecules while GluR2 AMPA receptors can accommodate three to four such water molecules (Armstrong *et al.*, 1998;Mayer, 2005a). Together, these characteristics are proposed to account for the greater stability of glutamate-bound KA receptors and clearly demonstrate that water act as surrogate molecules (Mayer, 2005a) that must be taken into account in any modeling strategy for iGluRs. Using this combined approach, we identify a number of new KA receptor ligands and utilize them to test the proposed correlation between domain closure and agonist efficacy.

Article title:

Functional characterisation and *in silico* docking of full and partial GluK2 kainate receptor agonists

ABSTRACT

Two structural models have been developed to explain how agonist binding leads to ionotropic glutamate receptor (iGluR) activation. At AMPA iGluRs, full and partial agonists close the agonist-binding domain (ABD) to different degrees whereas agonist-induced domain closure is apparently fixed at NMDARs. Although Kainate (KA) iGluRs are thought to behave like AMPARs, the issue has not been formally tested due to the paucity of available receptor agonists. Here we identify a series of structurally-related full and partial agonists at GluK2 (formerly GluR6) KARs and predict their docking mode using the *in silico* ligand-docking program, FITTED. As expected, the neurotransmitter, L-Glu, behaved as a full agonist but modest reduction (e.g. L-serine or L-aspartate) or elongation (e.g. L-α-aminoadipate) in chain length generated weak partial agonists. Interestingly, *in silico* ligand-docking predicted that most partial agonists select for the closed and not, as expected, the open or intermediate conformations of the GluK2 ABD. Experiments using concanavalin-A to directly report conformations in the intact GluK2 receptor support this prediction with the full agonist, L-Glu, indistinguishable in this regard from weak partial agonists, D- and L-Asp. Exceptions to this were KA and domoate which failed to elicit full closure due to steric hindrance by a key tyrosine residue. Our data suggests that alternative structural models need to be considered to describe agonist behaviour at KARs. Finally, our study identifies the responsiveness of several neurotransmitter candidates establishing the possibility that endogenous amino-acids other than L-Glu may regulate native KARs at central synapses.

INTRODUCTION

iGluRs mediate the vast majority of excitatory neurotransmission in the mammalian brain and have been implicated in numerous CNS disorders (Bowie, 2008b). Given this, much effort has focused on their structure-function properties since, amongst other benefits, it provides a rational approach to drug discovery. Insight into their structure was first advanced by homology modeling using the bi-lobed domain of bacterial amino-acid binding proteins as a template (Stern-Bach et al., 1994b). Subsequently, the agonist-binding domain (ABD) of the GluA2 (Collingridge *et al.*, 2009) (formerly GluR2 or GluRB) AMPAR was crystallized, revealing the predicted clamshell-like structure of globular domains 1 and 2 that close upon agonist binding (Armstrong & Gouaux, 2000b). Since then, a similar approach has permitted the atomic resolution of ABDs of all iGluR family members including the KAR (Nanao et al., 2005b;Mayer, 2005b), NMDAR (Inanobe et al., 2005a) and more recently, the delta-2 orphan iGluR (Naur et al., 2007a). From these studies, two structural models of agonist behavior have emerged. At the NR1 NMDAR subunit, full and partial agonists differ little in the conformational change they elicit in the ABD (Inanobe et al., 2005a). In contrast, at AMPARs agonist efficacy is thought to reside in the conformations adopted by the ABD with full agonists more effective at promoting domain closure than partial agonists (Armstrong & Gouaux, 2000b;Jin *et al.*, 2003b).

Although KARs are thought to behave like AMPARs, the structural basis of agonist efficacy of this receptor family has not been firmly established for several reasons. First and foremost, there are fewer agonist-bound crystal structures available to make the comparison. To date, the ABD of GluK1 and/or GluK2 bound by the full agonist, L-Glu, and partial agonists KA and domoic acid (Dom) have been resolved at atomic resolution (Mayer, 2005b;Nanao et al., 2005b). Other structures for quisqualic acid (QA) and SYM 2081 have also been described (Mayer, 2005b) but it is not yet clear whether they act as full or partial agonists. Second, the extent of domain closure elicited by the full agonist, L-Glu, differs from partial agonist, KA, by only 3^{o} (Mayer, 2005b) which is modest in comparisons with the effect of the same agonists at AMPARs (e.g. L-Glu vs. KA,

8^{o} difference) (Armstrong & Gouaux, 2000b). An added complication is that the apo state of the KAR ABD has yet to be resolved; therefore, the extent of domain closure is given with respect to the GluA2 AMPAR apo state. Third and finally, KARs require external anions and cations as well as the neurotransmitter L-glutamate for activation (Wong et al., 2006a); a property not shared by AMPARs (Bowie, 2002b). Given this, it is possible that the degree of activation of KARs is shaped not only by the agonist molecule but also external ions.

Here, we have tested the functionality of a range of L-Glu analogues as a first step in understanding the structural basis of agonist behavior at KARs. To complement this data, we also used the *in silico* ligand-docking program, FITTED, to predict the conformation of the ABD preferred by each agonist. Interestingly, this combined approach suggests unexpectedly that most partial agonists select for the closed and not the open or intermediate conformation of GluK2 ABD. This finding suggests that agonist efficacy at KARs may not be solely determined by the extent of closure in the GluK2 ABD and therefore, alternative structural models may need to be considered.

MATERIALS & METHODS

Cell Culture & Transfection. Techniques used to culture and transfect mammalian cells to express GluR6 KARs have been described in detail elsewhere (Bowie, 2002b) . Briefly, tsA201 cells, a transformed human kidney (HEK 293) cell line stably expressing an SV40 temperature sensitive T antigen (provided by R. Horn, Jefferson Medical College, PA, USA) were maintained at a confluency of 70–80% in minimal essential medium with Earle's salts, 2 mM glutamine and 10% fetal bovine serum supplemented with penicillin (100 units per ml) and streptomycin (100 μg per ml). After plating at low density (2×10^4 cells per ml) on plastic dishes, cells were transfected with cDNA encoding unedited rat glutamate receptor subunit 6 (GluR6Q) using the calcium phosphate technique as previously described (Bowie, 2002b). The cDNA for enhanced green fluorescent protein (EGFP S65T mutant) was routinely co-transfected to identify transfected cells. In this and all subsequent publications from our lab, we adopt the recommended change to iGluR nomenclature (Collingridge *et al.*, 2009). Consequently, GluR6 will be referred to as GluK2 and the GluR-B or GluR2 AMPAR subunit as GluA2.

Electrophysiological Solutions and Techniques. All ligands tested in this study were dissolved in external solutions containing 150 mM NaCl, 5 mM HEPES with low concentrations of $CaCl_2$ and $MgCl_2$ (0.1 mM each) to avoid divalent block. For dose-response relationships to D- and L-Asp (Fig. 2.3D), however, agonists were applied at concentrations (i.e. > 100 mM) which would cause a shifting in reversal potential due to changes in the driving force for the main permeant ion, Na^+. To avoid this, the ionic strength of all solutions was increased to 200 mM with the desired agonist concentration balanced by the appropriate amount of NaCl. All concentrated ligand solutions were adjusted to pH 7.3 with NaOH before being stored at -20^{o}C. Saturating agonist concentrations chosen for L-Glu (10 mM), kainate (1 mM), domoate (50 μM) were at least 5-fold larger than published EC_{50} values at GluK2 receptors. We confirmed empirically that these concentrations were saturating by doubling the agonist concentration in each

case and observing that peak response amplitudes were unchanged. For sulfur-containing amino acids, QA, SYM 2081 as well as L-α-aminoadipate, saturating levels were determined empirically by increasing concentrations until a maximal response was observed. In cases where millimolar concentrations of agonist were required for activation (e.g. 40 mM L-cysteic acid), the reported response amplitudes were corrected for the shift in the reversal potential observed. Internal pipette solution contained 115 mM NaCl, 10 mM NaF, 5 mM HEPES, 5 mM Na_4BAPTA, 0.5 mM $CaCl_2$, 1 mM $MgCl_2$ and 10 mM Na_2ATP to chelate endogenous polyamines. The pH and osmotic pressure of internal and external solutions were adjusted to 7.3 and 295 mOsm respectively. Concanavalin-A (Con-A) (Sigma, St. Louis) was prepared in glucose free saline solution and filtered (0.2 μm filter, Corning) immediately before use as described previously (Bowie et al., 2003b). All recordings were performed with an Axopatch 200B amplifier (Axon Instruments Inc., CA) using thin-walled borosilicate glass pipettes (2-5 MΩ) coated with dental wax to reduce electrical noise. Control and agonist solutions were rapidly applied to outside-out patches excised from transfected tsA201 cells as described previously (Bowie, 2002b). Solution exchange (10-90 % rise-time = 25-50 μs) was determined routinely at the end of the experiment by measuring the liquid junction current (or exchange current) between the control and agonist-containing solution in which total Na^+-content was reduced by 5%. Current records were filtered at 5 kHz, digitized at 25-50 kHz and series resistances (3-10 MΩ) compensated by 95 %. Most recordings were performed at -20 mV membrane potential to ensure adequate voltage clamp control of peak currents. Data acquisition was performed using pClamp9 software (Axon Instruments Inc., CA). All experiments were carried out at room temperature (22 – 23 °C).

Overview of the docking program, FITTED. Conformational changes in the ligand-binding domain of iGluRs have been investigated through X-ray crystallography. Previous X-ray data has revealed two fundamental features pertaining to the ligand-binding domain of iGluRs which has made it difficult to

accurately model these proteins. First, the model must allow for protein flexibility since it is well established that the ligand-binding domain can adopt a range of degree of clamshell closure. Moreover, given that water molecules have been shown to play a key role in stabilizing the ligand in the binding cleft of both AMPA and KARs (Mayer, 2005b), the docking program would have to allow for displacement and movement of waters. Until recently, docking software that simultaneously accounted for these features in their search algorithm was not available. However, the development of a genetic algorithm based docking program called FITTED 2.0 (Flexibility Induced Through Targeted Evolutionary Description) which performs all these functions has recently been described (Corbeil et al., 2007b). This docking tool can uniquely accommodate for displaceable bridging water molecules while treating the ligand/protein as a realistically dynamic system and therefore provides the most appropriate docking approach to investigate iGluRs. For data shown in this study we used FITTED version 2.0 using the semi-flexible docking option with displaceable waters and, in each case, the pharmacophore-oriented docking function was used (Corbeil et al., 2007b).

Protein and ligand structure preparation prior to docking. The X-ray structures of GluK2 complexes were retrieved from the Protein Data Bank (PDB codes: 1s50, 1s7y, 1sd3, 1s9t, 1tt1, 1yae) and hydrogen atoms were added with their position optimized through energy minimization. The result was visually inspected, as described previously to ensure the optimum hydrogen bond network (Corbeil et al., 2007b). Six bridging water molecules found to be conserved throughout most of the ligand-protein complexes were retained for the docking study. All proteins structures were prepared using ProCESS (a module of FITTED) and the ligands were fully ionized and prepared with SMART (a module of FITTED) (Corbeil et al., 2007b).

Docking amino acid ligands using FITTED. The data obtained from docking experiments are summarized in Table 2.1. Six protein structures initially resolved

with five different agonists were used as input files (i.e. 1s7y (L-Glu), 1s9t (QA), 1sd3 (SYM 2081), 1tt1 (kainate), 1yae_a (domoate, conformation 1) and 1yae_b (domoate, conformation 2). All the original PDB files pertain to KAR dimer structures solved with different ligands (Mayer, 2005b) with the exception of 1yae which was solved as a hexamer (Nanao et al., 2005b). To compare GluK2 monomers within a given polymer, protein superimposition was achieved by aligning the alpha carbons of the residues found with at least one atom within 10 Å from the ligand. With the exception of 1yae, all the monomers within a given polymer were identical. Therefore, only one of the monomers per dimer was retained for the docking studies. As for 1yae, two monomers (1yae_a and 1yae_b) were retained as input files to allow for greater protein fluctuations within the binding pocket. The five agonists (L-Glu, QA, SYM 2081, KA and domoate) were docked using six protein structures as input files (1s7y, 1s9t, 1sd3, 1tt1, 1yae_a and 1yae_b). As previously reported, comparison of the crystal structures reveals three distinct protein conformations which we will refer to as: closed, intermediate and open. Consistent with the identical degree of domain closure observed with the binding of L-Glu (1s7y), SYM 2081 (1sd3) or QA (1s9t) at GluK2 crystals, the computed root mean square deviation (RMSD) between the active site of all three protein structures were small (1s7y and 1sd3: 0.24 Å; 1s7y and 1s9t: 0.46 Å; 1sd3 and 1s9t: 0.51 Å). For the remainder of the text, the term "closed" conformation will be used to refer to any of these three protein conformations. In agreement with crystallographic studies (Nanao et al., 2005b), the "open" state will denote the conformation observed with domoate-bound crystals (RMSD between 1s7y and 1yae: 1.6 Å). Finally, the conformation adopted by the kainate-bound GluR6 crystal conformation will be termed "intermediate" (RMSD between 1s7y and 1tt1: 0.91 Å).

We assessed the validity of FITTED 2.0 for GluK2 KARs in several ways. First, we performed statistical analysis comparing the ligand bound in the actual crystal structures with the docked ligand predicted by FITTED. A ligand pose was considered successfully docked when the RMSD relative to the ligand bound in the actual crystal structure was below 2.0 Å (Table 2.1) (Corbeil et al., 2007b).

Second, the protein structure was considered to be accurately selected when the population favored that specific protein conformation over others (Corbeil et al., 2007b). Thirdly, we compared the number and position of water molecules in the crystal structure with that predicted by FITTED. In all cases, FITTED correctly predicted the number and position of water molecules. For each pose, FITTED used the RankScore function to yield a docking score, an estimation of the free energy of binding including entropic contributions (Table 2.1). Importantly, although the scoring function has been trained to reproduce free energies of binding, the accuracy level is not high enough to make highly accurate predictions within two orders of magnitude in K_i. In addition, the apparent agonist affinity (see Fig. 2.3) is not governed only by the free energy of agonist binding but also by multiple aspects of ion-channel behavior that include channel gating properties and desensitization. A minimum set of 10 runs was carried out for each ligand (Corbeil et al., 2007b). An initial population of 500 was enough for docking of all GluK2 KAR ligands to reach the convergence criterion. Moreover, a maximum of 500 generations was used to reach convergence for each ligand.

TABLE 1

Functional and structural properties of GluK2 KAR agonists

Functional properties of responses elicited by the sixteen GluK2 receptor agonists examined in this study. Structural information obtained with FITTED are also provided. All data are expressed as the mean ± S.E.M.

Agonist, Concentration Range	Peak		Ligand Category	Conformation		RMSD	Docking Score (FITTED)
				Selected (FITTED)	Experimental (Crystallography)		
	%	*n*					
L-Glu,10 mM	100	43	Full	Closed	Closed	0.34	−5.73
SYM 2081, 1–3 mM	102.0 ± 7.7	3	Full	Closed	Closed	0.24	−6.52
QA, 1–3 mM	90.0 ± 1.1	3	Full	Closed	Closed	2.0	−8.70
L-Cys, 10–60 mM	73.6 ± 1.9	4	Partial	Closed			−5.60
SSC, 1–2 mM	64.1 ± 7.6	5	Partial	Closed			−8.06
HCSA, 10–40 mM	58.0 ± 5.4	5	Partial	Closed			−6.84
KA, 1–3 mM	39.1 ± 2.0	10	Partial	Intermediate	Intermediate	0.46	−8.33
HC, 10–40 mM	34.4. ± 3.4	8	Partial	Closed			−6.62
CSA, 10–40 mM	31.72 ± 3.75	3	Partial	Closed			−6.97
Dom, 50–150 μM	15.3 ± 1.9	8	Partial	Open	Open	1.2	−8.88
L-Aminoadipate, 10–40 mM	14.05 ± 0.19	4	Partial	Closed			−6.70
D-Asp, 10 mM	3.6 ± 1.15	6	Partial	Closed			−6.29
L-Asp, 10 mM	0.95 ± 0.39	5	Partial	Closed			−5.13
SOS, 1–20 mM	0.52 ± 0.7	3	Partial	Closed			−7.24
L-Ser, 10 mM	0.26 ± 0.3	3	Partial	Closed			−5.01
D-Ser, 10 mM	0.05 ± 0.5	3	Partial	Closed			−4.96

Table 2.1. Functional and structural properties of GluK2 KAR agonists. Table summarizing the functional properties of responses elicited by the sixteen GluK2 receptor agonists examined in this study. Structural information obtained with FITTED are also provided. All data are expressed as the Mean ± S.E.M.

Assumptions of molecular docking strategy. To perform molecular ligand docking experiments, four assumptions were made. Firstly, our modeling strategy

pertained to transposing the information obtained from resolved crystal with the behavior of the mature receptor under physiological conditions. Our electrophysiological recordings from GluK2 KARs were performed under physiological pH (7.3-7.4), while most of the GluK2 S1S2 isolated cores were crystallized under conditions that were significantly more acidic (ranging from 4.0 to 6.5). To assess the effect (if any) of these pH fluctuations, we compared the two GluK2 KAR crystals in complex with L-Glu that were co-crystallized at distinct pH (1s50, pH = 8.0; 1s7y, pH = 4.8) (Mayer, 2005b). Visual inspection of the superimposed protein-ligand complexes revealed no significant differences between the two structures. We therefore used the 1s7y structure and did not further consider 1s50 in our analysis. Secondly, our modeling experiments assumed that all the amino acids tested bind in the same cavity between the S1S2 domains (i.e. the orthosteric site) as previously reported for other ligands co-crystallized with GluK2 KARs. Thirdly, since the apo state of GluK2 KARs has yet to be resolved, the degree of domain closure of the agonist-binding domain was obtained in comparison with the apo state of GluA2 AMPA receptor. Fourthly, we have assumed that L-Glu analogs bind to one of the three known GluK2 conformations identified through X-ray crystallography (*open*, *intermediate* and *closed*). The computed RMSD between the active sites of structures for L-Glu (1s7y), SYM 2081 (1sd3) and QA (1s9t) were small (1s7y and 1sd3: 0.24 Å; 1s7y and 1s9t: 0.46 Å; 1sd3 and 1s9t: 0.51 Å), and therefore their domain closure was considered indistinguishable in agreement with previous structural analysis (Mayer, 2005b).

RESULTS

L-Glu analogs exhibit a wide range of agonist activity at GluK2 receptors

In an effort to identify receptor ligands that exhibit the full range of agonist behavior, we studied the kinetic properties of a number of commercially available L-Glu analogs (see Methods). In all cases, agonists were applied at saturating concentrations and at frequencies that permit full recovery from desensitization. Figure 2.1 shows the extended structure of the selected amino acids all of which possess a common L-Glu backbone.

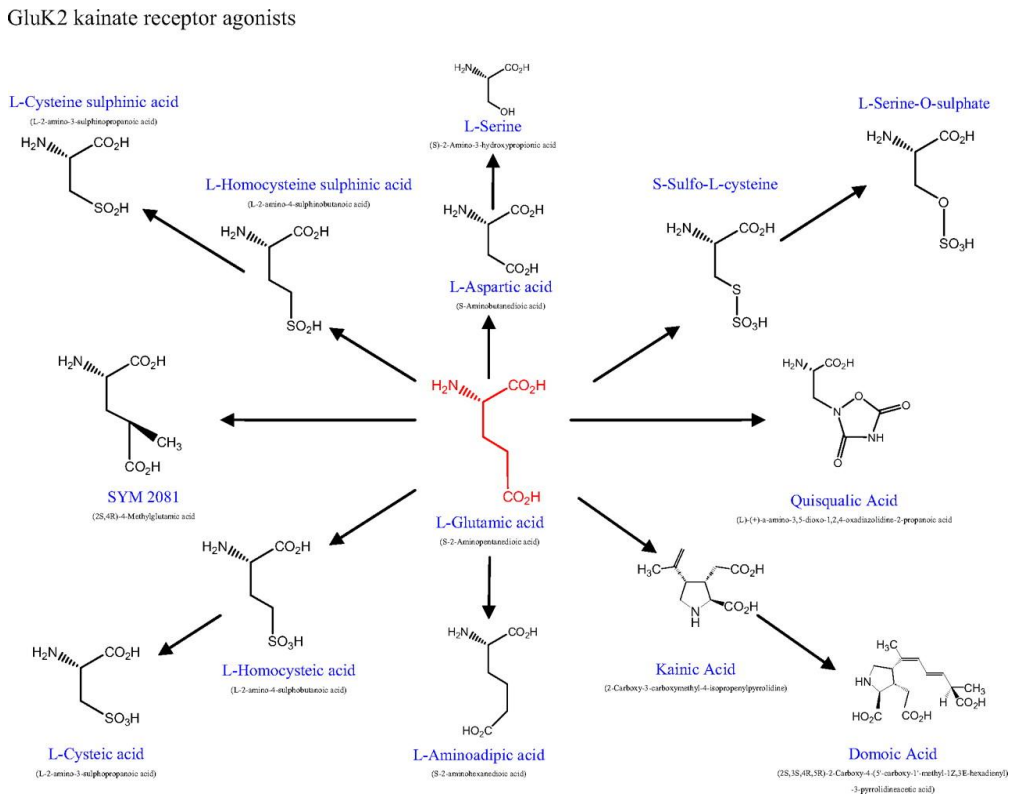


Figure 2.1 Extended structure of GluK2 receptor agonists. Schematic diagram showing the extended structure of all the amino acids selected for investigation. All amino acids are structural analogs of L-Glu and thus were chosen purposely to provide information on how changes in chain length, atom substitution, as well as the addition of side groups and/or sulfur groups affect agonist efficacy. Each structure is identified by both its common and IUPAC nomenclature.

We purposely chose this group of amino acids since they would provide information on how agonist efficacy is shaped by changes in chain length, atom

substitution, as well as the addition of side groups and/or sulfur groups. Agonist activity of several of these amino acids have been previously reported at AMPARs, NMDARs and metabotropic glutamate receptors (Patneau & Mayer, 1990b;Kingston *et al.*, 1998a), but not yet at KARs.

Almost all amino acids tested elicited membrane currents that consisted of a rapidly-rising peak response which declined in the presence of the agonist to a new equilibrium level (Fig. 2.2 upper, Table 2.1).

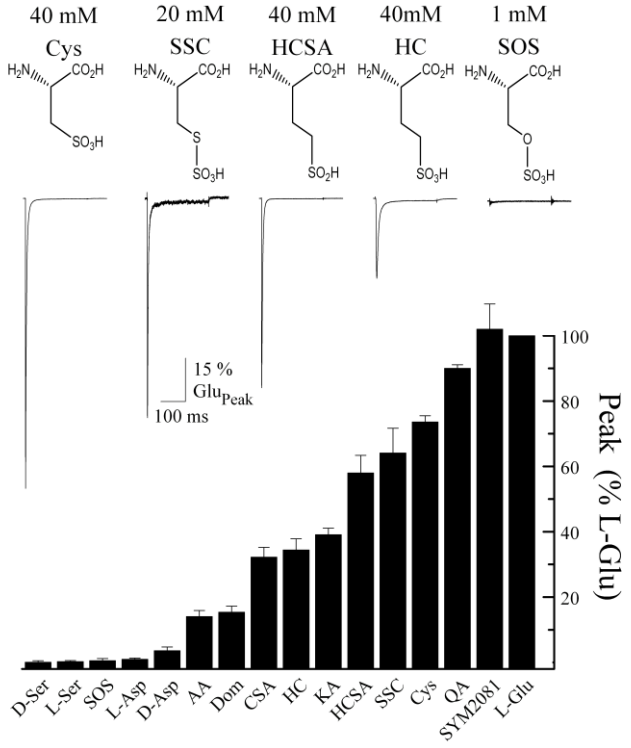


Figure 2.2 Response profile of an extended series of GluK2 kainate receptor agonists. (Upper Panel) Structure-function relationship of five sulfur-containing amino acids aligned in order of peak agonist responsiveness. To allow comparison between experiments, membrane currents were normalized to the peak L-Glu response in each recording. Patch #s were 04622p4 (Cys), 04629p2 (HCSA), 060720p1 (HC), 060720p4 (SSC) and 04621p1 (SOS). (Lower Panel) Summary bar graph comparing the peak response amplitude observed with saturating concentrations of each amino acid (n = 8 to 43 patch recordings). The data are arranged in increasing order of responsiveness, from very weak partial agonists (stereoisomers of serine and aspartate as well as SOS) to QA, SYM 2081 and L-Glu which are full agonists. All data are expressed as the mean ± S.E.M.

In some cases, as with L-serine-O-sulphate (SOS) and stereoisomers of serine (Ser) and aspartate (Asp), responses were difficult to resolve due to their small amplitude (even in high-expressing patches) which made detailed kinetic analysis problematic (Figs. 2.2 and 2.3). Nevertheless, a wide range of agonist efficacy was observed amongst all the amino acids tested (Fig. 2.2, lower panel). For example, five sulfur-containing amino acids exhibited the following rank order of efficacy: L-cysteic acid (Cys, 40 mM) > S-sulfo-L-cysteic acid (SSC, 20 mM) > L-homocysteine sulfinic acid (HCSA, 40 mM) > L-homocysteic acid (HC, 20 mM) > SOS (1 mM) based on peak response amplitude with saturating agonist concentrations (Fig. 2.2 upper). As mentioned above, SOS evoked barely detectable responses demonstrating that even modest changes to the agonist structure has pronounced effects on agonist efficacy (Fig. 2.2 upper). In this case, replacement of the sulphur atom at the ω-position with an oxygen converted the partial agonist, SSC, into the poorly stimulating SOS. Except for SYM2081 and QA, all other agonists tested were partial agonists since they elicited peak responses smaller than that observed with L-Glu (one sample t-test, $p < 0.01$; Fig. 2.2 lower). Finally, modest reduction (e.g. L-serine and L-aspartate) or elongation (e.g. L-α-aminoadipate) in chain length of the L-Glu structure generates weak partial agonists suggesting that the KAR ABD is optimized for the binding of this amino acid.

Desensitization does not profoundly affect estimates of peak response amplitude

Although solution exchanges performed in this study were rapid, relative rates of activation and desensitization may vary amongst different agonists. Consequently, agonists designated as poorly conducting (i.e. weak partial agonists) may, in fact, behave as full agonists if studied in the absence of desensitization. To address this issue, we looked more closely at SOS and the stereoisomers (i.e. D and L) of both Asp and Ser which were ideal for this purpose since these ligands represent the five weakest responding agonists which, as explained above, may reflect genuine partial agonist activity or result from rapid rates into desensitization. To delineate between these two possibilities, we

examined agonist responses following treatment with the plant lectin, concanavalin-A (Con-A).

While Con-A does not block desensitization or shift apparent agonist affinity, it irreversibly increases current flow through GluK2 KARs (Bowie et al., 2003b). We reasoned that this property would permit better resolution of responses elicited by weakly responding agonists. Prior to Con-A treatment, typical responses elicited by each of these agonists were small in amplitude which made accurate analysis of their kinetic properties problematic as shown in figure 2.3A for 10 mM L-Asp and SOS. To allow comparison, membrane currents elicited by the full agonist, L-Glu (10 mM), in the same patch recording are shown superimposed (Fig. 2.3A). As anticipated, Con-A treatment (10 μM, 3-5 mins) increased current flow through GluK2 receptors activated by SOS and stereoisomers of both Asp and Ser making it possible to routinely study their peak responses (Fig. 2.3B). From detailed analysis of the stereoisomers of Asp, two important characteristics of their response were revealed that unequivocally demonstrate that they behave as partial agonists. First, stereoisomers of Asp elicited rapidly-rising, non-desensitizing membrane currents showing that these agonists are not weakly-responding due to the rapid onset of desensitization (Fig. 2.3B). Second, construction of activation curves for each agonist revealed that maximal responses in each case were significantly smaller than with L-Glu (Fig. 2.3C). Compared to the maximal response elicited by L-Glu, responses to saturating concentrations of D- and L-Asp were 2.5 ± 0.1 % and 3.0 ± 0.3 % (n = 4-6) respectively. In addition, estimated EC_{50} values (Hill coefficient, n_H) for D-Asp and L-Asp were 1.2 ± 0.1 mM (n_H = 1.7 ± 0.3) and 19.4 ± 4.7 mM (n_H = 2.2 ± 1.1) respectively compared 0.5 ± 0.1 mM (n_H = 0.8 ± 0.1) for L-Glu (Fig. 2.3C & D). Taken together, these observations directly demonstrate that D- and L-isomers of Asp elicit responses of small amplitude since they are partial agonists and not due to the rapid onset of desensitization.

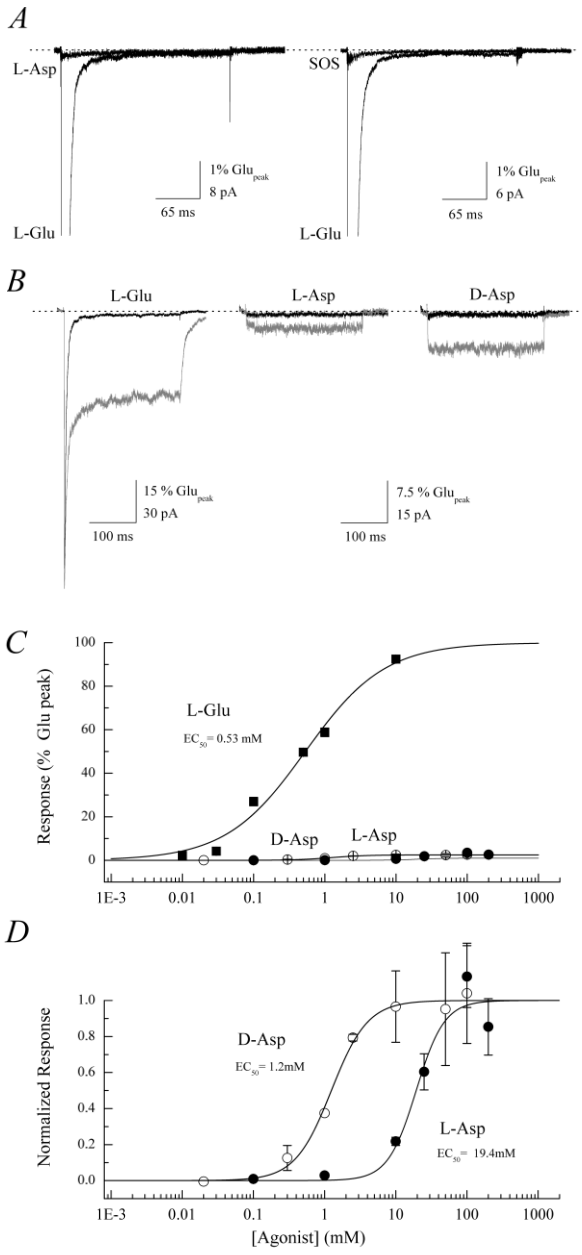


Figure 2.3 Stereoisomers of aspartate are partial agonists at GluK2 kainate receptors. (A) Representative membrane currents elicited by 10 mM L-Glu, 10 mM L-Asp and 1 mM SOS (Patch # 080425p2). The dotted line denotes the zero current level. (B) Typical electrophysiological recordings elicited by 10 mM L-Glu, L- and D-Asp (10 mM each) before (black line) and after (grey line) Concanavalin-A (3 min) treatment in the same patch (Patch # 080425p2). Con-A treatment reveals that both D- and L-Asp elicit rapidly-rising, non-desensitizing membrane currents that quickly deactivate upon cessation of the agonist application. (C & D) Activation curves to L-Glu as well as D- and L-Asp reveal that stereoisomers of Asp are weak partial agonists with significantly lower affinity than with the full agonist, L-Glu.

In silico ligand-docking correctly identifies conformations adopted by the GluK2 agonist-binding domain

To combine this functional data with *in silico* ligand-docking using FITTED, we first focused on receptor agonists previously co-crystallized with the isolated ligand binding core of GluK2 (Mayer, 2005b;Nanao et al., 2005b). From electrophysiology recordings, we already identified kainate (1 mM KA) and domoate (50 μM Dom) as partial agonists at GluK2 receptors with L-glutamate

(10 mM L-Glu), SYM 2081 (3 mM) and quisqualate (3 mM QA) all behaving as full agonists when applied at saturating concentrations (Fig. 2.4A & B). Peak KA and Dom responses were 39.1 ± 2.0 % (n = 10) and 15.3 ± 1.9 % (n = 8) respectively of the maximal full agonist response (Figure 2.4B, Table 2.1). Previous structural work has shown that Dom induces domain closure of 12.3 ^{o}, KA elicits an intermediate closure of 23.3^{o} whilst the degree of domain closure with SYM 2081, QA and L-Glu are between 26.2° – 26.6° (Mayer, 2005b;Nanao et al., 2005b). Consequently, our electrophysiological data supports the current view that agonist efficacy is determined by the degree of closure in the GluK2 ABD (Mayer, 2005b;Nanao et al., 2005b).

To look at domain closure and binding mode, we performed *in silico* ligand-docking with the same series of receptor agonists using FITTED (Fig. 2.4D – F). FITTED is a suite of programs which is unique in that the fitting process permits flexibility in macromolecules (side chains and main chains) and the presence of bridging water molecules while treating protein/ligand complexes as realistic dynamic systems (Corbeil et al., 2007b). These characteristics are particularly relevant to the iGluR ABD since ligand and protein flexibility as well as water molecule mobility are critical determinants of agonist behavior (Arinaminpathy et al., 2006a). In practical terms, agonists were docked to previously published structures of GluK2 that together represent the closed, intermediate or open conformation of the ABD (see Methods for details). It is important to emphasize that the final structure only ever represents a composite of these input structures and that FITTED cannot predict a completely novel structure. Upon convergence of the fitting process, we were able to assign a preferred conformation of the GluK2 ABD to each agonist. In agreement with published X-ray crystal structures (Mayer, 2005b;Nanao et al., 2005b), the full agonist, L-Glu, selected the closed conformation (Fig. 2.4D) whereas the partial agonists, KA and Dom, selected intermediate and open conformations respectively. Superimposition of the agonist-receptor complexes observed with FITTED and published X-ray crystal structures reveal that the structures obtained by each approach were indistinguishable (Fig. 2.4D – F). In support of this, comparison of the computed

RMSDs between the crystal and docked structures for L-Glu, KA and Dom were 0.34 Å, 0.46 Å and 1.2 Å respectively (Table 2.1) indicating that the ligand pose was accurately selected for each agonist.

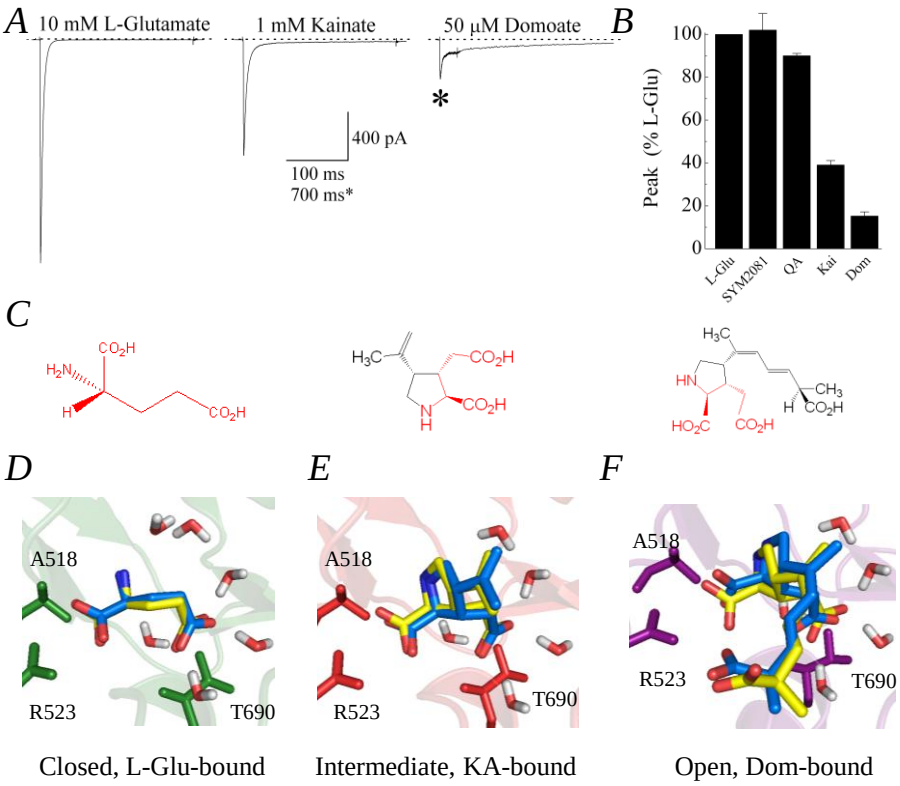


Figure 2.4. FITTED accurately predicts conformations adopted by the GluK2 agonist-binding domain. A, membrane currents evoked by L-Glu (10 mM, 250-ms duration, Hp = -20 mV), KA (1 mM), and Dom (50 µM) in the same outside-out patch containing homomeric GluK2 channels (patch number 030724p2). *, Dom response is drawn on a different time base. B, summary plot showing peak responses evoked by five agonists, all of which have been cocrystallized with the GluK2 KAR: L-Glu (n = 13), SYM 2081 (n = 3), QA (n = 3), KA (n = 13), and Dom (n = 8). All data are expressed as the mean +/- S.E.M. C, extended molecular structures showing that kainate and domoate have a common L-Glu backbone (red labeling). D to F, superimposition of the GluK2 agonist-binding pocket containing L-Glu, KA, and Dom where the solved crystal structures are compared with that docked by FITTED. In this and subsequent figures, the numbering of amino acid residues begins at the start site of the open reading frame and therefore includes the signal peptide. The solved crystal structures are shown in yellow, whereas the modeled structures are in blue. Key residues as well as agonist and water molecules are shown as sticks. L-Glu, KA, and Dom selected the closed (green), intermediate (red), and the open conformations (purple), respectively. Note that binding of KA and Dom displaces one of the key surrogate water molecules, which are present in the L-Glu-bound crystal. Nonpolar hydrogens are omitted for clarity.

A closer view of the GluK2 ligand-binding pocket (Fig 2.4D - F) reveals key water molecules and selected amino acid residues involved in ligand recognition. For example, Arg523 and Ala518 are involved in H-bonding with the α-carboxyl group of all ligands. In contrast, Thr690 is involved in both direct hydrogen bonding with the γ-carboxyl group and indirect interactions through surrogate water molecules. Two other full agonists previously crystallized, SYM 2081 and QA, also selected the closed clamshell conformation with small computed RMSDs (SYM 2081: 0.24 Å and QA: 2.0 Å, Table 2.1). Taken together, our findings validate the use of FITTED in providing information on the conformational state adopted by the GluK2 ABD bound by different receptor agonists.

Agonist efficacy and predictions of domain closure do not correlate

We next broadened our analysis to include all L-Glu analogs. With the exception of KA and Dom, FITTED predicted that all amino acids bind preferentially to the *closed* conformation suggesting that agonist efficacy and the degree of closure in the GluK2 ABD are apparently not correlated (Fig. 2.5). At first glance, this result was perplexing since it suggested that weak partial agonists, such as stereoisomers of Asp or Ser, elicit similar degrees of conformational change as L-Glu (Fig. 2.5B). Our immediate concern was that the outcome of the modeling represented a local minimum in the fitting process that is nonsensical from a biological perspective. However, we excluded this on two counts. First, FITTED already predicted the correct docking orientation of ligands previously crystallized with the GluK2 ABD (c.f. Fig. 2.4). Second, the binding mode of all other docked agonists was comparable to the binding orientation observed with L-Glu as would be expected. Typical binding orientation is illustrated by a visual inspection of the GluK2 ligand-binding pocket docked with L-α-aminoadipate (AA) and L-homocysteine sulfinic (CSA) (Fig. 2.5A). In each case, the α-carboxyl groups of both partial agonists are predicted to form H-bonds with Ala518, Arg523 and AlaA689 (Fig. 2.5A) while the α-amino group is predicted to interact with Pro516 and Glu738 (not shown).

As expected, FITTED predicts that the terminal-carbon interacts with Thr690 via direct H-bonding and surrogate water molecules.

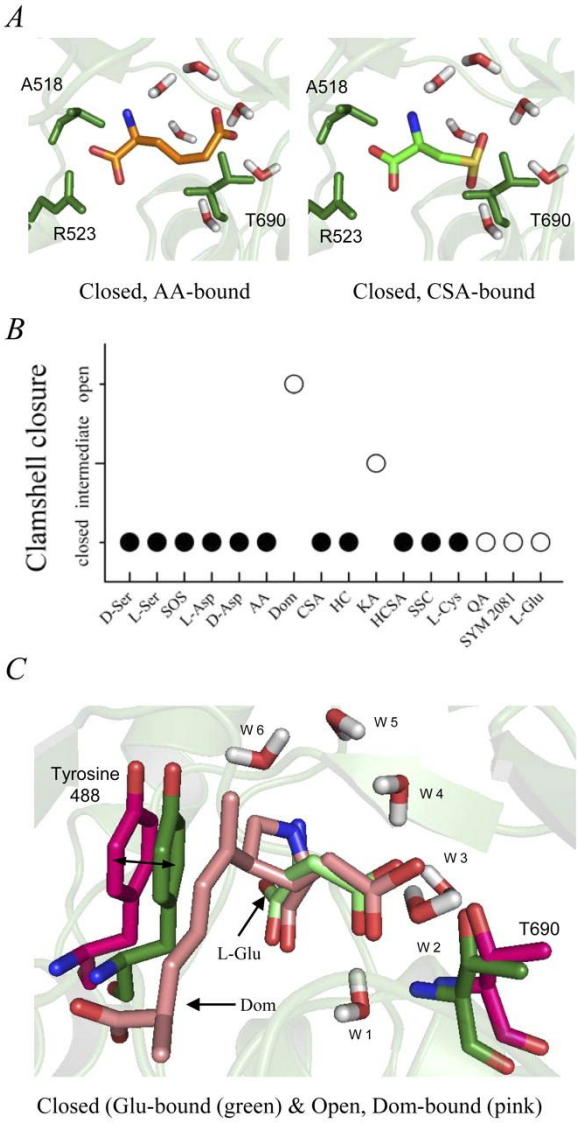


Figure 2.5 Tyrosine 488 prevents full cleft closure with domoate and kainate. (A) Docking of L-aminoadipate (AA) (left panel) and CSA (right panel) to GluK2 KARs using FITTED selects the *closed* conformation (green) in each case. The modeled structures are shown in orange and green, respectively. (B) Summary plot showing the conformation selected by each L-Glu analog using FITTED. Agonists previously co-crystallized with GluK2 are labeled as open circles whereas the conformation selected by newly identified ligands is denoted by a filled circle. (C) Superimposition of the GluK2 ABD in complex with L-Glu (green) and Dom (pink). Note that different shading intensities have been used to distinguish between amino acid residues in the GluK2 ABD from the agonist molecule. In addition, only the protein backbone of the *closed* conformation is illustrated. Note the pyrrolidine ring of Dom elicits a displacement of the Tyr488 residue as well as a water molecule (W6) normally found in the L-Glu-bound crystal structure. Interestingly, KA has the same effect though to a lesser extent due to its smaller side-chain that extends from the pyrrolidine ring. Agonists, water molecules and selected key residues are shown as sticks. Non-polar hydrogens have been omitted for clarity.

An additional concern was that the limited number of structures of the KAR ABD may bias the outcome of our analysis with FITTED. Although important to consider, we feel that this issue is not critical in our case since the structures we have used cover an appreciable range of cleft closure in the GluK2 ABD from 12.3 ^{o} for Dom to 26.2° – 26.6° for L-Glu (Mayer, 2005b;Nanao et al., 2005b). Furthermore, these structures represent the preferred conformations of full agonists (i.e. L-Glu, SYM 2081 & QA) to moderate and weak partial agonists

(e.g. KA, Dom). FITTED does not provide information on whether the ABD adopts discrete or a limitless range of conformations following agonist binding. Nor does it identify any putative twist motion proposed from molecular dynamics to occur with partial agonists acting on GluA2 AMPARs (Bjerrum & Biggin, 2008a). However given these limitations, FITTED still permits us to examine the more general issue of whether there is any proposed relationship between cleft closure and agonist efficacy.

It is not wholly surprising that FITTED predicts that weak partial agonists, such as Asp or Ser, elicit the same degree of domain closure as full agonists, such as L-Glu, especially since almost all ligands used in this study have compact structures. Consequently, it is reasonable that most of the agonists we have docked using FITTED prefer the closed rather than the open or intermediate conformation of the KAR ABD. This conclusion is supported by recent work on AMPARs that has established the precedence that agonist efficacy need not be correlated with the degree of cleft closure (Zhang et al., 2008b). Specifically, Zhang *et al* found that mutation of the Thr686 residue of the GluA2 AMPAR renders L-Glu a partial agonist but yet structural changes elicited are indistinguishable from wildtype receptors (Zhang et al., 2008b). The exceptions to this at GluK2 receptors are KA and Dom which prefer the intermediate and open conformations. However, as explained below, this observation can be simply accounted for by steric hindrance within the KAR ABD that limits the closure achieved by more bulky ligands such as KA and Dom.

Domain closure is determined by ligand interaction with Tyrosine 488

If the degree of closure in the ABD is not correlated with agonist efficacy, what is the basis for differences in closure observed with some agonists? Visual inspection of the ligand-bound complexes predicted by FITTED reveals an important property of the GluK2 ABD unique to Dom- and KA-bound structures (Fig. 2.5C). Specifically, the large side chain that extends from position 4 on the pyrrolidine ring of Dom causes a translational motion of Tyr488 which prevents complete closure of the GluK2 ABD. Likewise, the shorter side chain extending

from the pyrrolidine ring of KA also causes steric hindrance but to a lesser extent accounting for the intermediate closure of the ABD. In contrast all other amino acids tested, including the full agonist, L-Glu, do not interact directly with Tyr488 and due to their compact structure allow complete closure of the agonist-binding pocket (Figure 2.5B). The exception to this is QA which possesses a bulky oxadiazolidine ring (Fig. 2.1). In this case, however, the ring structure of QA occupies a different region of the GluK2 ABD from the pyrrolidine ring of Dom and KA. Consequently, QA binds to GluK2 permitting complete closure of the ABD.

Conformational changes elicited by D- and L-Asp are indistinguishable from L-Glu

Although docking experiments with FITTED predicts that weak partial agonists, such as D- and L-Asp, bind to the closed conformation of the GluK2 ABD, it was nevertheless important to demonstrate this experimentally. To do this, we examined GluK2 responses following pre-treatment with Con-A (Fig. 2.6). Con-A binds to a number of N-glycosylated residues in and around the GluK2 ABD (Fay & Bowie, 2006b). In the resting or apo state of the GluK2 ABD, access to these sites is unrestricted, as a result, Con-A can bind to the receptor. Con-A binding in turn leads to the upregulation of GluK2 responses as we have described previously (Bowie *et al.*, 2003b;Fay & Bowie, 2006b). A typical experiment showing this effect is illustrated in figure 2.6A. Note that the equilibrium / peak response ratio to 10 mM L-Glu increased to 21.8 ± 2.9 % following pre-treatment with Con-A (10 μM, 3 mins) (Fig. 2.6A & C). Conversely, if appreciable conformational changes are induced in the GluK2 ABD, such as occurs following L-Glu binding (Fay & Bowie, 2006b), Con-A's access to its binding sites are significantly restricted. As a consequence, pre-treatment with Con-A has only a modest effect on the GluK2 response. In the example shown in figure 2.6A, the equilibrium / peak response ratio to L-Glu observed following pre-treatment with Con-A was increased only to 6.7 ±1.8 % (Fig. 2.6A & C).

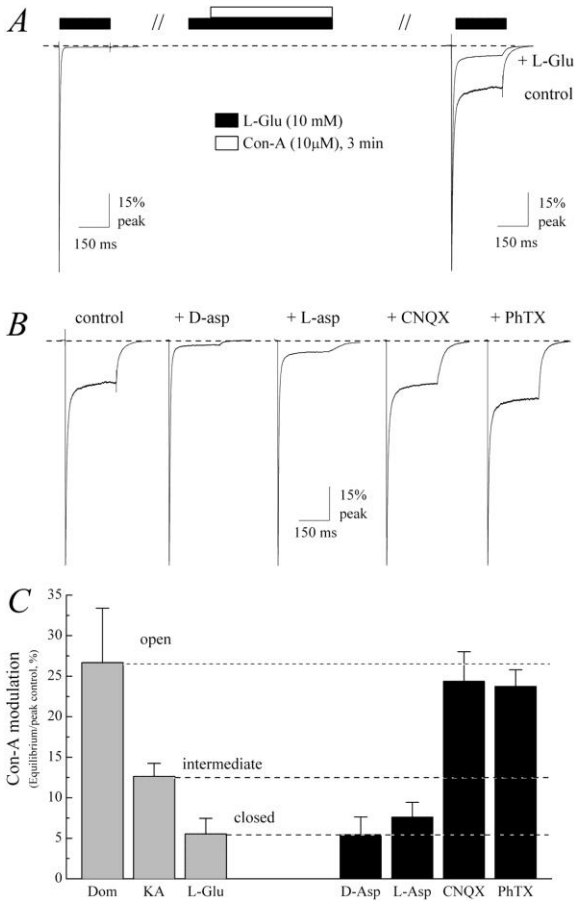


Figure 2.6 Conformational changes elicited by L-Glu and stereoisomers of Asp to the GluK2 agonist-binding pocket are indistinguishable. (A) Typical experiment showing how modulation by Con-A reports conformational changes in the GluK2 ABD (see (Fay and Bowie, 2006) for details). Con-A binds to a number of N-glycosylated residues in and around the GluK2 ABD. If agonist is bound, access to these sites is restricted, as a result, Con-A has a much weaker effect on the L-Glu equilibrium response. In the example shown, the equilibrium response is much smaller following co-application of Con-A and L-Glu (10 mM, Patch # 030724p2) than when Con-A is applied alone (Control, Patch # 01817p6). Filled and open bars indicate the application period of 10 mM L-Glu and 10 μM Con-A, respectively. The dotted line denotes the zero current level. The first and third applications of 10 mM Glu had a duration of 250 ms. (B) Experimental traces showing the extent of Con-A modulation as described in (A) with D-Asp (Patch # 071018p3), L-Asp (Patch # 07906p1), CNQX (Patch # 07913p2) and philanthotoxin (PhTX, Patch # 07104p1) compared to control. (C) Summary bar graphs showing the extent of Con-A modulation after co-treatment with various pharmacological agents (L-Glu, n =13; D-Asp, n = 4; L-Asp, n = 4; CNQX, n = 3; PhTX, n = 3). The dotted lines on the graph denote the extent of Con-A modulation observed for the open, intermediate and closed conformations of the GluK2 ABD which we have described previously (Fay and Bowie, 2006). CNQX and PhTX adopt the open conformation of the GluK2 ABD since their degree of Con-A modulation exactly matches that observed with Dom. In contrast, both D- and L-Asp adopt the closed conformation since modulation with Con-A is statistically indistinguishable from that observed with L-Glu. All data are expressed as the mean ± S.E.M.

State-dependent modulation by Con-A was therefore used to report the conformational changes elicited by D- and L-Asp. As positive controls, we compared the amount of modulation observed when GluK2 receptors were pre-incubated with Con-A and one of 3 agonists (i.e. 10 mM L-Glu, 1 mM KA or 50 μM Dom) (Fig. 2.6A and C). We have shown previously that Con-A modulation of GluK2 receptors pre-incubated with Glu, KA or Dom corresponds to the

closed, intermediate or open states of the ABD respectively (Fay & Bowie, 2006b) (Fig. 2.6C). As negative controls, we examined pharmacological compounds that would not be expected to induce significant closure of the GluK2 ABD which were the competitive antagonist, 6-Cyano-7-nitroquinoxaline-2,3-dione (CNQX), as well as the ion-channel blocker, philanthotoxin (PhTX). Although CNQX induces modest closure in the AMPAR ABD by acting as a partial agonist (Menuz et al., 2007a), this effect has not been observed at KARs consequently, we have assumed it behaves as a competitive antagonist.

As expected, pre-incubation with CNQX or PhTX did not interfere with the degree of modulation of GluK2 receptors by Con-A (Fig. 2.6B & C). In support of this, the degree of Con-A modulation observed with CNQX or PhTX was similar to that observed for the open conformation of the GluK2 ABD but statistically distinct from the closed or intermediate (Table 2.2). These findings suggest that occupancy of the pore with a channel blocker or the ABD with a competitive antagonist does not evoke appreciable closure of the GluK2 ABD. In contrast, pre-incubation with either D- or L-Asp significantly reduced the degree of Con-A modulation (Fig. 2.6B & C). In support of this, the degree of Con-A modulation observed with D-Asp or L-Asp was similar to that observed for the closed conformation of the GluK2 ABD but statistically distinct from the open or intermediate (Table 2.2). This finding further supports the central tenet of our study that weak partial agonists, such as D- and L-Asp, elicit conformational changes in the GluK2 ABD that are indistinguishable from conformations elicited by the full agonist, L-Glu.

Although these observations are consistent with Con-A reporting conformational changes in the GluK2 ABD, it was nevertheless important to evaluate alternate explanations. For example, it is possible that Con-A modulation reveals that stereoisomers of Asp adopt a similar desensitized conformation to L-Glu instead of reporting the extent of cleft closure. This possibility, however, is unlikely for three main reasons.

Table 2.2

Ligand	Glutamate (closed)	Kainate (intermediate)	Domoate (open)
D-Asp	n.s.	*	*
L-Asp	n.s.	*	*
PhTX	**	**	n.s.
CNQX	**	**	n.s.

** t significant at $p < 0.01$
* t significant at $p < 0.05$
n.s. means not significant

Table 2.2 Statistical comparisons between the degree of Con-A modulation observed with different GluK2 receptor ligands. he ability of stereoisomers of Asp, CNQX and PhTX to affect Con-A modulation of GluK2 receptors was compared with the modulation observed with L-Glu, KA and Dom using Student's *t*-test. The modulation observed by pre-incubating with stereoisomers of Asp was statistically significant from that observed with KA and Dom but indistinguishable from L-Glu. In contrast, the modulation observed by pre-incubating with CNQX or PhTX was statistically significant from that observed with L-Glu and KA but indistinguishable from Dom.

First, there is no available evidence to suggest that conformational changes in the dimer interface that accompany the onset of AMPA or KAR desensitization are agonist-dependent (Weston et al., 2006b;Armstrong et al., 2006b) which would be required to explain Con-A's effects. Second, Con-A binding and consequently modulation of GluK2 is almost entirely eliminated by mutation of 3 key N-terminal amino-acid residues that do not participate in forming the dimer interface (Fay & Bowie, 2006b). Although residues distant from the dimer interface may still regulate KAR desensitization, GluK2 receptors that lack the N-terminal desensitize normally (Plested & Mayer, 2007a) suggesting that this region of the intact receptor is not functionally coupled to the dimer interface. Third and finally, Con-A does not affect rates into or out of desensitization (Bowie *et al.*, 2003b;Fay & Bowie, 2006b) which would not be expected if lectin binding reports separation in the dimer interface. Given this, the most parsimonious explanation of our data is that Con-A reports conformational changes in the ABD of GluK2 receptor as discussed in detail elsewhere (Fay & Bowie, 2006b).

DISCUSSION

To our knowledge, this study is the first to identify a series of structurally-related amino-acids that exhibit the entire range of agonist behavior at KARs. Analysis of their structure-function relationship reveals that the agonist binding pocket of KARs is ideally suited to respond to the neurotransmitter, L-Glu, since modest changes in its chain length generates weak partial agonists. Using both *in silico* docking as well as measurements of conformations in the intact receptor, we show that the majority of full and partial agonists select for the closed conformation of the GluK2 ABD. Although this finding is not wholly surprising given the compact structures of most ligands tested, it is inconsistent with agonist efficacy being solely determined by the extent of closure in the KAR ABD. Exceptions to this were the partial agonists, KA and Dom, which select for the open and intermediate conformations respectively. However this finding can be simply explained by steric hindrance due to the Tyr 488 residue in domain 1 of the GluK2 ABD. Our findings suggest the value in looking more closely at the relationship between agonist efficacy and the extent of agonist-induced domain closure in KARs.

Can other mechanisms account for agonist efficacy at kainate receptors?

Although the view that agonist efficacy is governed by closure in the ABD has gained much popularity, recent work on AMPARs has identified a different mechanism (though not mutually exclusive) (Robert et al., 2005a;Zhang et al., 2008b) that may also account for full and partial agonist behavior at KARs. In essence, it is argued that the time the ABD remains in the closed conformation determines several gating properties of AMPARs including agonist efficacy, deactivation rates as well as apparent agonist affinity. For L-Glu, closed-cleft stability is optimized by direct and indirect interactions with domains 1 and 2 of the AMPAR ABD which permit L-Glu to attain full agonist activity whilst exhibiting rapid unbinding (Robert et al., 2005a;Zhang et al., 2008b); essential features for any fast-acting neurotransmitter. In the specific case of AMPARs, mutation of a key threonine (i.e. Thr686) residue in domain 2 of the GluR2 ABD,

disrupts the optimization established between the ligand and receptor. As a result, L-Glu is rendered a weak partial agonist with much lower affinity (Robert et al., 2005a).

There are several reasons to suggest that basic elements of the mechanism proposed by Zhang *et al* (2008) may also account for differences in efficacy between L-Glu and stereoisomers of Asp reported in this study.

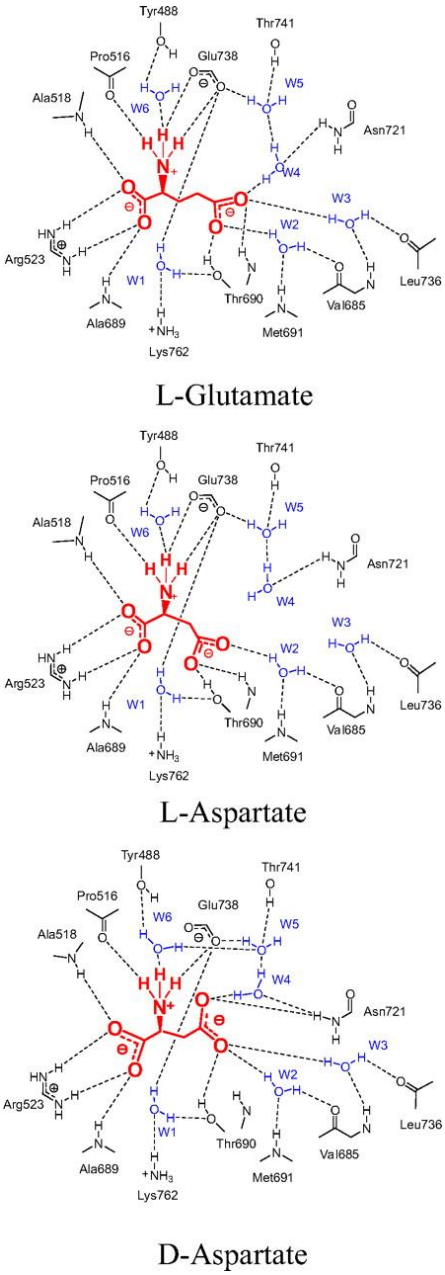


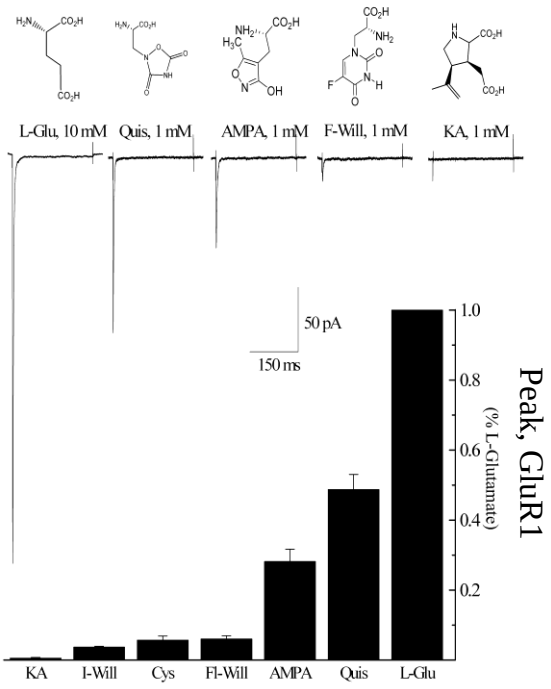
Figure 2.7 Stereoisomers of Asp establish fewer contact points with the GluK2 agonist binding pocket than L-Glu. 2-dimensional topographical maps of the GluK2 ABD shows that the number of contact points and the binding orientation of the full agonist, L-Glu, and partial agonists, D- and L-Asp. Topographical maps were deduced from structure complexes obtained with FITTED. Note the number of contact points made by D- and L-Asp was fewer than with L-Glu. In addition, the binding orientation is different between L-and D-Asp which would be expected for stereoisomers of the same amino acid.

First, activation curves of partial agonists, D- and L-Asp, are rightward shifted compared to the full agonist, L-Glu (Fig. 2.3C and D), suggesting that, in this case, agonist efficacy and affinity may be tightly correlated. Second, deactivation rates for stereoisomers of Asp (e.g. D-Asp, $\tau = 1.2 \pm 0.3$ ms) were faster than with L-Glu ($\tau = 2.6 \pm 0.2$ ms) (Bowie, 2002b). Third and finally, the more extended structure of L-Glu permits more contact points (direct and indirect) to be established with the GluK2 ABD than with D- or L-Asp (Fig. 2.7). This difference in agonist binding would be expected to weaken the stability of the closed GluK2 ABD. Notably, L-Asp formed considerably less contacts than D-Asp or L-Glu which may explain its weaker responsiveness based on analysis of activation curves (Fig. 2.3D). Although more work is required to rigorously test this model, it provides a valuable framework for future work on agonist behavior at KARs.

Are amino acids other than L-Glu suitable neurotransmitter candidates at kainate receptors?

Several of the amino acids examined in this study are endogenous to the CNS and have been previously evaluated as neurotransmitter candidates at glutamatergic synapses. The candidature of sulfur-containing amino acids, which include L-Cys, HC, SSC and HCSA, was considered after mechanisms that lead to their release, uptake and responsiveness (see below) were identified (Do et al., 1986b;Bouvier et al., 1991a). Recent attention has focused on their potent activation of metabotropic glutamate receptors (e.g. (Kingston et al., 1998a)). However, earlier work demonstrated that they also activate iGluRs (Thompson & Kilpatrick, 1996a). At the time, most investigators argued for their greater ability to activate NMDARs than AMPARs (Patneau & Mayer, 1990b) however, their effect on KARs was never tested. The reason being that evidence for the existence of this iGluR subclass had yet to emerge (Bowie, 2008b). In view of this, our data on homomeric GluK2 receptors suggests the value in testing the responsiveness of native KARs to sulfur-containing amino acids. Interestingly, the most potent sulfur-containing amino acid in our experiments, L-Cys, is a very weak partial

agonist on homomeric GluR1 AMPARs (Supplementary Figure S2). Therefore it would be interesting in future work to determine if different non-NMDA receptor subtypes discriminate amongst sulfur-containing amino acids.



S2: Supplementary Figure 2. Response profile of amino acids acting at GluR1 AMPA receptors. (Upper Panel) Structure-function relationship of five L-Glu analogs aligned in order of peak agonist efficacy. To allow comparison between experiements, membrane currents were normalized to the peak L-Glu in each recording. (Lower Panel) Summary bar graph comparing the peak responses amplitude observed with each agonist at the concentration indicated above. Note that L-cysteic, is a very weak partial agonist on homomeric GluR1 AMPARs, but a strong partial agonist at GluR6 KARs (c.f. Figure 2.2).

In comparison, there is more compelling evidence linking the stereoisomers of both serine and aspartate to roles in glutamatergic transmission (Boehning & Snyder, 2003a). D-Ser was considered in this capacity only after it was shown to act as a co-agonist at the glycine binding site of NMDARs (McBain et al., 1989b). Since D-serine is expressed in discrete populations of glial cells opposed to NMDARs (Schell *et al.*, 1997a), it has been categorized as a gliotransmitter (Mothet et al., 2000a;Panatier et al., 2006a). The role of D-Asp is more elusive

although it is found in the developing and adult brain (Schell et al., 1997c). Accumulation of D-Asp in CNS tissue has marked behavioral consequences, such as impaired motor coordination (Weil et al., 2006a), which is consistent with its putative role as a transmitter at the climbing fibers of the cerebellum (Wiklund et al., 1982a). Similarly, L-Asp's role in neurotransmission has centered on NMDARs (Fleck et al., 1993a), although, it elicits a high calcium conductance in cerebellar Purkinje cells that apparently involves a novel iGluR (Yuzaki et al., 1996a). Our study shows that D- and L-forms of each amino acid are weak partial agonists and, although these properties are not normally expected of a neurotransmitter candidate, it may be interesting to evaluate their roles at native KAR-containing synapses.

Conclusion

It is puzzling that not all iGluR subunits respond to the neurotransmitter, L-Glu. In fact, neither the NR1 NMDAR subunit nor the orphan-class delta-2 (δ2) subunit even bind L-Glu. Since the ancestral iGluR, GluR0, possesses a L-Glu binding pocket (Chen et al., 1999b), it is conceivable that evolving NR1 and δ2 subunits sacrificed this ability in order to serve more specialized roles in the mammalian CNS. In this regard, it is interesting that NMDARs (McBain et al., 1989b) and orphan-class δ2 iGluR (Naur et al., 2007a) retained their ability to bind D-Ser. Likewise, AMPARs (Brown & Bowie, unpublished observation) and KARs (present study) are also gated by D-Ser (and D-Asp) suggesting that these naturally occurring D-amino acids discriminate little amongst iGluR families. Whether this observation is a peculiarity of iGluRs that holds little biological significance or hints at a broader role for D-amino acids at glutamatergic synapses awaits future investigation.

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FOOTNOTES

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CHAPTER 3:

ARE EXTERNAL IONS AN ABSOLUTE REQUIREMENT FOR KAINATE RECEPTOR ACTIVATION?

PREFACE TO CHAPTER 3

We have shown in **CHAPTERS 1** and **2** that KA receptor responses are modulated by the binding of the plant lectin Con-A to KA receptors, an effect which is both state- and agonist-dependent (Fay & Bowie, 2006a;Fay *et al.*, 2009). Although this effect is mediated via a large glycoprotein, much smaller molecules such as single atoms have also been shown to affect KAR responses. In fact, our laboratory has previously shown that the external cations and anions modulate response amplitude and decay kinetics in a concomitant manner (Bowie & Lange, 2002;Bowie, 2002a). This interesting observation raised one main unresolved issue, namely, what is the mechanism by which external ions govern the kinetics of KARs? There are two possibilities which we will test in this *Chapter* using a heterologous expression system. One possibility is that ions simply **modulate the basal gating properties of KARs** in much the same way that phosphorylation regulates KAR kinetics. An alternative explanation is **that ions are an absolute requirement for kainate receptor activation**. This section of the chapter was published as a research article in the *Journal of Neuroscience* (Wong et al., 2006).

Article title:

External Ions Are Coactivators of Kainate Receptors

 This chapter was published as a research article in Molecular Pharmacology and was highlighted as the cover illustration for the May issue 2009 (Fay et al., 2009).

ABSTRACT

The activation of ligand-gated ion channels is thought to depend solely on the binding of chemical neurotransmitters. In this study, we demonstrate that kainate (KA) ionotropic glutamate receptors (iGluRs) require not only the neurotransmitter L-glutamate (L-Glu) but also external sodium and chloride ions for activation. Removal of external ions traps KA receptors (KARs) in a novel inactive state that binds L-Glu with picomolar affinity. Moreover, occupancy of KARs by L-Glu precludes external ion binding, demonstrating crosstalk between ligand- and ion-binding sites. AMPA iGluRs function normally in the absence of external ions, revealing that even closely related iGluR subfamilies operate by distinct gating mechanisms. This behavior is interchangeable via a single amino acid residue that operates as a molecular switch to confer AMPA receptor behavior onto KARs. Our findings identify a novel allosteric site that singles out KARs from all other ligand-gated ion channels.

Key words: agonist; glutamate receptor; desensitization; gating; epilepsy; activation

INTRODUCTION

In the vertebrate brain, ligand-gated ion channels are an important class of signaling protein designed to respond to a specific chemical neurotransmitter such as acetylcholine (ACh) (Colquhoun and Sakmann, 1998), L-glutamate (L-Glu) (Erreger et al., 2004), glycine (Lynch, 2004), or GABA (Maconochie et al., 1994). Although neurotransmitter substances are numerous in number (Krnjevic, 1974), all ligand-gated ion channels are thought to undergo conformations into the activated state by harnessing the energy from neurotransmitter binding (Colquhoun, 1998). There are no exceptions to this rule, although it has been recognized for some time that basal ion-channel activity is regulated by other factors such as phosphorylation and, more recently, by interactions with scaffolding proteins. Previously, we have shown that external anions and cations regulate both the response amplitude and channel kinetics of kainate (KA) ionotropic glutamate receptors (iGluRs) (Bowie, 2002; Bowie and Lange, 2002). Specifically, the rate of channel closure (i.e., deactivation) is ion dependent, suggesting that the stability of the activated/open state of the receptor is regulated by external ions as well as neurotransmitter binding. As yet, the molecular basis of this effect is not understood, although closely related AMPA receptors (AMPARs) are insensitive to external anions and cations (Bowie, 2002; Bowie and Lange, 2002; Paternain et al., 2003).

Here, we tested two opposing mechanisms to account for the effect of external ions on KA receptors (KARs). Experiments in ion-free solutions reveal that external anions and cations do not simply modulate basal receptor activity but instead are an absolute requirement for activation. This observation demonstrates unequivocally that external ions are coactivators of KARs. Furthermore, we identify allosteric cooperativity between ligand- and ion-binding sites and show that KARs enter into a novel inactive state when external sodium and chloride ions are absent.

MATERIALS & METHODS

Cell culture. tsA201 cells were transiently cotransfected with cDNA encoding wild-type (wt) or mutant GluR6 or GluR1 subunits and enhanced green fluorescent protein (eGFPS65T) as described previously (Bowie, 2002; Bowie and Lange, 2002). After transfection for 8–10 h (GluR6) or 12 h (GluR1), cells were washed and maintained in fresh medium. Electrophysiological recordings were performed 24–48 h later.

Mutagenesis. Mutation of GluR6(Q) was performed using the Stratagene (La Jolla, CA) Quickchange II XL site-directed mutagenesis kit. Mutant cDNAs were amplified, purified, and initially identified by restriction digest and confirmed by automated DNA sequencing of the entire GluR6 coding region (McGill University and Genome Quebec Innovation Center, Montreal, Québec, Canada).

Electrophysiology. Experiments were performed on outside-out patches, and agonist solutions were applied using a piezo-stack-driven perfusion system (Bowie, 2002; Bowie and Lange, 2002; Bowie et al., 2003). Solution exchange (10–90% rise time, 25–50 μs) was determined at the end of each experiment by measuring the liquid junction current. Recordings were performed with an Axopatch 200B amplifier (Molecular Devices, Palo Alto, CA) using borosilicate glass pipettes (4–6 Ω (Evans et al., 1982)) coated with dental wax. Current records were filtered at 10 kHz and digitized at 50–100 kHz, and series resistances (7–12 Ω (Evans et al., 1982)) were compensated by 95%. The reference electrode was connected to the bath via a 3 M KCl agar bridge. Data acquisition was performed using pClamp9 (Molecular Devices) and illustrated using Origin 7 (Microcal, Northampton, MA). All experiments were performed at room temperature.

Solutions. External solutions contained the following: 5 mM HEPES, 0.1 mM $CaCl_2$, 0.1 mM $MgCl_2$, and 2% phenol red, to which 1–405 mM NaCl was added as required. For solutions containing 150 mM external NaCl or less, the osmotic

pressure was adjusted to 290 mOsm using sucrose. For solutions with higher NaCl (>150 mM), the osmotic pressure was adjusted to 760 mOsm. pH was adjusted to 7.3 using 5N NaOH, with the exception of experiments presented in Figure 3.1 in which different cations were compared. In this case, pH was adjusted with the corresponding hydroxide solution (e.g., LiOH for LiCl). The internal solution contained the following (in mM): 115 NaCl, 10 NaF, 5 HEPES, 5 Na_4BAPTA, 0.5 $CaCl_2$, 1 $MgCl_2$, and 10 Na_2ATP, pH was adjusted to 7.3 with 5N NaOH, and the osmotic pressure was adjusted with sucrose to correspond with external solutions.

In experiments shown in Figures 3.2 and 3.3, solutions lacking external NaCl contained 100 µM each of $CaCl_2$ and $MgCl_2$ to improve patch stability, sucrose to maintain the osmotic pressure at 290 mOsm, and 5 mM Tris or 5 mM ammonium bicarbonate (NH_5CO_3) as pH buffers. With Tris, the pH was adjusted to 7.4 using 5N HCl, whereas the pH of NH_5CO_3-containing solutions was maintained by a gas mixture of 95%O_2/5%CO_2. For agonist solutions, the free acid of L-glutamate was dissolved in NaCl-free solution, and the pH was adjusted using 2.5 M Tris (for Tris buffer) or 2 M NH_5CO_3 (for NH_5CO_3-buffered solution).

Analysis. Concentration–response curve to external NaCl (see Fig. 3.1d, middle) was fit with the following equation: $\tau_{NaCl} = \tau_{max}/1 + (EC_{50}/[NaCl])^N$, where τ_{NaCl} represents the observed decay kinetics at any concentration of NaCl, τ_{max} is the slowest time constant for the fast decay component, which assumes that NaCl has a saturable effect, EC_{50} is the concentration of NaCl that elicits half-maximal decay kinetics, and *N* is the slope. Inhibition curves shown in Figure 3.4 were fit with a single- and double-binding site isotherm of the following forms: (for single) $I_{Glu} = I_{max}/1 + ([Glu]/IC_{50})^N$ and (for double) $I_{Glu} = I_{max(High)}/1 + ([Glu]/IC_{50(High)})N + (1 - I_{max(High)})/1 + ([Glu]/IC_{50(Low)})^N$, where I_{max} is the response to 10 mM L-Glu in the absence of preapplied L-Glu, IC_{50} is the concentration of L-Glu that elicits half-maximal inhibition, *N* is the slope, and the low-affinity component (I_{Low}) of inhibition by L-Glu is $1 - I_{max(High)}$. Data in all experiments are expressed as mean ± SEM from at least five patches.

RESULTS

External anions and cations modulate KAR amplitude and channel kinetics

Figure 3.1a shows typical effects of external monovalent ions on electrophysiological responses mediated by recombinant GluR6 KARs. Replacement of external Na^+ with an equimolar equivalent of either Li^+ or Cs^+ elicits a reduction in peak response amplitude as well as acceleration in decay (i.e., desensitization) kinetics (Fig. 3.1a, left). Similar findings were observed with an extended series of monovalent cations in which the degree of modulation was dependent on ion species (Fig. 3.1a, middle). Interestingly, substitution of external Cl^- with equimolar concentrations of other anions had a comparable effect to cation replacement (Fig. 3.1a, middle). Together, these data suggest that the chemical nature of the external solution and not its ionic strength regulates KAR gating behavior (Bowie, 2002).

The conventional explanation for these observations as described for other voltage- and ligand-gated ion channels (Yellen, 1997) is that external ions regulate the basal gating behavior of GluR6 KARs but are not an absolute requirement. In this case, the effect of external Cs^+ or propionate ions is to reduce channel activity, whereas it is increased by external Na^+ or Cl^-. An alternative is that GluR6 KARs exhibit an absolute requirement for external anions and cations; that is, external ions act as coactivators of KARs. In this case, external Na^+ or Cl^- ions are more effective in stabilizing KARs in the open state compared with Cs^+ or propionate ions. We will use the term "coactivator" throughout to include two possible mechanisms: (1) that ions affect KARs simply by binding or (2) ion binding causes conformational changes in the receptor, which affects function. To test whether external ions are coactivators of KARs, we compared GluR6 responses in 150 and 405 mM external ion solutions (Fig. 3.1a, right). Increasing external ion concentration to 405 mM prolonged GluR6 decay kinetics with each external anion (Cl^-, $NO3^-$) or cation (Na^+, Li^+, Cs^+) tested (Fig. 3.1a, right). Interestingly, a parallel shift in the relationship between response amplitude and decay kinetics was observed with the rank order of potency for each ion unchanged (Fig. 3.1a, right).

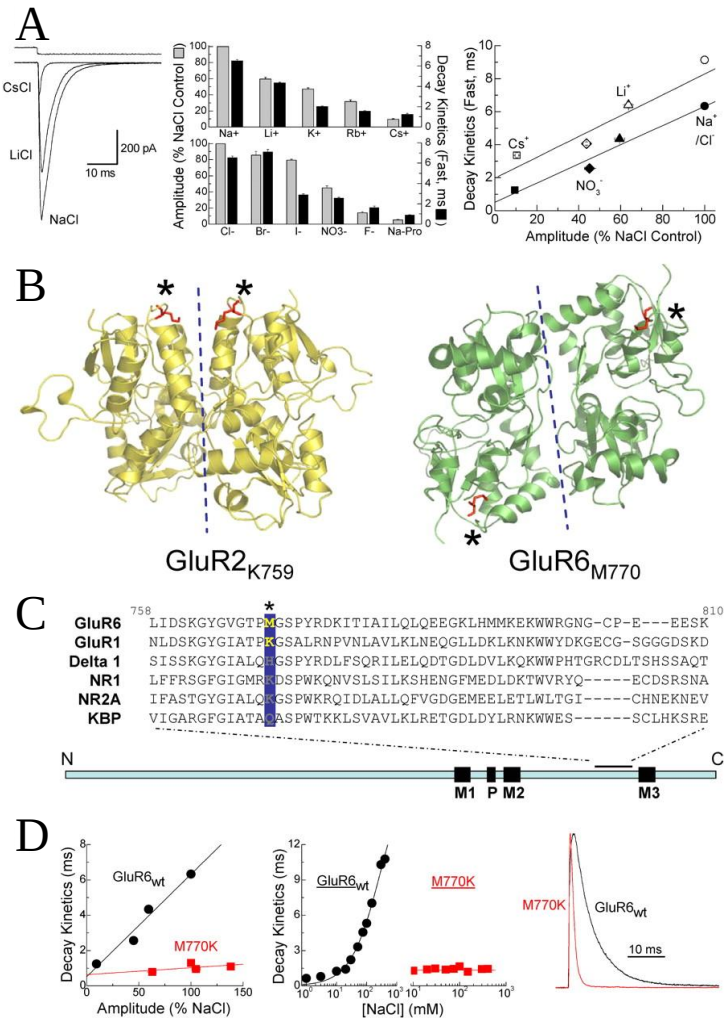

Figure 3.1 External anions and cations regulate GluR6 KARs. ***a***, Left, Membrane currents elicited by 10 mM L-Glu in 150 mM Na^+, Li^+, or Cs^+, with top trace showing solution exchange (patch 040419p2). Middle, Summary bar graph of external ion effects on GluR6 amplitude (gray bars) and decay kinetics (black bars). Right, Comparison between the amplitude and decay kinetics of GluR6 in different ion concentrations (150 mM, filled symbols; 405 mM, open symbols). Solid lines represent linear regression fits of the data at each ion concentration. ***b***, Crystal dimer structures drawn using Pymol show GluR2 (Protein Data Bank number 1FTJ) and GluR6 (Protein Data Bank number 1S7Y), with M770 and K759 in red and marked by asterisks. Dotted lines show dimer interface. ***c***, Sequence alignment of several iGluRs at the extracellular M2–M3 linker region, with the GluR6 M770 position highlighted in yellow. ***d***, $GluR6_{M770K}$ is unaffected by ion type [left, 150 mM Na^+ (Cl^-), Li^+, NO_3^-, and Cs^+] or concentration (middle, 1–405 mM NaCl) and has faster decay kinetics than $GluR6_{wt}$ (right, 150 mM NaCl).

This observation is inconsistent with a mechanism whereby external ions modulate the basal gating properties of KARs. In this case, Cs^+ ions would be expected to further accelerate decay kinetics at higher concentrations (i.e., 405 mM). However, the slowing of decay kinetics with all ion species tested supports the hypothesis that external ions are coactivators of KARs.

A single amino acid residue delineates between channel kinetics and response amplitude

It is interesting that KARs are the only iGluR whose response amplitude and decay kinetics is regulated by external anions and cations (Bowie, 2002; Bowie and Lange, 2002) despite structural (Mayer, 2005b) and functional (Dingledine et al., 1999) similarities with other family members, particularly AMPARs. In view of this, we further hypothesized that the gating mechanism of other iGluRs, such as AMPARs, do not have an absolute requirement for external ions; that is, agonist-induced conformational changes into the open state can still occur in the absence of external ions. Furthermore, given their considerable homology, we reasoned that it should be possible to interconvert the gating behavior of KA and AMPARs. In support of this, it has been shown that replacement of methionine-770 (M770) in GluR6 with its equivalent lysine (K752 for GluR1) residue in AMPARs (Fig. 3.1b,c) blocks ion modulation of the KAR response amplitude (Paternain et al., 2003). Interestingly, recent x-ray diffraction studies of GluR6 KAR (Mayer, 2005a) and GluR2 AMPAR (Armstrong and Gouaux, 2000) dimers places these residues in different locales of the quaternary structure (Fig. 3.1b, red labels and asterisks). An important caveat, however, is that another group has suggested a different dimer organization closer to that of GluR2 AMPARs (Nanao et al., 2005). Although the precise nature of the dimer interface awaits additional study, the structure reported by Mayer (2005) is consistent with KARs possessing a unique ion-binding site(s) that regulates the peak response amplitude. Given the concomitant effect of external ions on amplitude and decay kinetics (Bowie, 2002), we were therefore interested in testing whether M770K also affects GluR6 decay kinetics.

Figure 3.1d summarizes a series of experiments in which desensitization kinetics of GluR6M770K and GluR6wt were compared. In contrast to GluR6wt, the decay kinetics of GluR6M770K were almost identical for all external anions (i.e., Cl^- or $NO3^-$) and cations (i.e., Na^+, Li^+, and Cs^+) tested (Fig. 3.1d, left), suggesting that inclusion of a positively charged lysine at the 770 site is sufficient in abolishing ion-sensitive effects on channel kinetics. Unexpectedly, external anions and cations continued to regulate the peak response amplitude of GluR6M770K, contrary to Paternain et al. (2003). In this case, the rank order of potency was different between GluR6M770K [$NO_3^- > Na^+$ (or Cl^-) ~ $Li^+ > Cs^+$] and GluR6wt [Na^+ (or Cl^-) > $Li^+ > NO3^- > Cs^+$] (Fig. 3.1d, left). GluR6M770K mutant also blocked the effect of changing the external Na^+ and Cl^- ion concentration on desensitization kinetics (Bowie, 2002; Bowie and Lange, 2002) (Fig. 3.1d, middle), further supporting the pivotal role of M770 in controlling KAR gating behavior. Interestingly, in 150 mM external NaCl, the decay kinetics of GluR6M770K were several-fold faster than GluR6wt (Fig. 3.1d, right) or GluR1 AMPARs (data not shown), suggesting that amino acid residues other than the M/K site may be involved in endowing KARs with ion-dependent gating. As reported by others (Paternain et al., 2003), mutant AMPA receptors containing a Met residue instead of Lys express poorly, and, therefore, we were unable to examine the ion sensitivity of GluR1K752M.

External anions and cations are an absolute requirement for KAR activation

To directly test whether KAR activation has an absolute requirement for external ions, we recorded GluR6 responses in the absence of external NaCl at a range of membrane potentials (–100 to +110 mV, 15 mV increments) (Fig. 3.2). To do this, experiments were performed using the free acid of L-glutamate and either Tris or bicarbonate buffers to maintain an external pH of 7.3 (see Materials and Methods). For comparison, we repeated experiments on GluR1 AMPARs and the KAR mutant GluR6M770K. GluR6wt were entirely unresponsive in the absence of external NaCl at all membrane potentials tested (Fig. 3.2, left), consistent with our hypothesis that external ions are coactivators of KARs. Figure 3.2 (bottom,

left) shows the current–voltage (I–V) relationships observed in 0 mM NaCl (filled circles) compared with I–V plots in 10 mM (open squares) and 150 mM (open triangles) external NaCl. In contrast, GluR1 AMPARs were fully responsive in the absence of external NaCl (Fig. 3.2, middle), suggesting that external ions are not an absolute requirement for the gating behavior of this iGluR subfamily. The membrane current observed represents the outward movement of permeating ions (i.e., Na^+) from the internal solution of the patch pipette. Interestingly, GluR6M770K was also responsive in solutions lacking external NaCl (Fig. 3.2, right), supporting the pivotal role of the M/K site in determining KAR gating behavior. Moreover, this observation eliminates the possibility that the functional effects observed in low ionic strength solutions are not attributable to denaturation of the quaternary structure of the intact KARs.

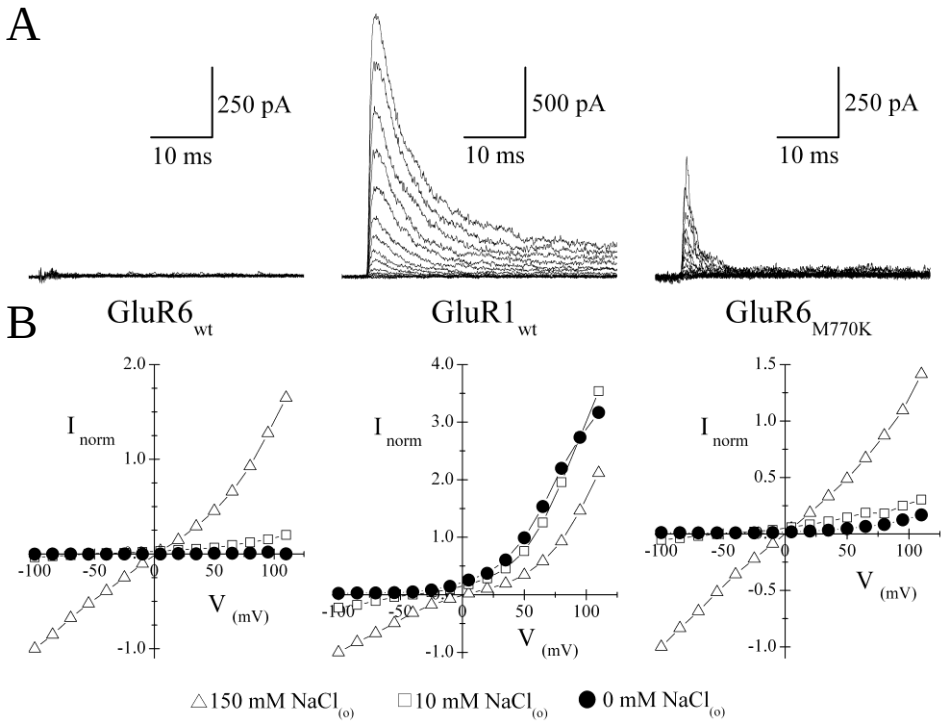

Figure 3.2 GluR6 KARs have an absolute requirement for external ions. ***a***, Superimposed family of membrane currents evoked by 1 mM L-Glu acting on $GluR6_{wt}$ (patch 050311p1), $GluR1_{wt}$ (patch 050321p2), and $GluR6_{M770K}$ (patch 050405p2) receptors in solutions lacking external NaCl (range, –100 to +110 mV, 15 mV increments). ***b***, Averaged current–voltage plots in 0 mM (filled circles), 10 mM (open squares), and 150 mM (open triangles) NaCl for each iGluR tested.

Crosstalk between agonist- and ion-binding sites

Our observations cannot be explained by the failure of agonist binding to KARs in NaCl-free solutions. In support of this, GluR6 KARs failed to respond to both NaCl and L-Glu when preincubated in solutions lacking external ions but containing L-Glu (Fig. 3.3a).

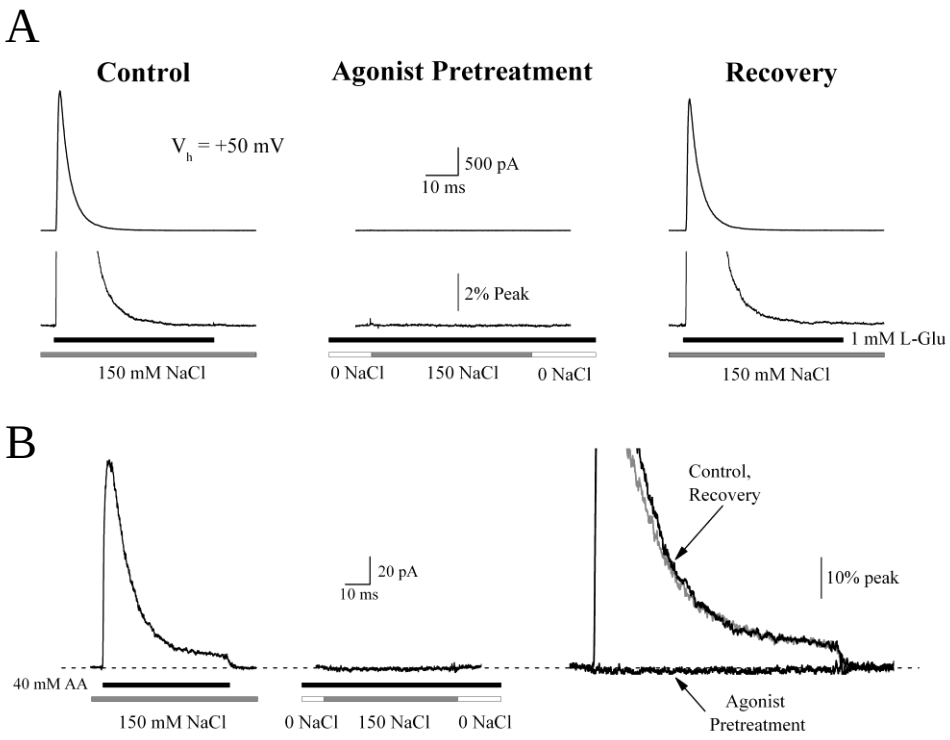

Figure 3.3 External ions are not a prerequisite for agonist binding to KARs. ***a***, Typical experimental traces (V_h of +50 mV) in which the effect of preincubating GluR6 KARs in NaCl-lacking solutions but containing 1 mM L-Glu was tested (patch 050711p1). Left, GluR6 receptors elicited robust responses to 1 mM L-Glu when preincubated in 150 mM external NaCl. Middle, In contrast, GluR6 receptors were unresponsive to NaCl when pretreated in 0 mm NaCl and 1 mM L-Glu. Right, The response was fully recovered during the addition of 150 mM NaCl to external solutions. ***b***, Same experiment as in ***a*** using 40 mM AA instead of L-Glu (patch 050818p1). ***c***, Experimental traces from ***b*** superimposed for comparison.

This demonstrates that agonist binding (and subsequent receptor desensitization) can occur in the absence of external ions. If agonist binding had not occurred, GluR6 receptors would be expected to respond to the application of 150 mM NaCl (Fig. 3.3a, middle). Unexpectedly, however, we did not observe an

equilibrium current typically associated with desensitized GluR6 receptors (Bowie and Lange, 2002; Bowie et al., 2003). We therefore hypothesized that agonist binding precludes the ability of external ions to bind. Because it was difficult to resolve equilibrium responses with L-Glu, we repeated the experiments using L-aminoadipate (AA) (40 mM), which elicits larger equilibrium responses (Fig. 3.3b, left). As with L-Glu, application of NaCl failed to elicit an equilibrium response after pretreatment with AA (Fig. 3.3b, right, c). Together, we have shown that, although external ions are required for KAR functionality, GluR6 receptors are able to bind agonists in solutions lacking external ions. Furthermore, conformational changes elicited by agonist binding prevent subsequent ion binding. To explain the failure of GluR6 receptors to respond in solutions lacking external ions, we show below that their removal accumulates KARs in a novel inactive state with high agonist affinity.

Identification of a novel inactive state with picomolar agonist affinity

Figure 3.4, a and b, shows a typical experiment in which the occupancy of the desensitized states was determined from inhibition of 10 mM L-Glu responses after incubation in L-Glu (0.1–50 µM). Similar experiments were also performed in 5, 10, 75, 150, and 405 mM external NaCl. A family of curves observed in different concentrations of NaCl were then fit with a single- or double-binding site model of inhibition as shown in Figure 3.4c. The inhibition of L-Glu responses in 150 and 405 mM NaCl (Fig. 3.4c, open and filled circles) were best fit with a single binding site isotherm estimating the IC_{50} to be 0.49 ± 0.04 and 0.56 ± 0.09 µM, respectively, in good agreement with previous studies of GluR6 (Wilding and Huettner, 1997; Paternain et al., 1998). At lower NaCl levels, inhibition plots were biphasic, revealing a high affinity, NaCl-dependent binding site with IC_{50} values of 50 ± 20 pM in 5 mM NaCl and 0.8 ± 0.6 nM in 10 mM NaCl (Fig. 3.4c,d). Extending our observations, <5 mM NaCl was not possible because membrane currents were small in amplitude, making measurement and analysis difficult.

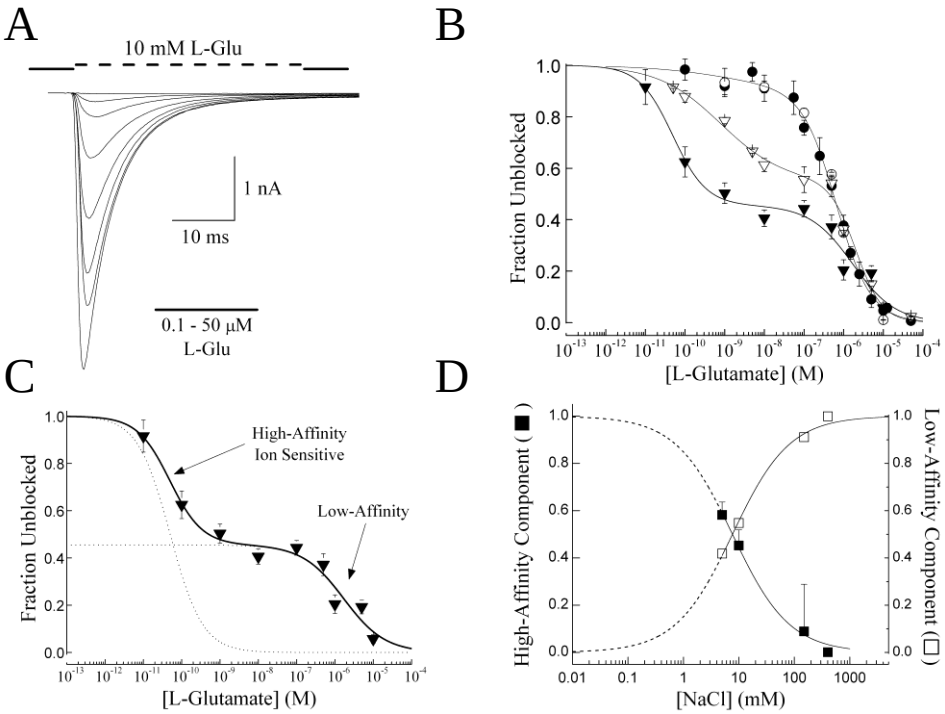

Figure 3.4. External ions regulate occupancy of a novel, high-affinity inactive state. ***a***, Typical experiment showing the onset of and recovery from inhibition of peak GluR6 responses by L-Glu. ***b***, Individual traces shown in ***a*** superimposed to show response profile in detail (patch 030613p1). ***c***, Family of inhibition curves to L-Glu in 5 mM (filled triangles), 10 mM (open triangles), 150 mM (filled circles), and 405 mM (open circles) NaCl. Solid lines are fits to single- or double-binding site isotherms. The IC_{50} of the low-affinity state was concentration independent (1.5 µM), whereas the Hill coefficient, n_h, was 0.8 in 5 mM NaCl and 1.1 in 10–405 mM NaCl. The IC_{50} for the high-affinity inactive state was concentration dependent, being 70 nM (n_h = 0.5) in 10 mM NaCl and 10 pM (n_h = 1.0) in 5 mM NaCl. ***d***, Inhibition curve observed in 5 mM external NaCl in more detail showing contribution of high- and low-affinity states (dotted curves). ***e***, Effect of NaCl on the fractional occupancy of high- and low-affinity states. Dotted lines represent fit extrapolations.

Moreover, patch stability was compromised in low ionic strength solutions. However, extrapolated fits of occupancy of the high- and low-affinity states (Fig. 3.4d) revealed that GluR6 receptors accumulate into this high-affinity inactive state as external ions are lowered. This finding explains the failure of KARs to gate in the absence of external NaCl (Fig. 3.2a, left).

DISCUSSION

Classically, it has been thought that ligand-gated ion channels depend solely on chemical neurotransmitters for activation. This is exemplified by work on nicotinic ACh receptors in which channel lifetime is solely dependent on the nature of the ligand (Colquhoun and Sakmann, 1985). Here we show that external ions are coactivators of KARs, suggesting, unexpectedly, that channel lifetime is not only controlled by the ligand but also by ions. As yet, it is not clear whether external ions control KARs simply by binding or whether an additional conformational change is required in much the same way that glycine acts as a coagonist at NMDARs (Kleckner and Dingledine, 1988). It is also unclear whether M770 represents the ion-binding site(s) or a residue critical in the transduction process. Intuitively, it would be expected that anions and cations bind to discrete sites to satisfy electrostatic principles; however, other mechanisms, such as the establishment of a dipole, are possible (Bowie, 2002). Differentiating between these mechanisms can only be resolved through additional structure–function analysis of KARs.

How ligand and external ions determine the stability of the open state is not clear, but two possibilities may be considered. First, if external ions have a lower affinity (i.e., shorter residency time) than ligands, the time the KAR ion channel remains in the open state will be dependent on the rate of ion unbinding. This mechanism, however, fails to account for the slowing of channel kinetics at elevated ion levels (Bowie, 2002). Moreover, because agonist occupancy prevents ion rebinding (Fig. 3.3), KARs would also be expected to accumulate into the novel inactive state during prolonged agonist application. In this case, a decline in the equilibrium response would occur as ions unbind. However, we observe a well maintained equilibrium response, arguing against this mechanism (Fig. 3.3b). Alternatively, external ions may stabilize the ligand-binding cleft, which would account for the slowing of KAR deactivation and desensitization at high ion concentrations as well as the sustained equilibrium response. However, this mechanism seems inconsistent with the observation that L-Glu activation curves are weakly ion dependent (Bowie, 2002). Clearly, if KAR gating is to be

elucidated, an important step will be to resolve the interplay between ligand and external ions.

Although the M770 residue is restricted to GluR6 and GluR7 subunits, equivalent residues in other KAR subunits also confer sensitivity to external Cs^+ block (Paternain et al., 2003). In contrast, all AMPA and NMDA iGluRs have a conserved lysine residue in the M/K position (Fig. 3.1c), suggesting that only members of the KAR family are coactivated by external ions. Recent crystallographic work has indicated that full and partial agonists elicit different degrees of closure in the ligand-binding core of GluR6 KARs (Mayer, 2005a; Nanao et al., 2005). From work on AMPAR crystal structures, the degree of domain closure has been shown to be directly correlated to agonist efficacy (Jin et al., 2003). Our results, however, suggest that agonist behavior is not governed solely by conformations in the agonist-binding domain but that occupancy of a novel ion-binding site(s) must also be considered.

Finally, KARs are therapeutic targets in the treatment of several neurological diseases, including neuropathic pain (Palecek et al., 2004) and epilepsy (Smolders et al., 2002). Although some success has been achieved in developing selective KAR antagonists, a recurrent obstacle is that most, if not all, strategies rely on exploiting differences between the agonist-binding domain of iGluR subtypes (Bleakman et al., 2002). This is particularly problematic for AMPARs and KARs whose agonist-binding domains have overlapping pharmacology (Dingledine et al., 1999), which is expected given their significant structural homology (Mayer and Armstrong, 2004; Mayer, 2005a; Nanao et al., 2005) (Fig. 3.1). In principle, drug selectivity is best achieved when a unique pharmacological target can be identified. Our findings point to a mechanism that may be exploited to design drugs with a high selectivity for KARs that are useful in the treatment of neurological conditions.

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FOOTNOTES

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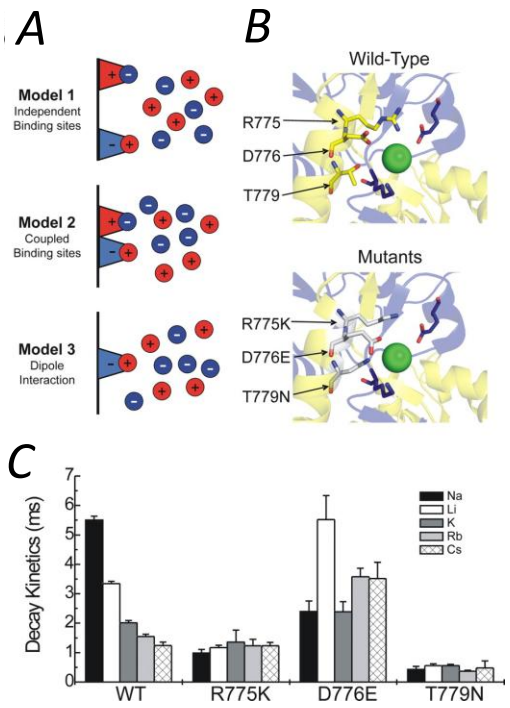
UPDATED DISCUSSION ON CHAPTER 3

In continuation to the study presented in this Chapter, a great deal of effort has been dedicated to: (1) identify the curious effect of cations and anions alike and (2) elucidate the nature of the ion binding site at KA receptors. Surpisingly, the initial breakthrough in this pursuit was the elucidation of a single anion atom binding site in a cavity established at the interface of two KAR subunits (Plested & Mayer, 2007b). This finding was unexpected given that we have shown in **CHAPTER 3** that only a positively-charged Lys tethered to the putative cation binding site was necessary to restore channel function (Wong *et al.*, 2006b).

To examine this apparent discrepancy, findings from our laboratory examined possible mechanisms described previously (see 4.5.2.3) for monovalent interaction at KA receptors (Wong *et al.*, 2007, see Supplementary Figure S3). Briefly, the three following simple models were considered: (1) independent binding sites, (2) coupled binding and (3) dipole interaction, with the latter being supported by experimental findings with a series of receptor mutants of key amino acids in the putative cation binding site.



S3: Supplementary Figure 3. Possible mechanisms for monovalent ion interactions at KARs. ***a***, Schematic showing three distinct models to explain the effect of monovalent anions and cations on GluR6 KARs. ***b***, Top, Crystal structure showing critical amino acid residues that constitute the proposed anion binding site (Protein Data Bank number 2F34). Although only one dimer is shown, the ion is also conjugated by the corresponding amino acids from the adjacent subunit. ***b***, Bottom, Proposed anion binding site containing point mutations (R775K, D776E, and T779N) that interfere with both anion and cation modulation of GluR KARs. Note that each mutation elicits an enlargement of the anion binding pocket through changes in the orientation of each residue. ***c***, The effect of changing external cation species with the various anion binding site mutants. Both R775K and T779N abolish cation modulation, whereas cation modulation is markedly reduced in the D776E mutant. Data are mean SEM of at least three patches per mutant in each cation. Reproduced from Wong et al. (2007) Journal of Neuroscience. 27 (25): 6800-6809.

Disruption of the putative cation binding pocket was shown to significantly affect anion binding (Wong et al., 2007). Similarly, disruption of the identified anion binding site (Plested & Mayer, 2007b) was shown to alter cation binding (Wong et al., 2007), indicating a clear coupling between the anion and putative cation binding. Soon afterward, the Mayer laboratory elucidated the cation binding pocket (Plested et al., 2008), revealing that the location of the cation binding site was distinct from that of anions. They confirmed the predicted proximity of the Met770 residue with a number of exposed carbonyl residues, thus creating an overall electrostatic binding pocket for the cation (Wong et al., 2007). Together, these findings revealed that the KA receptor dimer interface binds Na^+ and Cl^- ions at a stoichiometry of 2:1 (Plested et al., 2008) where anion and cation binding pockets are structurally distinct, though functionally coupled (Wong et al., 2007). Interestingly, estimates of anion and cation affinity revealed that only anions are occupied at physiological pH (Plested & Mayer, 2007b;Plested *et al.*, 2008). These findings raise the obvious question as to why KA receptors are sensitive to external ions, an issue which has been addressed in detail in a later section (see Discussion & Conclusions - 2.1 and 2.2).

PART THREE

DISCUSSION

&

CONCLUSIONS

GENERAL REMARKS

Disorders of the CNS have puzzled society since early historical times and continue to represent a major challenge for modern medicine. The scientific study of the nervous system underwent significant progress in the second half of the twentieth century, mainly because of technical breakthroughs in the areas of molecular biology, imaging, electrophysiology and computational neuroscience. With these tools, it is becoming possible to understand, in great detail, the complex processes occurring at the level of single ion-channels at synapses. The glutamatergic system is the prominent pathway for excitatory information in the brain, and is thus essential for its normal function. As elaborated in the review of literature, KA iGluRs are key components of the glutamatergic system.

This aim of this section is to focus on the implications of our results for future work and, in some cases, suggest specific experiments required to move the field forward. First, I will consider the significance of the lack of correlation between domain closure and agonist efficacy at KA receptors for other iGluRs subfamilies. Moreover, I will present testable models for receptor activation. Secondly, I will speculate on why KA receptors are sensitive to ions by considering their potential role as detectors of ions fluxes. Thirdly, I will explore the possibility that Con-A may mimic the behavior of an endogenous protein. Finally, I will consider the hypothesis that amino acids, other than L-glutamate, may be transmitters at central synapses.

1. KAINATE RECEPTOR ACTIVATION

1.1. Domain Closure and Agonist Efficacy at Kainate Receptors

As suggested for AMPA receptors (Jin *et al.*, 2003a), the separation between the two *lobes* of the S1S2 construct was recently argued to correspond positively with the extent of receptor activation at KA receptors (Mayer, 2005a). As discussed below, a number of studies (including the work presented here (Wong *et al.*, 2006b;Fay *et al.*, 2009)) have identified complicating factors, which bring into question this model that has almost become dogma. Here, I discuss distinct

lines of evidence suggesting that the proposed relationship does not explain agonist efficacy at KA receptors.

The missing crystal. First of all, an important issue to consider in measuring and interpreting lobe closure is the missing apo crystal. Although the agonist-binding domain of the GluR5 and GluR6 KA receptor subunits have been crystallized with different ligands and ions (Nanao *et al.*, 2005a;Mayer, 2005a;Naur *et al.*, 2005;Hald *et al.*, 2007;Mayer *et al.*, 2006;Plested *et al.*, 2008;Frydenvang *et al.*, 2009), it has yet to be crystallized in the absence of any ligand (i.e., apo state). In fact, the domain closure calculations have been entirely based on the apo state of AMPA receptors (Naur *et al.*, 2005;Nanao *et al.*, 2005a;Mayer, 2005a).

To illustrate the important limitations associated with this, recent studies have shown that the agonist-binding domain of KA receptors appears to be capable of hyper-flexibility compared to AMPA receptors (Mayer et al., 2006;Du et al., 2008;Hald et al., 2007). Consistent with this, using molecular dynamics, Lau and Roux (2007) have shown that the apo S1S2 AMPA receptor easily accesses low-energy conformations that are more open than the observed crystallographic data.

Likewise, luminescence resonance energy transfer (LRET)-based measurements revealed that the ligand-binding domain GluR6 in the apo state adopts a conformation which is much more open than anticipated from AMPA receptor crystals (Du et al., 2008). In fact, the distance between the apo-state and the L-glutamate bound GluR6 complex was similar to that observed between the glutamate and the competitive antagonist UBP-310 complexes with the GluR5 agonist-binding domain (Du et al., 2008;Mayer et al., 2006). This evidence suggests that binding of full agonists at KA receptors induces cleft closure of 29°-30° (and not 26.6° as previously proposed from comparison with the apo state of GluR2 (Mayer, 2005a)), representing one of the largest measured within the iGluR family (Du et al., 2008). Though these findings are consistent with the notion that closure of the agonist-binding domain may be one of the factors controlling efficacy, it illustrates the constraints of the static crystals.

External ions are co-activators. Secondly, external ions must bind to the Met770 site to allow activation of GluR6 KA receptors (Wong *et al.*, 2006b;Plested *et al.*, 2008). Curiously, this mutant receptor does not lose the ability to bind L-glutamate (Wong *et al.*, 2006b), suggesting that the ligand-binding domain may adopt a closed conformation similar to that observed in L-glutamate-bound crystals (Mayer, 2005a). Clearly, the ion-binding site plays a critical role in determining agonist behavior at KA (but not AMPA) receptors and suggests that closure alone cannot predict agonist efficacy.

New kainate receptor agonists. A third complicating issue is illustrated by the work presented in **CHAPTER 2**, where we have shown that domain closure of the agonist-binding domain of the GluR6 KA receptor does not correlate with agonist efficacy (Fay et al., 2009). Since we have used an *in silico* approach, there is clearly an urgent need to resolve GluR6 structures with these new ligands to test if static snapshots of these complexes also reveal a comparable conformation. Excitingly, this work is currently in progress in our laboratory. It is perhaps not wholly surprising that we have found a different result than proposed by Mayer since his conclusion was based on only four agonist-GluR6 complexes (Mayer, 2005a). Here, we have compared 16 ligands at KA receptors and demonstrate that its ligand-binding domain adopts comparable conformations when bound with full agonists and partial agonists at KARs (Fay et al., 2009).

One possibility to explain these findings is that the time the ligand-binding domain remains in the closed conformation governs channel properties such as agonist efficacy and affinity, as proposed by Zhang et al. (Zhang *et al.*, 2008a). Essentially, serine and aspartate are expected to make fewer and weaker interactions with residues in the binding pocket which would be translated into a shorter residency time in the closed cleft conformation than L-glutamate, even though these agonists induce comparable extent of domain closure (Fay et al., 2009).

This explanation cannot account for the behavior of the partial agonists, KA and domoate which adopt intermediate and open clamshell conformations, respectively (Mayer, 2005a;Nanao *et al.*, 2005a). Interestingly, these neurotoxins display more complex interaction networks than L-glutamate within the binding site and their pyrrolidine ring structure occludes complete binding of the clamshell through steric hindrance via tyrosine 488 (Fay et al., 2009). Thus, a combination of factors render L-glutamate exquisitely designed to act as a full agonist at these receptors.

A kainate receptor antagonist induces full domain closure. Consistent with this idea, Kastrup and colleagues subsequently resolved GluR5 crystal structures in complex with the weak partial agonist, dyshiberbaine, and the functional antagonist, 8,9-dideoxy-neodysiherbaine (MSVIII-19) which both induced full closure of the ligand-binding domain (Frydenvang et al., 2009). Both ligands exhibited a degree of relative domain closure (~30 degrees) that was similar to that of the full agonist L-glutamate, thus directly challenging the prevailing model of KA receptor activation.

The question is, why should only one simple factor (i.e., domain closure) dictate agonist efficacy. Moreover, how and why do ligands elicit distinct degrees of desensitization (i.e., efficacy at equilibrium)? As detailed in a later section, it is very likely that agonist efficacy at iGluRs is multi-factorial. Finally, if this is true for KA receptors, then what does this imply for other iGluRs? Examination of the literature reveals several issues that limit and/or complicate the interpretation of the prevailing model at NMDA and AMPA receptors.

1.2. Domain Closure and Agonist Efficacy at Other iGluR Families

1.2.1. NMDA Receptors

At NMDA receptors, crystal structures reveal no correlation between domain closure and agonist efficacy. In support of this, comparison of the NR1 NMDA receptor subunit in complex with full (glycine/ D-serine) or partial agonists (D-cycloserine, 1-aminocyclopropane-1-carboxylic acid (ACPC), 1-aminocyclobutane-1-carboxylic acid (ACBC) revealed no difference in the degree of domain closure (Furukawa & Gouaux, 2003;Inanobe *et al.*, 2005b). In contrast, the NR1 ligand-binding domain in complex with NR1 antagonists such as 5,7-dichlorokynurenic acid (DCKA) or 1-aminocyclopentane-1-carboxylic acid (cycloleucine) was shown to adopt an open conformation (Inanobe *et al.*, 2005b;Furukawa & Gouaux, 2003). Accordingly, NMDA receptor agonist behavior was described by differences in the ability of the bound-agonist to shift the equilibrium between the closed and open state of the channel, as described by the two-state model introduced previously (Figure 6, section 4.1.1.1).

This conclusion, however, must be tempered by at least two qualifications. First, since only five agonist-bound NR1 complexes have been resolved, additional ligands (McBain *et al.*, 1989a) need to be co-crystallized with this subunit to further evaluate this prediction. Secondly, one cannot neglect that NMDA receptors require both glycine and glutamate for activation with NR1 and NR2 forming glycine and glutamate sites, respectively (Benveniste & Mayer, 1991;Clements & Westbrook, 1991;Furukawa *et al.*, 2005). However, the NR2 X-ray crystal structure has yet to be resolved with ligands other than L-glutamate (Furukawa et al., 2005) and the nature of the isolated binding core cannot address intersubunit constraints present in the mature receptor.

1.2.2. AMPA Receptors

At AMPA receptors, the S1S2 domain has been crystallized with over ten ligands (Erreger et al., 2004) and agonist efficacy is thought to be correlated with clamshell closure (Jin *et al.*, 2003a). Careful scrutiny of these experiments and

subsequent studies reveal that there are many important observations which cannot be explained by this model.

Nature of the partial agonists. A first concern is the nature of the agonists selected for the influential study on AMPA receptors which argued to demonstrate the structural basis of iGluR activation (Jin *et al.*, 2003a). The 5-substituted willardiines used, which vary by a single atom (Patneau et al., 1992), may have inherently biased the outcome of their study. In fact, since the uracil ring of these compounds produces substantial structural changes in the agonist-binding pocket, it is perhaps not surprising that an increase in the size of the 5-substitutent on the ring is associated with a graded increase in the cleft opening between the two lobes (Jin *et al.*, 2003a). Given this, a more relevant test of their hypothesis would be to use agonists which, though they share a common L-glutamate backbone, exhibit more structural diversity such as the ligands we have tested at KA receptors (Fay et al., 2009). Why should only one parameter, rotational movement of the two lobes together, account for agonist efficacy? In fact, testing agonists which are so closely related may not allow probing of other parts of the protein pivotal to agonist binding.

The lobes are not rigid structures. A second issue is that the angle of lobe closure may not be the only factor to consider and may not be fixed in all cases. In line with this, partial AMPA receptor agonists elicit a significant degree of twist in the agonist binding domain, which could not have been anticipated using a single rotational measurement between the apo and agonist-bound state (Bjerrum & Biggin, 2008b). Moreover, molecular dynamics simulations has suggested that the two lobes showed more movement during the apo-state compared to agonist-bound for AMPA receptors (Arinaminpathy *et al.*, 2006b). Interestingly, AMPA receptors bound with partial agonists displayed a significant increase in the extent of protein fluctuations, compared with the full agonist, L-glutamate (Arinaminpathy *et al.*, 2006b). This suggests that the degree of protein flexibility

in the agonist-binding domain may be used as one of the many indicators of agonist efficacy.

Importantly, the apo state of the AMPAR most likely occurs as an ensemble of states (Madden et al., 2005). Likewise, GluR2 in complex with various ligand types may be trapped in a spectrum of conformations of the solution at low temperature using NMR (Ahmed et al., 2007). Both these studies are consistent with measurements of dynamics using NMR spectroscopy on a range of timescales, suggesting that the agonist-binding domain of GluR2 is relatively flexible (McFeeters & Oswald, 2002). Specially, the β-sheets and the residues in the binding site which contacts the α-substitutents of the L-glutamate backbone in Lobe 1 were shown to exhibit very little motion on the μs-time scale. Conversely, both the β-sheets and the residues in the binding site which contacts the γ-substituents of the L-glutamate backbone in Lobe 2 displayed flexibility on a faster μs-time scale (McFeeters & Oswald, 2002). Although the lobes of the iGluR crystals are often considered as rigid, the situation is clearly more complex in the intact receptor under physiological conditions in solution.

Different crystal forms. Significantly, the GluR2 L650T mutation which partially removes steric constraints on lobe closure is found in different crystal forms (Armstrong et al., 2003). Despite the fact that AMPA is partial agonist at this receptor, in complex with this mutant it crystallized as four fully closed constructs and one partially open, displaying a $\approx 10^{o}$ range in domain closure (Armstrong et al., 2003). Moreover, fluorescence resonance energy transfer (FRET)-based measurements also showed that this mutant GluR2 receptor was, in fact, more closed than expected from its efficacy (Ramanoudjame et al., 2006). In line with this, the GluR2 T686A alters agonist efficacy of both L-glutamate and quisqualate without changing the extent of domain closure observed in the isolated binding domain (Zhang *et al.*, 2008a). In sum, although lobe closure is often regarded as being fixed, these exemplify the notion that many complexes may be found in different crystal forms.

Changing the salt. Another critical issue is the curious necessity of zinc in solution to obtain a correlation between the extent of domain closure and agonist efficacy at AMPA receptors (Maltsev et al., 2008;Oswald et al., 2007). In fact, the willardiine series with a halogen substitution in complex with GluR2 exhibited a correlation with agonist efficacy only in the absence of zinc. Inclusion of zinc in the crystal solution yielded no correlation (Inanobe *et al.*, 2005b;Jin & Gouaux, 2003;Maltsev *et al.*, 2008). Although the molecular basis of this effect remains to be determined, crystallographic data suggest that histidine residues in the S1S2 domain of GluR2 are likely candidates for zinc binding sites in the mature receptor (Armstrong & Gouaux, 2000a). Vesicular zinc is released along with L-glutamate during electrical stimulation in the hippocampus and cerebellum (Perez-Clausell & Danscher, 1985). In both native (Mayer & Vyklicky, Jr., 1989) and recombinant systems (Dreixler & Leonard, 1994), micromolar zinc concentrations have been shown to potentiate kainate-induced currents, suggesting that that zinc binding may induce structural rearrangements that modulate receptor activity (Armstrong & Gouaux, 2000a). Whether this occurs in vivo and is important for function, or if the effects are just on crystal packing, awaits further studies.

Competitive antagonists. Finally, the ligand-binding domain of AMPARs in complex with competitive antagonists which display only modest changes in domain closure upon binding compared to the apo state, have raised important concerns in regards to the interpretation of the crystal structures. In fact, although the 2.5^{o}-6.0^{o} domain closure with CNQX and DNQX was not considered enough to cause channel activation of the wildtype receptor, more recent work has shown that transmembrane AMPA receptor regulatory protein (TARP) switches AMPA receptor antagonists into partial agonists (Menuz *et al.*, 2007b). This observation adds yet another level of complexity which cannot be reconciled with a simple model based on domain closure.

Given these observations, it is likely that the general idea that lobe closure acts as the initial trigger for channel opening is correct. Yet, the details of how this process leads to agonist efficacy remain to be determined and are likely to involve a combination of structural changes in the protein.

1.3. Potential Models for AMPA/ Kainate Receptor Activation

Since the development of the first homology model for iGluRs, it has been proposed that lobe closure may be the mechanism triggering channel gating (Sutcliffe et al., 1996). Subsequently, comparison of crystal structures combined with functional data further suggested a close correlation between lobe closure and agonist efficacy (Jin *et al.*, 2003a;Mayer, 2005a). Despite this, the amplitude of macroscopic current measurements is influenced by numerous factors including rapid desensitization, the number of channels activated, the single-channel conductance, the single-channel amplitude as well as the probability of channel opening.

Ultimately, according to this paradigm, the degree of lobe closure should correlate with an observable parameter. Perhaps the most obvious property would be single-channel conductance. That is to say, is the relative movement of the two lobes directly coupled to a property of the pore? As presented in the review of literature, work by Jin et al. has suggested that both full and partial agonists access the same series of subconductance states, but with different relative frequencies (Jin *et al.*, 2003a). In this scheme, full agonists preferentially activate higher conductances and partial agonists preferentially select lower conductances (Jin *et al.*, 2003a). To explain how the tetrameric receptor may function, the authors proposed a model in which each subunit contributes a gate and the number of opened gates determines the conductance level (Figure 7 from review of literature).

The activation model proposed by Jin et al. for AMPA receptors has left two major questions unanswered which we consider here (Oswald et al., 2007). The first question is: Are the reported single-channel recordings sufficiently resolved and appropriately performed to support the model? Although the channels were

heavily filtered in their study (Jin *et al.*, 2003a), better resolved AMPAR recordings also support the notion that both full and partial agonists access similar subconductance states, but with varying probabilities (Swanson et al., 1997). Another crucial issue which needs to be addressed is the use of CTZ to block AMPA receptor desensitization during the single-channel recordings evoked by the willardiine compounds (Jin *et al.*, 2003a). These data were then directly compared with crystal structures of the GluR2 receptor in complex with the agonists, in the absence of CTZ. Given this, the relevance of this comparison should have been carefully considered in the interpretation of the results.

Secondly, this work obviously raised the following question: What are the structural or conformational changes directly linked to channel gating? Unfortunately, this question was not addressed in their initial paper and cannot be answered without adequate information on the structure and dynamics of the entire receptor. Assuming that the S1S2 agonist-binding domain is one of the controlling factors that elicits dynamic change to allow the channel to gate, one can develop two potential scenarios to account for agonist behavior (Oswald et al., 2007).

(1) According to the *first model* (Figure 1), there is a direct link between that ligand-binding domain and the opening of the channel pore. It assumes that one channel gate only opens following full closure of one of the S1S2 lobes. In this case, since each subunit contributes to one gate, the conductance level would then be determined by the number of opened gates. While the S1S2 lobe could close to different degrees, the fully closed state would be favored by full agonists whilst partial agonist would adopt this conformation less frequently. This model presumes that lobe closure is a not a static property, but rather a dynamic process. Resolved crystal structures are thus thought to represent an ensemble of various structures exhibiting distinct degree of domain closure.

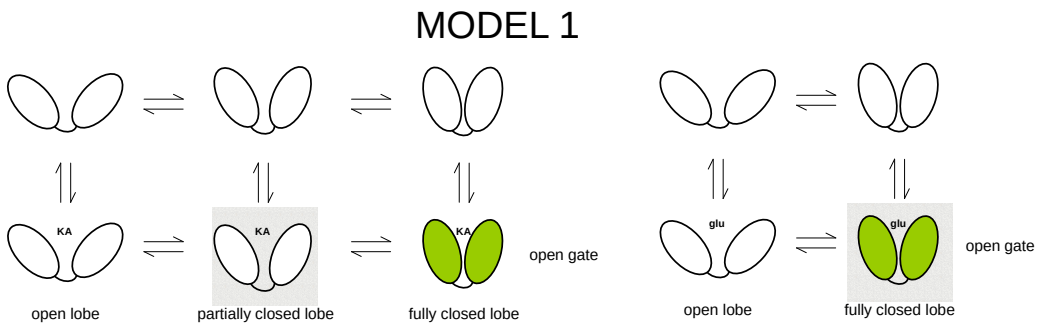


Figure 1. First plausible model of AMPA/KA receptor activation. The ligand-binding domain can exist in closed, open and partially open states. The most favorable state adopted by each agonist type is shown as a shaded box and the open gate form with the green-filled lobes. Although, all agonists can bind and the lobes can be either open or fully closed, full agonists (such as L-glu) favor the closed form which induces opening of the channel gate. In contrast, kainate and other partial agonists favor the partially open form, but only the fully closed form allows opening of the channel gate. Thus, a partial agonist elicits a smaller conductance, since (one average) more gates are closed than when a full agonist is bound. As proposed by Rosenmund et al. (1998) (see Figure 7 in the *Review of Literature*), the opening of individual gates causes an incremental increase in conductance. Modified from Oswald et al. (2007) Current Drug Targets. 8(5): 573-582.

(2) In the *second model*, closure of the S1S2 domain would only increase the probability that the gate would open (Figure 2). Though, not explicitly illustrated, it implies that the apo and agonist-bound receptor most likely exists in a number of conformations. Opening of the channel gate would be governed by other processes within the receptor.

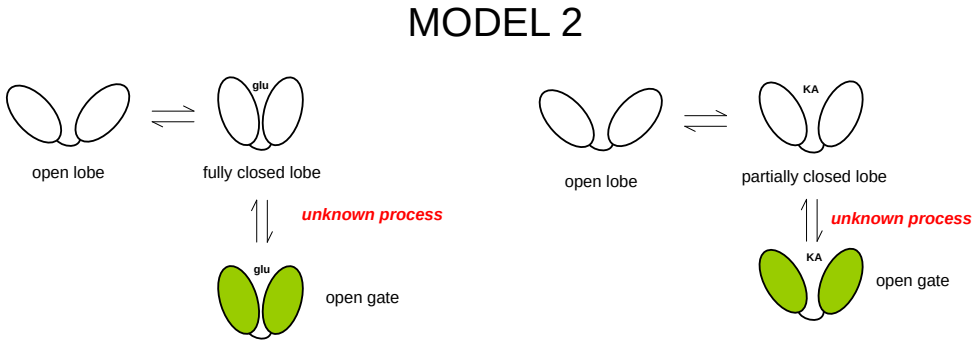


Figure 2. Second plausible model of AMPA/KA receptor activation. Opening of a gate can result from either partially or fully open lobes. The open gate is shown by the green-filled S1S2 lobes. Although the precise mechanism controlling gate opening have yet to be defined, the energy barrier leading to gate opening is lower for fully closed lobe than partial closed lobes. Regions outside the ligand-binding domain are also thought to govern channel gating. Modified from Oswald et al. (2007) Current Drug Targets. 8(5): 573-582.

Potential activation mechanisms may be gleaned from examining other ligand-gated ion channels. The mechanisms by which binding is translated into gating differed greatly across different ion-channels - from rotational movement for pentameric nAChRs (Miyazawa et al., 2003) and tetrameric cyclic-nucleotide gated channels (Flynn & Zagotta, 2001;Flynn & Zagotta, 2003) to bending of the gating hinge for K^+ channels. K^+ channels possess a gating hinge composed of a glycine residue that is essential for K^+ channel activation (Jiang et al., 2002)

Interestingly, K^+ channels and iGluRs are thought to have evolved from the same ancestral protein (Chen *et al.*, 1999a;Kuner *et al.*, 2003). By homology the M3 region of iGluRs also contains glycine residues, but replacing them has no obvious effect of receptor function, suggesting the two families may operate via distinct mechanisms, despite important similarities (Sobolevsky et al., 2004). Alternatively, here I consider other possibilities, which though not mutually exclusive, may account for these other processes in the receptor.

(a) One explanation is the link between the agonist-binding domain and the transmembrane domain is formed by Lobe 2, as well as putative flexible linkers between Lobe 2 and the TM segments (M1 and M3). Given this, the opening of the gate would require additional motion within one of these two regions (or both) or within the channel itself. Internal motion may account for differences in agonist efficacy where full domain closure may require less internal motion to open the gate than partial closure.

This model is particularly significant in light of our results presented in **CHAPTER 3**, where we have shown that binding of external ions are required for gating of KA, but not AMPA receptors (Wong *et al.*, 2006b). This finding highlights a critical difference between the gating machinery of these two iGluRs, which were essentially considered to be the same before the beginning of my thesis work (Weston *et al.*, 2006a;Lerma *et al.*, 1997). A second explanation considering this observation is that the nature of the bound ion may govern efficacy. To address this issue, future experiments recording single-channel from GluR6 receptor and the GluR6 M770K mutant in the presence of different external ions will be required.

(b) Alternatively, Zhang et al, have recently proposed that even though a conformational change in the agonist-binding domain is required for channel gating, agonist efficacy may be instead related to the amount of time it spends in the agonist-binding domain (ie., dissociation rate) (Robert *et al.*, 2005b;Zhang *et al.*, 2008a) rather than the extent of domain closure. To explain agonist efficacy at NMDA receptors, where full and partial agonists at the NR1 subunit adopt comparable degrees of domain closure, Gouaux and colleagues have proposed subtle differences in the complex stability of its agonist-binding domain (Furukawa & Gouaux, 2003;Inanobe *et al.*, 2005b).

Moreover, vibrational spectroscopy studies have found differences in the strengths of the interactions of the α-carboxylates and α-amines for full and partial agonists at AMPA receptors. Full agonists at GluR2, such as glutamate and AMPA, exhibited weak interactions of the α-carboxylates and strong interactions with its α-amines (Kubo & Ito, 2004). Conversely, the partial agonist kainate displayed strong interactions of the α-carboxylates and weak interactions with α-amines. Moreover, differences in the secondary structure of the protein upon binding of full and partial agonists were also observed (Cheng & Jayaraman, 2004;Du *et al.*, 2005). Consequently, one interesting hypothesis would be that strong interactions with the ligand's α-amine group and weaker interaction with the α-carboxylate group may govern the time the ligand spends in the ligand binding domain. Based on these studies, it is very likely that agonist behavior at iGluRs is governed not by only one protein movement (i.e.: domain closure), but rather multiple and perhaps more subtle factors.

Future directions. In summary, though X-ray structures of the agonist-binding domain of iGluRs have provided a wealth of information, important challenges still lie ahead. One of the main issues that had hampered the proper characterization of KA receptors was the paucity of KA receptor ligands (Huettner, 1990;Erreger et al., 2004). To this end, the identification of novel L-glutamate analogs that activate KA receptors (Fay et al., 2009) may prove to be useful in much the same way as the willardines have been at AMPA receptors

(Patneau *et al.*, 1992;Jin *et al.*, 2003a;Jin & Gouaux, 2003;Dolman *et al.*, 2006), with the advantage of being more structurally diverse. In fact, there has yet to be a detailed comparison of the single-channel properties of KA receptors with full and partial agonists. An important concern here is the fact that they exhibit very rapid desensitization (Chittajallu et al., 1999). Although stabilization of the binding domain dimer via intermolecular disulfide bonds apparently blocks KA receptor desensitization (Priel et al., 2006), as yet, there is no adequate pharmacological tool to remove this phenomenon. The resolution of single-channel analysis of KA receptors with ligands eliciting a range of response profiles is greatly needed and will provide pivotal and unprecedented insight into the gating mechanism governing agonist behavior at these receptors.

Another important consideration is that several key ligand-binding domains have yet to be resolved. These include: the GluR6 with the novel agonists identified here (Fay & Bowie, 2006a), the high affinity KA receptor subunits (i.e., KA-1 and KA-2), as well as the apo state of KA receptor subunits. Particularly, our current understanding of the mature tetrameric membrane-bound receptor is based solely on crystal structures, spectroscopic analysis and molecular dynamics simulations of the isolated ligand binding core. This is especially relevant given that most native KA receptors are thought to exist as heteromeric channels, displaying distinct pharmacological, biophysical and gating properties (Paschen *et al.*, 1994;Cui & Mayer, 1999;Alt *et al.*, 2004;Howe, 1996;Swanson *et al.*, 2002;Ruiz *et al.*, 2005). Thus, resolution of the structure of an intact receptor will surely mark a crucial step since it will provide insight into inter-subunit interactions. The difficulty will then be to develop an approach to measure conformational changes whilst simultaneously measuring single-channel activity. Expectantly, this will provide a very powerful context to study the basis of agonist behavior at iGluRs.

2. WHY ARE KAINATE RECEPTORS SENSITIVE TO IONS?

2.1. Ions as Potential Biosensors

Our novel finding that both cations and anions are required for kainate receptor gating (**CHAPTER 3**) raises a number of issues, three of which are discussed here. One important question that emerged as a result of this study was whether protons and divalent ions also regulated the time the channel remains in the open state, as shown for monovalent cations (Wong *et al.*, 2006b). To answer this, Wong et al. subsequently showed that divalent ions, but not protons, compete with Na^+ to stabilize the open state (Wong et al., 2007).

Another outstanding question was to elucidate the nature of the ion binding site. Do cations and anions bind to discrete sites in accordance with electrostatic principles or do the ions establish a dipole, as considered previously (Bowie, 2002a)? Using a series of cleverly-designed GluR6 receptor mutants, they predict that external ions co-activate KA receptors by setting up a functional dipole where the cation binds first, followed by the anion (Wong et al., 2007). The structural basis of this effect was predicted to occur via exposed carbonyl oxygen atoms (Wong et al., 2007), which was further confirmed by crystallography (Plested et al., 2008).

A third obvious question that emerged from the finding that kainate receptor activation is dependent upon external ions (Wong *et al.*, 2006b) is whether a physiological or pathophysiological condition exists where fluctuations in external ions can alter KA receptor activity. Given that synaptic membrane-bound proteins function in a chemically polarized ionic environment with high extracellular Na^+ and Cl^- concentration and high intracellular K^+, the existence of a cation-binding site in KA receptors is likely to have important functional significance (Plested et al., 2008). In fact, it is well established that during intense neuronal activity and pathological spreading depression there are important changes in extracellular ion concentrations (Somjen, 2001). More specifically, both *in vitro* and *in vivo* measurements of extracellular ions have shown that

extracellular Na^+ concentration drops by approximately 100 mM in 2-3 sec (Herreras & Somjen, 1993), thus reaching levels as low as 40-60 mM during spreading depression. At these concentrations, the response of kainate receptors should be significantly depressed (Bowie, 2002a).

Although it is more difficult to assess the changes in external concentration of Na^+ during normal repetitive neuronal activity, experiments using ion-sensitive electrodes have reported extracellular Na^+ drops of up to 20 mM (Dietzel & Heinemann, 1986;Dietzel *et al.*, 1982). Yet, Na^+ depletion within the synaptic cleft and in the vicinity of kainate receptors is likely to be much more profound. Although during intense neuronal activity, cations are not exchanged one for one and the Na^+ decrease is greater than the increase in external K^+, changes in the latter may also have important functional consequences. In fact, normal K^+ levels are about 3 mM in the extracellular space, but have been found to be as high as 20 mM (Connors & Ransom, 1984). The ionic intracellular environment (i.e., high K^+ and low Cl^-) thus favors KA receptor desensitization, which is required for efficient trafficking of these receptors to cell membranes (Priel et al., 2006).

Future Directions. Although our work has been performed, by necessity, on homomeric recombinant receptors (Wong *et al.*, 2006b), the behavior of native and heteromeric receptors remains to be established. Preliminary work has suggested that native kainate receptors in the hippocampus are also modulated by external cations (Paternain et al., 2003). Moreover, this regulation is likely to be working together with other modulations of the receptor such as association with auxiliary proteins (Zhang et al., 2009). Clearly, more work is required to have a thorough understanding of the implication of ion regulation at different combinations of kainate receptors. Again, resolution of single-channel currents will be central to directly test the effect of ions on the microscopic properties of KA receptors. It will be interesting to determine if ions govern macroscopic peak amplitude through open probability, channel conductance or both. Moreover, since desensitization kinetics are governed by the channel open time, it is also

expected that this parameter will be ion-dependent (Bowie, 2002a;Wong *et al.*, 2006b).

2.2. The Binding Site Hints at the Evolution Tree

Evolutionary Role. Comparison of gene sequences of mammalian kainate receptors with putative kainate receptors from chick, zebrafish, *Xenopus* and possibly *Drospholia* predicts conservation of the cation-binding site and suggests early evolution (Plested et al., 2008). Moreover, it has been speculated that *Caenorhabditis elegans* and *Caenorhabditis briggsae* have a primitive iGluR-like sequence that has a Lys residue at the homologous 770 position, which suggests that KA receptors may have evolved from an ancestral iGluR protein that behaved more like AMPA receptors (Wong et al., 2007).

Interestingly, the GluRδ2 lurcher mutation is potentiated by extracellular Ca^{2+} (Wollmuth et al., 2000) and the GluRδ2 subunit crystallizes as a dimer which binds Ca^{2+} ions (Naur *et al.*, 2007b). Thus, both kainate and GluRδ2 receptors possess an ion-binding pocket, suggesting a conserved mechanism despite distinct ion-binding sites (Plested et al., 2008). Given this, the ion-binding site may be used as an evolution marker.

That is, one possibility is that KA receptors first evolved from AMPA receptors. KA receptors may have lost a charged residue found at AMPA receptors through evolution, but remained functional since binding of external ions to this critical site provided an appropriate substitute for the positively charged lysine at AMPA receptors. In fact, all kainate receptors possess a nonpolar residue (ie., methionine, isoleucine, valine or leucine) at the M770 site of the GluR6 subunit. The GluRδ2 may have subsequently evolved from KA receptors, conserving an ion-binding site, but losing their ability to bind the endogenous neurotransmitter, L-glutamate – to fulfill a specialized function in the CNS. Curiously, the orphan glutamate-like receptor GluRδ2 subunit is exclusively expressed in Purkinje cells of the CNS and has the ability to bind D-serine (Naur *et al.*, 2007b), but not L-glutamate, KA or AMPA (Lomeli et al., 1993;Mayat et

al., 1995). Interestingly, we have shown here that GluR6 KA receptors are also activated by D-serine (**CHAPTER 2**).

Non-Canonical Signaling of iGluRs. Although GluRδ2 does not form or activate homomeric channels when expressed alone in a recombinant system with other glutamate receptor subunits (Yuzaki, 2003), its corresponding KO mice exhibit pronounced neurological deficits, demonstrating a functional role for GluRδ2 (Kurihara et al., 1997;Kashiwabuchi et al., 1995). These effects can be rescued by knocking in GluRδ2 wt or even mutant into the KO mutant (Kakegawa et al., 2007), suggesting that GluRδ2 may not signal only through ion permeation as expected, but rather through a metabotropic effect.

This proposed mechanism is reminiscent of the signaling properties of the high-affinity KA-2 subunit. Similarly, the KA-2 does not form homomeric channels, but must be assembled with other low-affinity KA receptor subunits (Dingledine et al., 1999). Using both genetic and pharmacological tools, Ruiz et al dissociated the ionotropic and metabotropic effects at KA receptors, suggesting that GluR6 is the chaperone subunit that drives KA-2 to the plasma membrane, where this subunit can also play a role in the metabotropic action of KA receptors (Ruiz et al., 2005). They further demonstrated that KA receptors interact with a G-protein (labeled by an anti-$G\alpha_q$/11 antibody) via KA-2. Yet additional, biochemical experiments are required to determine whether $G\alpha_q$ directly or indirectly binds to KA2 (Ruiz et al., 2005).

Likewise, the highly selective KA receptor agonist dysiherbaine (DH) and site-directed mutagenesis of KA receptor subunits have been used to demonstrate preferential activation of only the GluR5 subunits within a heteromeric GluR5/KA-2 KA receptor complex (Swanson et al., 2002). Together, these studies support the concept that occupancy of binding sites on individual iGluR subunits elicit distinct ionotropic and metabotropic actions (Swanson et al., 2002;Ruiz et al., 2005). Given this, one potential explanation for the role of these amino acids is that they act on "modulatory" glutamate receptor subunits (i.e., subunits that do form homomeric channels), such as KA-2 or GluRδ2 to mediate

metabotropic effects. Proteomic screening may be also be useful in identifying auxiliary or accessory proteins required for the assembly of the GluRδ2 by itself or with other glutamate subunits.

3. Are Amino Acids Other Than L-Glutamate, Transmitters at Central Synapses?

Although the precise implication of the newly identified L-glutamate analogs (Fay et al., 2009) as putative endogenous excitatory neurotransmitters or gliotransmitters at KA receptors remains elusive, here I speculate on the physiological and/or pathophysiological implications of these findings. First, I will focus on the sulfur-containing amino acids and potential implication in Alzheimer's disease and glioblastoma followed by a discussion on the stereoisomers of serine and aspartate in glutamatergic transmission.

3.1. Sulfur-Containing Amino Acids

Candidature as Transmitters in the Mammalian Central Nervous System. Several of the amino acids examined in this study are endogenous to the CNS and have been previously evaluated as neurotransmitter candidates at excitatory synapses. In fact, leading up to the 1990s, many researchers devoted their efforts to determine if sulfur containing amino acids were also endogenous excitatory neurotransmitters (Oja et al., 2007;Recasens et al., 1983;Mewett et al., 1983). Evidence for such a role was centered on demonstrating that they could be released in a calcium-dependent manner, (Do *et al.*, 1986a;Do *et al.*, 1986c), possessed an uptake mechanism (Bouvier *et al.*, 1991b) and could induce receptor activation (Do *et al.*, 1986a;Neal & Cunningham, 1992). For example, Do and colleagues have shown that L-CSA and L-HCA can be released following high

frequency stimulation to induce LTP in hippocampal slices (Klancnik et al., 1992).

Although growing evidence suggested that sulfur-containing amino acids fulfilled the criteria of neurotransmitters (reviewed in Thompson & Kilpatrick, 1996b), three anomalies were apparent when their properties were compared to that of L-glutamate and L-aspartate. First, the sulfur-containing amino acids were found at much lower levels than L-glutamate. Secondly, these amino acids did not seem to have a receptor class of their own, but rather activated many glutamate receptor families including metabotropic (Kingston *et al.*, 1998b;Shi *et al.*, 2003) and ionotropic receptors (Linn & Massey, 1996;Thompson & Kilpatrick, 1996b). One possibility to be explored is that some of the sulfur-containing amino acids may activate only a subset of GluRs, such as KA receptors. Consistent with this, the most potent of these amino acids at GluR6 peak responses, L-Cys, is a very weak partial agonist on homomeric GluR1 AMPARs (see Supplementary Figure S2, page 134).

Lastly, because sulfur-containing amino acids were also found and released from glial cells (Ortega et al., 1994), their action could not be readily described in the context of classical neurotransmission and were thus thought to differ from the conventional excitatory neurotransmitter, L-glutamate. Although this has been an important criterion in dismissing their action (Oja et al., 2007), an astrocyte-derived, nonsynaptic source of L-glutamate has been shown to represent a signaling pathway (Jourdain et al., 2007) that can activate neuronal kainate receptors in the hippocampus (Liu et al., 2004). This finding further supports the notion that it is becoming increasingly difficult to adequately define the term neurotransmitter (Boehning & Snyder, 2003b).

Potential Implication in Pathologies. Interestingly, some of the sulfur amino acids tested have been implicated in a number of pathologies, though their mechanism of action remains elusive. For instance, hyperhomocysteinemia (i.e., abnormally high levels of HC in plasma) is associated with a number of neurological conditions such as Alzheimer's Disease (Miller, 2000) and

schizophrenia (Levine et al., 2002). Both physiological (10μM) and pathophysiological levels (16-100μM) of HC in the adult (Shi et al., 2003) are much lower than the concentrations needed to activate GluR6 KA receptor (in mM) (Fay et al., 2009). However, since the properties of recombinant and native KA receptors are different, due in part to their assembly with other subunits (Alt *et al.*, 2004;Cui & Mayer, 1999;Howe, 1996) and their association with auxillary proteins such as NETO2 (Zhang et al., 2009), it will be important to measure the effect of these amino acids in native KA receptors.

Here, I have provided the first evidence that HC activates kainate receptors (Fay et al., 2009). Interestingly, of all the 16 amino acids tested, the partial agonist HC (34.4. ± 3.4 % glu_{peak}) evoked the second largest equilibrium response (2.8 ± 0.45% HC_{peak}, compared to the 0.31 ± 0.004 % peak evoked by L-glutamate). One possibility is that hyperhomocysteinemia may induce excitotoxicity through iGluRs that is characteristic of an Alzheimer's Disease brain (Wenk, 2006). Further work is needed to determine if it also activates native heteromeric KA receptors.

Consistent with this idea, domoic acid, also a partial KA receptor agonist (15.3 ± 1.9% glu_{peak}) also elicits larger sustained response at kainate receptors (34.8 ± 5.4% Dom_{peak}) than L-glutamate. This neurotoxin is known for having serious neurotoxic effect. Specifically, consumption of shellfish that have accumulated domoic acid causes amnesic seashell poisoning (Jeffery et al., 2004). Domoic acid is known to induce neuronal degeneration and necrosis in discrete regions in the hippocampus (Tryphonas & Iverson, 1990;Jeffery *et al.*, 2004). A serious outbreak of domoic poisoning in humans took place in Eastern Canada in 1987 with symptoms including hallucinations, memory loss, coma and even death in humans. Interestingly, hallucination and memory loss are reminiscent of ALZ and schizophrenia which have been associated with high levels of HC (Levine et al., 2002;Miller, 2000;Morris et al., 2001). It is thus tempting to suggest that HC may be acting through a similar mechanism as domoic acid, which may include abnormal iGluR receptor activation.

Another potential role for these amino acids is in the pathogenesis of glioblastoma, in which the malignant transformation of astrocytes, oligodendrocytes or their progenitor cells gives rise to tumors that are collectively called glioma (Sontheimer, 2003). One particularity of glioblastomas is that they lack excitatory amino-acid transporters and therefore release L-glutamate via a cysteine-glutamate exchanger which, in turn, causes excitotoxicity in neighboring tissue, thus allowing for further tumor expansion. Specifically, the pump has also been shown to bind HC (as well as quisqualate and l-α-aminoadipate) (McBean, 2002), suggesting that high levels of these amino acids may also contribute to neurotoxicity. Furthermore, application of HC on glioblastomas showed important toxic responses compared to neuroblastomas (Parsons et al., 1998), implying that toxic responses are cell-specific for HC.

3.2. Stereoisomers of Aspartate and Serine

Compared to the sulfur-containing amino acids, there is stronger evidence supporting a role for stereoisomers of both serine and aspartate in glutamatergic transmission (Boehning & Snyder, 2003b). Although the role of L-aspartate in neurotransmission has focused on NMDA receptors (Fleck *et al.*, 1993b), a high calcium conductance in cerebellar Purkinje cells that apparently involves a novel iGluR has been identified (Yuzaki *et al.*, 1996b). Similarly, though the functional role of D-aspartate is still unclear, it is found in both the developing and mature adult brain (Schell *et al.*, 1997d). Moreover, accumulation of D-aspartate in CNS tissue has significant behavioral consequences, such as impaired motor coordination (Weil *et al.*, 2006b), which is consistent with its putative role as a transmitter at the climbing fibers of the cerebellum (Wiklund *et al.*, 1982b).

The initial evidence supporting the pharmacological action of D-serine at iGluRs came from McBain and colleagues, who reported that D-serine was a co-agonist at the glycine binding site of NMDA receptors (McBain *et al.*, 1989a). Subsequently, functional evidence has shown that it acts as a gliotransmitter (Mothet *et al.*, 2000b) critical for synaptic memory (Panatier *et al.*, 2006b), which is consistent with previous studies localizing this amino acid in distinct populations of glial cells opposing NMDA receptors (Schell *et al.*, 1997b). One

possibility is that some of the ligands identified in our study (Fay et al., 2009) may act as gliotransmitters. Given that an astrocyte-derived source of glutamate has been shown to represent an important glutamate signaling pathway (Jourdain et al., 2007;Liu et al., 2004), this raises the possibility that D-serine may also act in a similar fashion (Mothet *et al.*, 2000b;Panatier *et al.*, 2006b). Although I have shown that both steroisomers of serine and aspartate are weak partial agonists compared to L-glutamate, it will be interesting to further test the precise role of the amino acids at central synapses expressing KA receptors.

Another possibility is that these amino acids may act on extrasynaptic iGluRs. In support of this, extrasynaptic $GABA_A$ receptors containing the δ-subunit have a particularily high affinity for GABA compared to synaptic receptors and are key mediators of non-synaptic inhibition (Semyanov *et al.*, 2004;Haas & Macdonald, 1999). Given this, it is possible that stereoisomers of aspartate and serine may activate KA-2-containing KA receptors which have also been shown to display a higher affinity for their endogenous neurotransmitter, L-glutamate (Dingledine et al., 1999). Though additional electrophysiological experiments are required to address this issue, another pressing matter that requires high-priority consideration in order to properly address this question, is to determine the stoichiometry of heteromeric KA receptors. As yet, whether KA receptors assemble in a fixed or random manner awaits further investigation.

4. DOES CONCANAVLIN-A MIMIC THE BEHAVIOR OF AN ENDOGENOUS PROTEIN?

4.1. Reporting Conformational Changes at Kainate Receptors

I have demonstrated in **CHAPTERS 1** and **2** that Con-A is a useful tool to report conformational changes in KA receptors (Fay & Bowie, 2006a;Fay *et al.*, 2009). There are some limitations to this approach, however, since Con-A is a rather large molecule (Edelman et al., 1972), which may cause steric hindrance. Despite this, the rate into and out of desensitization remains unchanged following lectin treatment, suggesting it does not interfere with this process. In line with

this, the substituted cysteine accessibility method (SCAM) (Sobolevsky et al., 2004;Matulef et al., 1999) (Wo et al., 1999;Yelshansky et al., 2004;Sobolevsky et al., 2002) has also been used to show that the mutated A684C residue in the ligand binding domain of GluR6 KA receptors becomes inaccessible during binding of the agonists (L-glutamate and KA), but not the antagonist (CNQX) tested (Basiry et al., 1999). This is consistent with our observation that Con-A's accessibility to some of its binding sites is restricted following ligands that induce a significant degree of domain closure compared to the apo or antagonist-bound structures (Fay *et al.*, 2009;Fay & Bowie, 2006a).

Furthermore, the structure of native GluR2 AMPA receptors was determined using single-particle electron microscopy in complex with: DNQX (a competitive antagonist) glutamate (a full agonist), CTZ (a modulator), as well as L-glutamate and CTZ (Nakagawa et al., 2005). Curiously, a negative correlation between the degree of desensitization and the extent of separation in the N-terminal domains was observed. Understanding how these changes in the N-terminal domain are indicative of conformational changes in the agonist-binding domain remains to be explored. This technique does not allow discrimination between the ion-conducting and ligand-free state of AMPA receptors. Nevertheless, it effectively suggests that different conformational changes are related to distinct functional states of the channel (Nakagawa et al., 2005).

Another concern is that since Con-A reports conformational changes at equilibrium (i.e., desensitized state) (Fay & Bowie, 2006a), it remains to be determined if the ligand-binding domain also adopts the same conformation in the activated state. Consequently, this section will briefly discuss our results in light of other approaches that have been used to examine conformational changes in the mature receptor. In an attempt to determine whether the ligand-binding domain of GluR4 receptors adopts the same conformation in the activated and desensitized state, Du et al have used FRET (Du et al., 2005) and reported no changes in the emission for the activated and desensitized state, suggesting there is no change in cleft closure associated with this process. This supports previous findings

indicating that desensitization is mainly due to interaction between subunits (Tichelaar et al., 2004;Du et al., 2005).

The major caveat of this approach is similar to the ones that emerge from crystallographic studies; though these results are suggested to measure activation of the receptor, it is mentioned that FRET experiments probe the *equilibrium and end states,* which reveal nothing of peak measurements that are rapid and transient. It is therefore doubtful FRET could properly delineate between the activated and desensitized (i.e.: equilibrium) state. Furthermore, mutation of cysteine residues could potentially affect the tertiary structure of the protein. Besides, although not explicitly quantified, the mutant receptors appear to have different kinetic profiles (slower desensitization) than the wildtype (Du et al., 2005).

4.2. Does the Plant Lectin Concanavalin-A Mimic an Endogenous Protein?

Given that a large portion of the extracellular domain of KA receptors is not directly involved in agonist-binding, one attractive possibility is that this domain may allow interaction between receptors or with transmembrane proteins. This could make them sensitive to structural reorganization associated with key physiological processes such as synapse maturation and LTP (Hoffman et al., 1998). Specifically, one way that KA receptors may interact with other proteins is through its N-glycosylation sites. Even though the function of N-glycosylation sites at KA receptors remains elusive, examination of the role of these sites at other proteins may be useful.

General role of N-glycosylation sites. As described here, the role of N-glycosylation sites for the proper interaction between transynaptic proteins has been clearly demonstrated. One example of this is the neurexin-neuroligin system, which are pre- and postsynaptic cell surface proteins, respectively, that function as trans-synaptic cell adhesion molecules (reviewed in Yamagata et al., 2003). Interestingly, the interaction between a splice variant of neuroligin and α-neurexin

is N-glycosylation-dependent. In support of this, this particular variant has an insert sequence containing the consensus N-glycosylation sequence, and the deglycosylation of neuroligin with PNGase F restored the interaction with α-neurexin (Boucard et al., 2005), suggesting that N-glycosylation sites play a critical role in regulating trans-synaptic signaling by this complex.

In addition, N-glycosylation regulates the interaction between various members of the immunoglobulin superfamily, such as synaptic cell adhesion molecules which have been implicated as synapse organizing molecules involved in promoting excitatory and inhibitory neurotransmission (Fogel et al., 2007). Both SynCAM 1 and 2 function as cell adhesion molecules that assemble with each other across the synaptic cleft into a specific, transsynaptic SynCAM 1/2 complex. In this case also, the interaction of SynCAM 1 and 2 is regulated by N-glycosylation, also curiously found to be developmentally regulated through a mechanism that has yet to be elucidated (Fogel et al., 2007). Taken together, these findings raise the possibility that these sugar-binding motifs may be important for synaptic formation and anchoring of iGluRs.

Synapse formation and anchoring of ionotropic glutamate receptors. The neuronal activity-regulated pentraxin (Narp), which has a sugar-binding motif strongly homologous to Con-A's secondary and tertiary structure, was found to play a critical role in excitatory synaptogenesis involving AMPA receptors, provides the first example of this (O'Brien et al., 1999). Though the interaction between the GluR1 AMPA subunit and Narp was not abolished following incubation with the N-glycosylation blocker tunicamycin (1 μM) as expected if this effect was mediated via these sites (Duksin & Mahoney, 1982;Kawamoto *et al.*, 1994), the concentration used was up to ten-fold less than what has been used in other studies (Kawamoto et al., 1994;Everts et al., 1999), suggesting further work is required to conclusively determine the role of the N-glycosylation sites. As yet, no such Narp-like endogenous molecules have been identified that bind KA receptors, but future proteomic screening may be also be useful in identifying

endogenous accessory or transynaptic proteins and provide additional clues into native roles of N-glycosylation sites.

Another example hinting at the role of N-glycosylation sites at iGluRs comes from a study where the N-terminal domain of GluRδ2 was shown to be necessary and sufficient for synaptogenesis at the parallel fiber-Purkinje cell synapse. Interestingly, its N-terminal possesses an N-glycosylation consensus sequence at position 293-295, suggesting this effect may be mediated through this site (Kakegawa et al., 2009). Additional experiments including abolishing N-glycosylation sites through site-directed mutagenesis or with the use of deglycosylation agents are needed to further explore this possibility. Although, N-glycosylation sites are not essential for KA receptor function (Everts et al., 1997), one possibility is that they are involved in synaptogenesis.

Lastly, KA receptors are thought to be involved in the maturation of mossy fiber excitatory neurotransmission in CA3 pyramidal cells (Marchal & Mulle, 2004). Postsynaptic GluR6 KA receptors interact with cell-adhesison proteins of the cadherin/catenin complex (Coussen et al., 2002), which, together with the nectin-afidin adhesion system, are implicated in synaptogenesis (Tepass et al., 2000;Mizoguchi et al., 2002). These findings open up the possibility that KA receptors may have an important role in the formation of the mossy fiber synapse through their interaction with cell-adhesion proteins located both pre- and postsynaptically. Further work examining potential interactions of these proteins with KA receptors through affinity chromatography, coimmunoprecipitation experiments and fluorescence microscopy using distinctly-tagged proteins to analyze cell-to-cell interactions in both HEK cells and *in vivo* is clearly required (Fogel et al., 2007).

5. CONCLUDING REMARKS

In summary, this thesis has elucidated some of the basic properties of kainate receptors – which were unexpectedly significantly different from the closely-related AMPA receptors. Importantly, single-channel recordings in the presence of full and partial agonists and in different ionic conditions are needed to fill in the relatively scant information available about KA receptors compared to other iGluR subfamilies. Until these issues are resolved, we will continue to make slow progress.

The studies presented here support the hypothesis that the molecular and structural events governing kainate receptor activation are multi-factorial. First, I have shown that closure of the agonist-binding domain at KA receptors does not predict agonist efficacy, as had been previously thought. I have also identified a series of novel KA receptor agonists which could provide a good starting point for rational drug design for subunit selective agonists & antagonists. Though it is likely that co-crystallization of these new agonists with the KA receptor will shed some light on (at least) one of the conformations adopted by the agonist-binding domain bound with these agonists, surely more work will elucidate the determinants of agonist behavior. In support of this, I have also demonstrated that closure of the ligand-binding domain is not sufficient to activate KA receptors, but that external ions are co-activators at KA, but not AMPA receptors. Finally, as elaborated in this **DISCUSSION & CONCLUSIONS**, several ideas emerged from the work presented in this thesis which could be useful in the planning of future experiments.

SUMMARY OF ORIGINAL CONTRIBUTIONS

I. I showed that the lectin, concanavalin-A, can be used as a pharmacological tool to report conformational changes in the agonist-binding domain of intact kainate receptors (*Chapter 1*).

II. I examined the conformation adopted by the agonist-binding domain of intact kainate and report that it is both state- and agonist-dependent (*Chapter 1*).

III. I reported that three N-glycosylated amino acid residues in close proximity to the agonist-binding domain are important in mediating the effect of concanavalin-A on kainate receptor responses (*Chapter 1*).

IV. Using three kainate receptor agonists, I showed that binding of a full agonist induced a more pronounced conformational change in the agonist-binding domain than partial agonists (*Chapter 1*).

V. Using electrophysiological recordings combined with an ultra-fast drug application system, I identified a series of L-glutamate analogs that display a wide range of agonist efficacy at kainate receptors. Particularly, I determined the relative efficacy of quisqualate and SYM 2081, which had been previously co-crystallized with GluR6 kainate receptors (*Chapter 2*).

VI. I have validated the use of the computational ligand docking program, FITTED, for the agonist-binding domain of kainate ionotropic glutamate receptors using the previously published co-crystal structures (*Chapter 2*).

VII. Combining our electrophysiological assay and data obtained with the docking software, I revealed that closure of the agonist-binding domain does not correlate with agonist efficacy at kainate receptors (*Chapter 2*).

VIII. Using electrophysiological recording and mutagenesis experiments, we have demonstrated that ions are co-activators at kainate, but not AMPA receptors (*Chapter 3*).

IX. We showed that the M770 site conveys ion-sensitivity at KA receptors and that mutation to a positively charged residue restores receptor gating in the absence of external ions (*Chapter 3*).

X. We have showed that occupancy of kainate receptors by L-glutamate prevents ion-binding, thereby identifying cross-talk between the agonist-binding and ion-binding sites (*Chapter 3*).

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APPENDIX

ARTICLE REPRINTS

1- J PHYSIOLOGY (2006)

2- MOL PHARMACOLOGY (2009)

3- J NEUROSCIENCE (2006)

REPRINT OF PUBLISHED ARTICLE NO 1

Concanavalin-A reports agonist-induced conformational changes in the intact GluR6 kainate receptor

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The agonist-binding domain of ionotropic glutamate receptors (iGluRs) has recently been crystallized as two polypeptide chains with a linker region. Although work on the structure of this isolated ligand-binding core has been invaluable, there is debate over how it relates to conformations adopted by intact receptors. iGluR crystals are proposed to represent the activated state as their degree of domain closure correlates well with agonist efficacy. However, iGluR crystals exhibit high agonist affinity that more closely matches that of desensitized receptors. Consequently, conformations adopted by iGluR crystals may represent this state. To test this, we have employed the plant lectin, concanavalin-A (Con-A) to report conformational changes elicited by kainate (KA) iGluR agonists during desensitization. When GluR6 KA receptors (KARs) were pre-incubated with Con-A, equilibrium responses evoked by the full agonist, L-glutamate (L-Glu), increased almost 30-fold. However, in the continued presence of L-Glu, Con-A exerted no effect suggesting that it has restricted access to its binding sites when the agonist is bound. However, Con-A does not discriminate well between agonist-bound or -unbound states with the weak partial agonist, domoate. Accessibility experiments with KA were intermediate in nature consistent with its equilibrium efficacy at GluR6 KARs. Our results suggest that full and partial agonists elicit distinct conformational changes in KARs during desensitization. This finding can be reconciled with crystallographic data if the agonist-binding domain adopts the same conformation in the activated and desensitized states. However, other interpretations are possible suggesting future work is required if this issue is to be resolved.

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The concept that agonist molecules act on allosteric proteins such as ligand-gated ion channels with different efficacy was first recognized almost 50 years ago (Ariens, 1954; Stephenson, 1956; del Castillo & Katz, 1957). At fully occupied receptors, agonists that elicit the maximum response are referred to as full agonists whereas partial agonists evoke submaximal responses. Two distinct models have been developed to account for agonist behaviour: the concerted (Monod *et al.* 1965) and multi-state (Koshland *et al.* 1958, 1966) models. In the concerted model, full and partial agonists evoke identical conformational changes in protein structure, but differ in their ability to activate channel openings. Nicotinic acetylcholine receptors (nAChRs) exemplify this behaviour since membrane currents elicited by full and partial nAChR agonists have identical single-channel conductance but differ in open-channel probability (Gardner *et al.* 1984). Moreover, this gating behaviour is widespread amongst other signalling proteins such as glycine, $GABA_A$ and NMDA receptors, as well as cyclic-nucleotide-gated channels (Zagotta & Siegelbaum, 1996; Colquhoun & Sivilotti, 2004; Lynch, 2004; Auerbach & Zhou, 2005). In the multi-state model, full and partial agonists elicit distinct conformational changes in protein structure. Contrary to the concerted model, single-channel recordings reveal that conformations in protein structure are governed by agonist concentration (Rosenmund *et al.* 1998; Smith & Howe, 2000) as well as agonist type (Swanson *et al.* 1997; Jin *et al.* 2003). Although few ligand-gated ion channels operate by this mechanism, recent work on the agonist-binding domain of AMPA and KA iGluRs has suggested that their agonist behaviour is best described by this model.

Detailed X-ray analysis of iGluR subtypes has been possible since their agonist-binding domains can be reconstituted as two polypeptide chains using a linker peptide to replace transmembrane regions (Armstrong *et al.* 1998; Furukawa & Gouaux, 2003; Mayer, 2005). From work on AMPA iGluRs, it is proposed that agonist binding promotes closure of the isolated ligand-binding

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core which in the intact receptor would lead to channel opening (Armstrong *et al.* 1998; Armstrong & Gouaux, 2000). Therefore, conformations adopted by the isolated ligand-binding core are understood to represent the activated state. In support of this, full and partial AMPAR agonists elicit complete and partial cleft closure, respectively, correlating well with agonist efficacy (Armstrong *et al.* 2003; Jin *et al.* 2003). Ligand-binding constructs of KAR iGluRs apparently behave similarly since full and partial agonists also promote distinct conformations (Mayer, 2005) consistent with the multi-state model already proposed from functional analysis of intact KARs (Bowie & Lange, 2002; Swanson *et al.* 2002). However, a potential caveat is that unitary current measurements indicate that single AMPA and KAR activations are short-lived, lasting only a few milliseconds (Swanson *et al.* 1996, 1997; Howe, 1996). Consequently, X-ray crystal structures may represent another protein conformation that is more thermodynamically stable, such as the desensitized state(s).

Here we have characterized the state-dependent modulation of GluR6 KARs by Con-A. Previous work from our laboratory has established that this plant lectin selectively regulates desensitized GluR6 receptors (Bowie *et al.* 2003). We have used this property of Con-A to test if full and partial agonists elicit distinct conformations in the extracellular domain of *intact* GluR6 KARs during desensitization. In agreement with recent work on GluR6 crystal structures, we show that different agonists evoke distinct conformations in intact receptors. This finding further establishes that agonist efficacy at KARs is best explained by a multi-state model. Our observations on desensitized channels can be reconciled with crystallographical data if the activated and desensitized states adopt comparable conformations. However, as discussed below, alternative interpretations are possible suggesting that future structure–function analysis of KA iGluRs must address this issue.

Methods

Cell culture and transfection

Techniques used to culture and transfect mammalian cells to express GluR6 KARs have already been described in detail elsewhere (Bowie, 2002, 2003; Bowie & Lange, 2002). Briefly, tsA201 cells, a transformed human kidney (HEK 293) cell line stably expressing on SV40 temperature sensitive T antigen (provided by R. Horn, Jefferson Medical College, PA, USA) were maintained at a confluency of 70–80% in minimal essential medium with Earle's salts, 2 mM glutamine and 10% fetal bovine serum supplemented with penicillin (100 units ml^{-1}) and streptomycin (100 $\mu g\,ml^{-1}$). After plating at low density (2×10^4 cells ml^{-1}) on plastic dishes, cells were transfected with cDNA encoding unedited wild-type glutamate receptor subunit 6 (GluR6Q) or mutant GluR6Q receptor subunits using the calcium phosphate technique as previously described (Bowie *et al.* 1998). The cDNA for enhanced green fluorescent protein (EGFP S65T mutant) was routinely cotransfected to help identify transfected cells.

Site-directed mutagenesis

Mutation of *N*-glycosylated residues located in close proximity to the agonist-binding domain of GluR6 KARs was performed to disrupt lectin modulation (Fig. 4). To generate mutants, three of the *N*-glycosylated consensus sites (N-X-S/T, where X ≠ P) in the GluR6 sequence were changed from an S/T to an A and will be referred to as GluR6(Q)ΔNG5,6,7 according to the nomenclature of Everts *et al.* (1999) (Fig. 4*A*). Alanine substitutions of T414 (NG5), T425 (NG6) and S432 (NG7) were performed in two steps using the Quickchange II XL site-directed mutagenesis kit (Stratagene, La Jolla, CA, USA) using *PfuUltra* DNA polymerase and custom primers (Alpha DNA, Montreal, Quebec, Canada). Mutant cDNAs were amplified using XL10-Gold ultra-competent cells (Stratagene), purified with the QIAprep Spin Miniprep kit (Qiagen Inc., Mississauga, Ontario, Canada), initially identified by restriction digest using *Bam*H I or *Sac* I (New England Biolabs, Beverly, MA, USA) and later confirmed by automated sequencing (McGill University and Genome Quebec Innovation Center, Montreal, Quebec, Canada). To obtain larger quantities of mutant cDNA, GluR6 mutants were amplified in bacterial cultures (Top10 cells, Invitrogen) and the cDNA purified using QIAfilter Maxiprep kits (Qiagen Inc.).

Electrophysiological solutions and techniques

Excitatory amino acid agonists were dissolved in external solutions containing 150 mM NaCl, 5 mM Hepes and 0.1 mM each of $CaCl_2$ and $MgCl_2$. All concentrated agonist stocks were adjusted to pH 7.3 with NaOH before being stored at −20°C. Saturating agonist concentrations chosen for L-glutamate (10 mM), KA (1 mM) and domoate (50 μM) were at least 5-fold larger than published EC_{50} values at GluR6 receptors (Köhler *et al.* 1993; Tygesen *et al.* 1994; Jones *et al.* 1997; Donevan *et al.* 1998; Bowie, 2002; Alt *et al.* 2004). We empirically confirmed that these concentrations were saturating by doubling the agonist concentration in each case and observing that peak response amplitudes were unchanged. The internal solution was composed of 115 mM NaCl, 10 mM NaF, 5 mM Hepes, 5 mM Na_4BAPTA, 0.5 mM $CaCl_2$, 1 mM $MgCl_2$ and 10 mM Na_2ATP to chelate endogenous polyamines (Bähring *et al.* 1997; Bowie *et al.* 1998). The

pH and osmolarity of internal and external solutions were adjusted to 7.3 and 295 mosmol l^{-1}, respectively. Con-A and succinyl Con-A (Sigma, St Louis, MO, USA) were prepared in glucose-free saline solution and filtered (0.2 μm filter, Corning) immediately before use as previously described (Bowie *et al.* 2003). All recordings were performed with an Axopatch 200B amplifier (Axon Instruments Inc., CA, USA) using thin-walled borosilicate glass pipettes (2–5 MΩ) coated with dental wax to reduce electrical noise. Control and agonist solutions were rapidly applied to outside-out patches excised from transfected tsA201 cells as previously described (Bowie *et al.* 1998, 2002; Bowie & Lange, 2002). Solution exchange (10–90% rise time = 25–50 μs) was determined routinely at the end of the experiment by measuring the liquid junction current (or exchange current) between the control and agonist-containing solution in which total Na^+ content was reduced by 5%. Current records were filtered at 5 kHz, digitized at 25–50 kHz and series resistances (3–10 MΩ) compensated by 95%. Recordings were performed at −20 mV membrane potential to ensure adequate voltage clamp control of peak currents. Data acquisition was performed using pCLAMP9 software (Axon Instruments Inc.). All experiments were performed at room temperature.

Results

iGluR molecular rearrangements and structural information has been inferred from the state-dependent behaviour of a number of pharmacological agents including channel blockers (Benveniste & Mayer, 1995; Bähring & Mayer, 1998; Bowie *et al.* 1998) and the accessibility of substituted cysteine residues (Kuner *et al.* 1996, 2001). At KARs, the binding and modulatory effect of Con-A is also state dependent (Everts *et al.* 1999). We speculated that this property may be useful in probing gating conformations elicited by full and partial KAR agonists. Therefore, our initial experiments were designed to further characterize the nature of state-dependent modulation of KARs by Con-A.

Con-A modulation of GluR6 KARs is state dependent

Previous work on invertebrate iGluRs has suggested that Con-A binding sites are masked during desensitization (Evans & Usherwood, 1985) whereas more recent studies on mammalian GluR6 receptors has proposed that binding can occur (Everts *et al.* 1999). However, in the latter study the authors did not exclude the possibility that incubation with desensitizing concentrations of agonists still permit Con-A to bind to GluR6 receptors recycling through the open state (Everts *et al.* 1999). In such conditions, recycling through the open state would occur with low probability and the onset of Con-A's effects would develop slowly. Since the authors did not determine the time course of modulation (Everts *et al.* 1999), it is possible that their observations reflect binding to open rather than desensitized channels.

To determine if Con-A is able to bind to desensitized states, GluR6 receptors were stimulated at two frequencies, 0.067 (every 15 s) and 0.33 (every 3 s) Hz, to vary the fraction of desensitized receptors. The time course of Con-A modulation was then compared at each frequency. Figure 1*A* and *B* shows typical patch recordings where the development of Con-A effects was compared using multiple applications of 10 mM L-glutamate (L-Glu, 250 ms duration, holding potential (V_h) = −20 mV) every 15 s or 3 s, respectively. In each case, L-Glu evoked a rapidly rising inward membrane current that desensitized in the continued presence of the agonist to reach a steady-state level. At 0.067 Hz, GluR6 channels recover fully from desensitization between agonist applications (Bowie & Lange, 2002) whereas at 0.33 Hz, 50–60% of the peak response is desensitized. Consequently, the peak agonist response at 0.067 Hz was unchanged (Fig. 1*A*), whereas at 0.33 Hz, the peak amplitude initially declined by almost 60% before a new peak level was established (Fig. 1*B*, see arrow). When peak amplitudes stabilized during a recording, the outside-out patch was treated with 10 μM Con-A as previously described (Bowie *et al.* 2003).

At both stimulation frequencies, Con-A did not significantly affect the peak amplitude but irreversibly potentiated the level of the equilibrium response (Fig. 1*A* and *B*). Since Con-A binding is irreversible, binding sites are saturated at any concentration where the total number of Con-A molecules is greater than or equal to the number of binding sites. In view of this, Con-A treatment modifies all GluR6 channels in each patch recording. At both stimulation frequencies, the time course for the onset of Con-A modulation reached a maximal effect after approximately 2–3 min of treatment (Fig. 1*D*–*F*). However, the effectiveness of Con-A on the equilibrium response was dependent on the stimulation frequency (Fig. 1*D*). The equilibrium responses observed at 0.067 and 0.33 Hz were 17.2 ± 2.1% (○, $n = 8$) and 5.7 ± 0.5% (●, $n = 10$) of the peak, respectively, representing a 3-fold difference in the effectiveness of Con-A (Fig. 1*D*). Taken together, these observations are not consistent with Con-A binding sites being masked by desensitization (Evans & Usherwood, 1985) since this mechanism would predict equi-effectiveness of Con-A at both stimulation rates but with a slower time course at 0.33 Hz. To account for the different degree of modulation, we propose that the number of glycosylated residues available for Con-A binding is restricted by desensitization.

Consistent with this, when we compared the rate and degree of modulation of GluR6 receptors with the lectin dimer, succinyl Con-A (sCon-A) (Gunther *et al.* 1973), the rate of onset was slower and the degree of

modulation was less (□, 5.7 ± 0.5% Peak, $n = 5$) (Fig 1*C*, *E* and *F*). Since Con-A and sCon-A possess a different number of carbohydrate binding sites, it is likely that differences in stoichiometry sterically hinder binding and/or cross-linking events essential for modulating GluR6 receptors. However, these initial experiments do not exclude the possibility that different modulatory effects of Con-A at 0.067 and 0.33 Hz reflect binding to the open state rather than the desensitized state. Experiments described below and illustrated in Fig. 2 resolve this issue.

Con-A modulation of GluR6 KARs is a multi-step process

Figure 2 shows the experimental protocol used to determine if Con-A binds to desensitized GluR6 receptors. In each experiment, control responses to 10 mM L-Glu (250 ms duration) were measured to establish the baseline amplitude of the equilibrium response (Fig. 2*A*, left panel). During the second, longer application of L-Glu (2–3 min), Con-A was continuously co-applied to the equilibrium response for a period previously shown to fully modulate GluR6 receptors (Bowie *et al.* 2003) (Fig. 2*A*, middle panel). The effect of Con-A was then determined by comparing the amplitude of the equilibrium responses at the beginning (Fig. 2*A*, △) and end of the treatment period (Fig. 2*A*, ▲). Interestingly, measurement of the equilibrium response at these two time points revealed that Con-A had almost no effect on equilibrium desensitization (End (▲): 0.44 ± 0.14% Peak, $n = 5$) when compared to control levels (Before (△): 0.47 ± 0.17% Peak, $n = 6$) (Fig. 2*C*). Similar results were also observed when patches were co-treated with L-Glu and Con-A for longer periods (e.g. > 5 min).

The lack of effect of Con-A on desensitized GluR6 receptors suggests one of two possibilities. Firstly, lectin

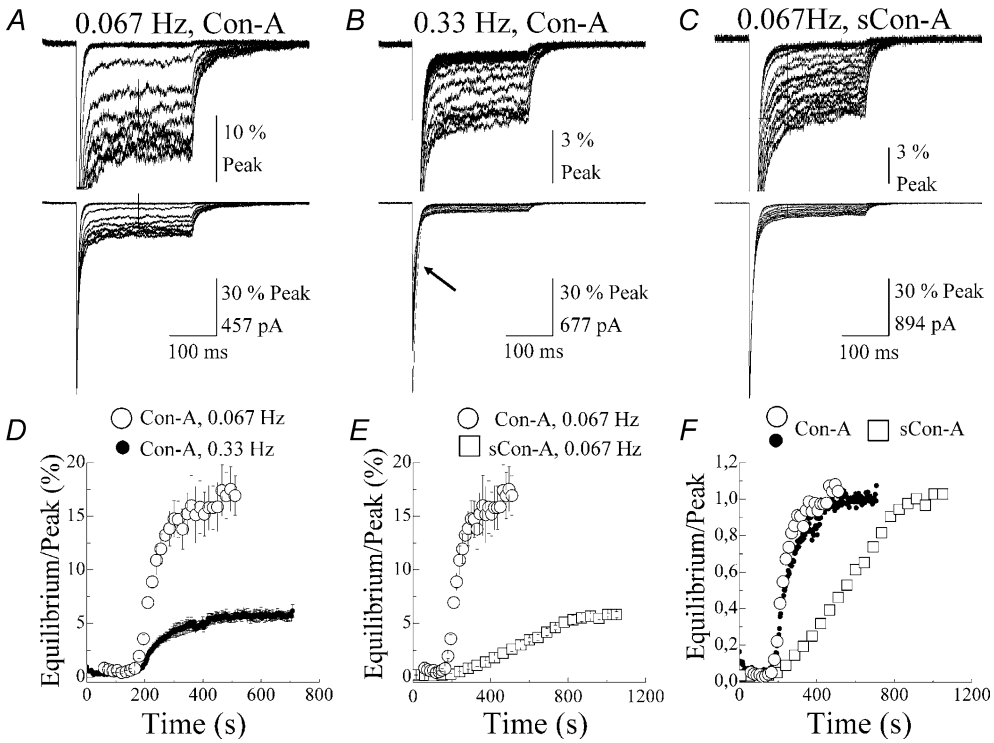

Figure 1. Determining the time course for lectin modulation of GluR6 receptors
A–C, time course for the onset of lectin modulation was determined by stimulating GluR6 receptors with 10 mM Glu (250 ms, $V_h = -20$ mV) every 15 s (*A* and *C*, 0.067 Hz patch numbers, 010327p2 and 010712p1) or 3 s (*B*, 0.33 Hz patch number, 010816p6) in the continuous presence of Con-A or succinyl Con-A (sCon-A). In each case, baseline control responses were first established before each patch was treated until a maximal effect on the equilibrium response was observed. Note, peak responses shown in *B* initially declined in amplitude when GluR6 receptors were stimulated at 0.33 Hz (see arrow). This effect, due to the onset of desensitization, was permitted to reach equilibrium before the patch was treated with Con-A. *D*, summary plot showing the development of modulation by Con-A of GluR6 receptors activated every 15 s (○, $n = 8$) or 3 s (●, $n = 10$). In each case, the rate of onset was similar, but the degree of modulation differed by more than 3-fold. *E*, plot comparing the time course for the onset of modulation by sCon-A (□, $n = 4$) and Con-A (○, $n = 10$) on GluR6 responses stimulated at 0.067 Hz. *F*, plot summarizing the data shown in *D* and *E*. In each case, the data were normalized to allow comparison between the onset of modulation at different stimulation frequencies and between different lectins. All data are expressed as mean ± S.E.M.

binding sites are masked by conformational events that occur during desensitization as suggested from work on invertebrate iGluRs (Evans & Usherwood, 1985). Alternatively, Con-A binding may have occurred but modulation requires an additional conformational step not permissible whilst receptors are desensitized (Fig. 2*B*). To distinguish between these two possibilities, co-treatment of the patch with 10 μM Con-A and agonist was terminated. The receptors were then allowed to fully recover from desensitization and a third 250 ms application of only 10 mM L-Glu was applied (Fig. 2*A*, right panel). Surprisingly, without subsequent Con-A treatment, the equilibrium response increased 14- to 15-fold to 6.74 ± 1.76% of the peak ($n = 4$) (Fig. 2*A*, ■). The increase in the equilibrium response represents only 30% of the modulation observed when GluR6 receptors were treated in the absence of agonist (Fig. 2*C*, Expected: 21.8 ± 2.9% Peak, $n = 29$). This experiment suggests that Con-A binds to desensitized channels but requires an additional step, involving agonist dissociation, before modulation is observed. It is unlikely that Con-A binds to GluR6 receptors recycling through the open state since the degree of modulation observed with the third L-Glu application is too large. Having established that Con-A can report agonist-induced conformations, we hypothesized that this behaviour may be useful in comparing structural changes evoked by full and partial agonists.

Con-A modulation of GluR6 KARs is agonist dependent

We initially compared the response profile of three structurally related agonist molecules recently crystallized in complex with the GluR6 KAR ligand-binding core (Mayer, 2005; Nanao *et al.* 2005). Figure 3*A* and *B* shows typical membrane currents evoked by rapid application of saturating concentrations of L-Glu (10 mM), KA (1 mM) and domoate (Dom, 50 μM) in the same patch recording before and after treatment with Con-A. Prior to Con-A treatment, peak responses to KA and Dom

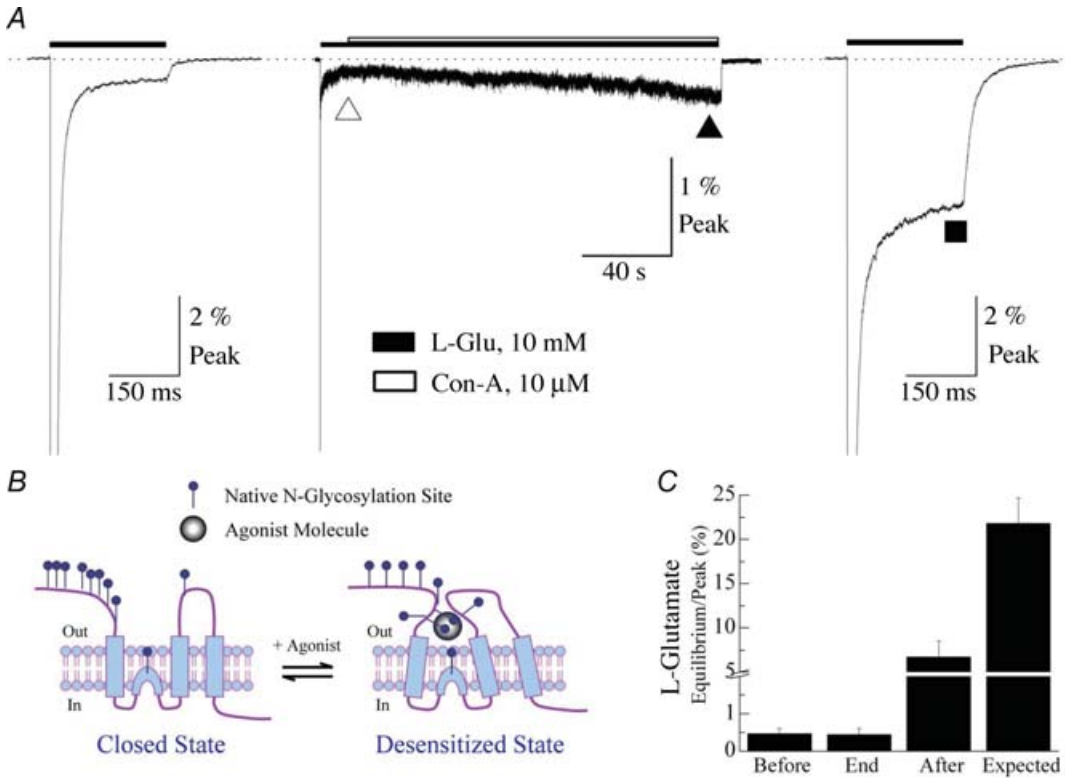

Figure 2. Con-A modulation of GluR6 receptors is a multi-step process
A, typical experiment showing the effect of Con-A when applied to predominantly desensitized channels. L-Glu (10 mM) was applied before (△), during (▲) and after (■) extensive Con-A treatment (200 s, $V_h = -20$ mV) to monitor changes in the equilibrium response amplitude (patch number, 010817p6). The filled and open bars indicate the application period of 10 mM Glu and 10 μM Con-A, respectively. The dotted line denotes the zero current level. The first and third applications of 10 mM Glu had a duration of 250 ms. *B*, schematic diagram illustrating how agonist-binding may prevent access of Con-A to a subset of *N*-glycosylated residues in the vicinity of the agonist-binding domain. *C*, summary plot of data from several patches ($n = 6$) where the amplitude of the equilibrium response was compared at various time points as exemplified by the experiment shown in *A*. The values for 'Expected' were taken from data shown in Fig. 3*C*. All data are expressed as the mean ± S.E.M.

were $44.9 \pm 2.2\%$ ($n = 13$) and $12.6 \pm 3.8\%$ ($n = 8$), respectively, of the L-Glu response ($n = 13$) (Fig. 3*A*) confirming that KA and Dom are partial agonists at GluR6 KARs. Although, Con-A increased the amplitude of the equilibrium response for all three agonists, the degree of modulation was agonist specific (Fig. 3*A*–*C*). For example, the equilibrium response elicited by L-Glu increased 30-fold from an equilibrium/peak ratio of $0.74 \pm 0.16\%$ in control conditions to $21.8 \pm 2.9\%$ ($n = 29$) following Con-A treatment (Fig. 3*C*). In contrast, equilibrium/peak ratio for Dom was $34.8 \pm 5.4\%$ ($n = 15$) in the control response compared to $195.7 \pm 6.8\%$ ($n = 3$) after Con-A treatment, representing a 5-fold change. Finally, consistent with the rank order of agonist efficacy observed in control conditions, modulation by Con-A of equilibrium KA responses was intermediate (Fig. 3*D*).

GluR6 equilibrium responses depend on the summed contribution of several subconductance states (Swanson *et al.* 1996; Howe, 1996) whose relative proportions may vary with full and partial agonists as recently proposed for AMPA receptors (Jin *et al.* 2003). We have shown that Con-A's effect on L-Glu responses is due to the up-regulation of a subset of conductance states (Bowie *et al.* 2003). Consequently, it is likely that Con-A affects KA and Dom equilibrium responses by modulating a different combination of subconductance levels. From a structural standpoint, irreversible binding of Con-A to *N*-glycosylated residues (Everts *et al.* 1997, 1999) may restrict conformational changes to a number of regions in the mature protein including the dimer interface, pore region or agonist-binding domain. Movement of the dimer interface governs the rate at which GluR6 receptors desensitize (Bowie & Lange, 2002; Horning & Mayer, 2004). Since Con-A does not affect GluR6 desensitization kinetics (Bowie *et al.* 2003) it is unlikely that lectin binding influences dimer–dimer interactions. Furthermore, Everts *et al.* (1999) have shown that *N*-glycosylated residues important for lectin binding are distant from the pore region (see Discussion) but located in and around the agonist-binding domain. Consequently, Con-A is unlikely to influence the pore region directly but may restrict conformations within the agonist-binding domain. To provide further experimental support for this, we performed mutational analysis of three *N*-glycosylated amino acid residues in close proximity to the agonist-binding domain.

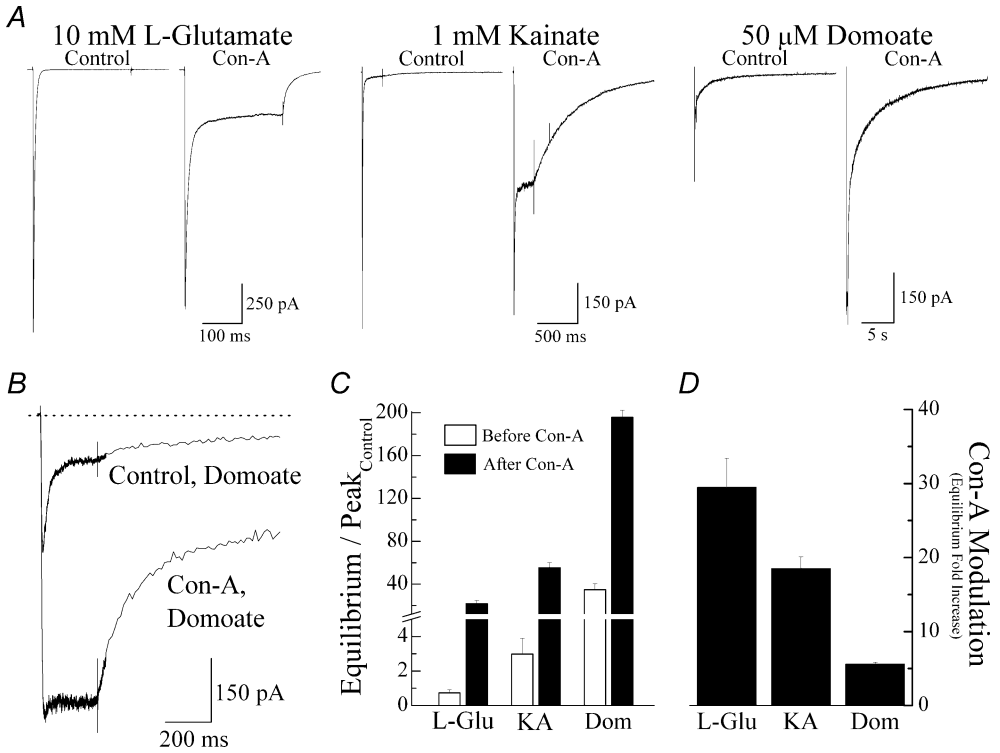

Figure 3. Modulation of GluR6 receptors by Con-A is agonist dependent
A, typical membrane currents (250 ms duration, $V_h = -20$ mV) elicited in the same patch by 10 mM L-Glu, 1 mM KA and 50 μM Dom before and after treatment with 10 μM Con-A (patch number, 030724p2). *B*, to show the early phase of the Dom response in more detail, agonist-evoked membrane currents prior to and after incubation with Con-A were superimposed. *C* and *D*, bar graphs summarizing the effect of Con-A treatment on equilibrium responses (*C*) and comparing its effects on different KA receptor agonists (*D*). All data are expressed as the mean ± S.E.M.

Mutation of Con-A binding sites in close proximity to the agonist-binding domain

Alanine substitution of three amino acid residues, T414A, T425A and S432A (Fig. 4*A*) was made since previous work had established that each residue was critical for Con-A modulation (Everts *et al.* 1999). The triple mutant will be referred to as GluR6(Q)ΔNG5,6,7 according to the nomenclature of Everts *et al.* (1999). We hypothesized that if full and partial agonists elicit distinct conformational changes during desensitization, the disruption of Con-A modulation by Con-A would be agonist specific.

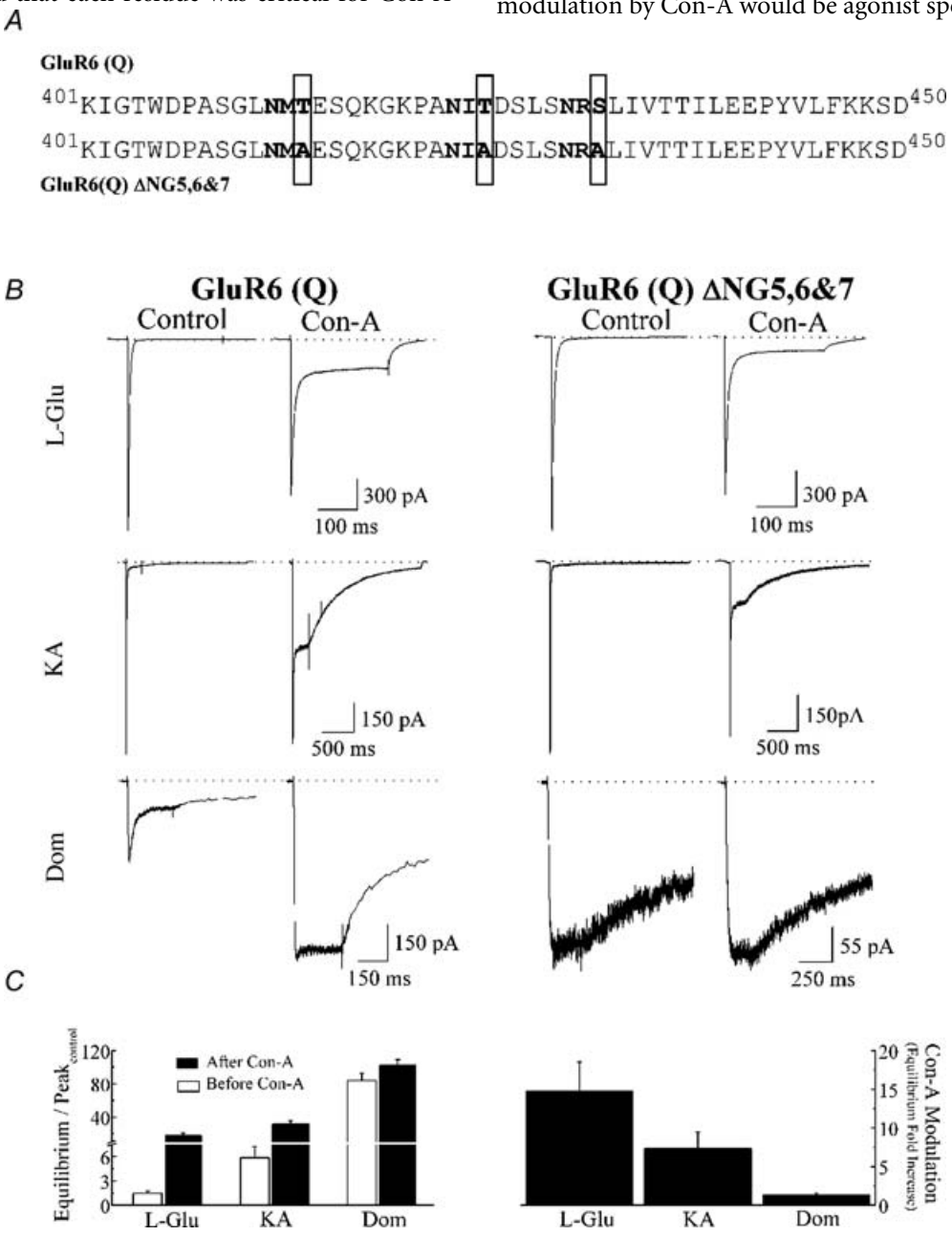

Figure 4. Disruption of Con-A binding sites interferes with Con-A modulation
A, amino acid sequence alignment of wild-type GluR6 and GluR6 (Q) ΔNG5,6 & 7 showing three *N*-glycosylation consensus sites (N-X-S/T, X ≠ P) highlighted in bold. Disruption of Con-A binding was achieved by replacing threonine (T) or serine (S) residues at these sites with alanines (A), as highlighted by grey boxes. *B*, comparison of the membrane currents evoked by L-Glu, KA and Dom at wild-type (patch number, 030724p2) and mutant (patch numbers, 041015p2 and 041008p2) GluR6 receptors before and after treatment with 10 μM Con-A. Although agonist responses evoked by wild-type and GluR6 ΔNG5,6,7 channels were comparable, the degree of modulation by Con-A was different. *C*: left panel, summary plot comparing the amplitude of the equilibrium response for GluR6 ΔNG5,6,7 channels with each agonist before and after treatment with Con-A; right panel, bar graph showing that the degree of modulation by Con-A of GluR6 ΔNG5,6,7 is agonist dependent but less than observed with wild-type receptors (cf. Fig. 3*D*).

Figure 4 compares experiments where wild-type and mutant GluR6 receptors were modulated by Con-A. As expected from previous work (Everts *et al.* 1997), removal of *N*-glycosylated residues did not significantly affect surface expression or the response profile of GluR6 receptor agonists (Fig. 4*B*). However, we did observe some variation in the Dom response. The majority of patches containing wild-type or GluR6(Q)ΔNG5,6,7 receptors exhibited a sustained response to Dom (Fig. 4*B*, right panel), but in some cases, the onset of desensitization was evident (Fig. 4*B*, left panel). This observation was labile in nature only appearing during the first but not subsequent applications of Dom making it difficult to study. Here, we have included both response types in our dataset since modulation by Con-A was indistinguishable. Compared to wild-type receptors, Con-A was less effective in modulating responses elicited by agonists acting on GluR6(Q)ΔNG5,6,7 receptors (Fig. 4*B* and *C*). Moreover, this disruption was agonist dependent. For example, Dom responses were rendered almost insensitive to treatment by Con-A in the triple mutant. The equilibrium/peak ratio observed after lectin treatment was only modestly increased compared to the equilibrium/peak ratio prior to Con-A (102.4 ± 6.8%, $n = 5$ and 84.2 ± 8.6%, $n = 5$, respectively). With L-Glu, equilibrium responses elicited by mutant receptors increased 15-fold (Fig. 4*C*) after Con-A treatment compared to the 30-fold increase observed in wild-type GluR6 (Fig. 3*D*). Finally, disruption of the modulation of KA responses was intermediate (Fig. 4*C*) consistent with the hypothesis that Con-A can be used to compare conformations elicited by agonists with different efficacies.

GluR6 agonists promote distinct conformational changes to intact KARs

To test if agonists cause distinct conformational changes during desensitization, we repeated experiments shown in Fig. 2 using prolonged applications of the partial agonists, KA and Dom (Fig. 5). Figure 5*A* and *C* shows representative experiments where treatment with Con-A was initiated only after responses evoked by 1 mM KA or 50 μM Dom reached equilibrium levels. As previously described (cf. Fig. 2), the amplitude of the equilibrium response before and at the end of treatment with Con-A was compared to assess lectin accessibility to the *N*-glycosylated sites (Fig. 5*B* and *D*). Unlike the full agonist L-Glu, Con-A modulated the equilibrium response

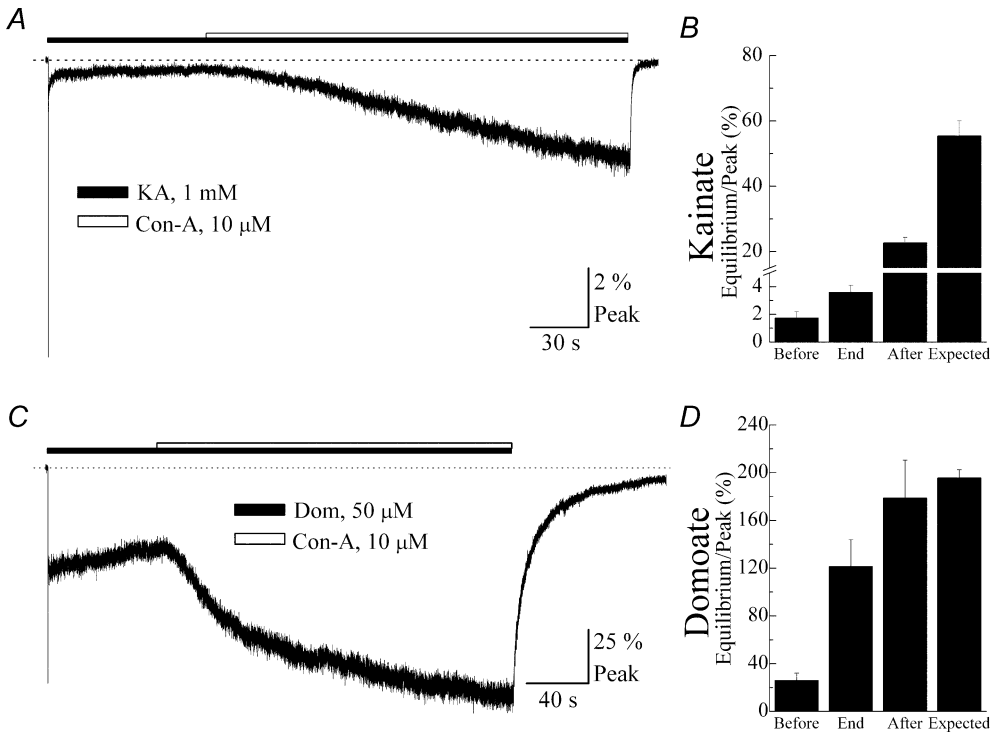

Figure 5. Accessibility of Con-A to its binding sites is increased by partial agonists
A and *C*, typical patch experiments where the effect of Con-A (10 μM) on the equilibrium response evoked by 1 mM KA (patch number, 031118p2) or 50 μM Dom (patch number, 031111p2) was tested. Filled and open bars indicate the application period of agonist and Con-A, respectively, and the dotted line denotes zero current level. Note that, unlike L-Glu, Con-A was able to modulate equilibrium responses elicited by each partial agonist. *B* and *D*, summary bar graphs showing the amplitude of the equilibrium response at various time points as described in Fig. 2. All data are expressed as the mean ± S.E.M.

elicited by partial agonists, KA and Dom (Fig. 5*A* and *C*). Moreover, it was possible to distinguish between partial agonists since the degree of Con-A modulation observed with Dom was greater than with KA. For example, Con-A treatment increased the equilibrium response (Before: $1.73 \pm 0.47\%$ Peak) evoked by KA approximately 2-fold when GluR6 receptors were pre-desensitized with the agonist (End: $3.60 \pm 0.51\%$ Peak) compared to an increase of 20- to 30-fold (Expected: $55.4 \pm 4.6\%$ Peak) when GluR6 receptors were treated in the absence of agonist (Figs 3*D* and 5*B*). In comparison, conformational events elicited by Dom only moderately restricted Con-A's accessibility. Here, Con-A increased the equilibrium response 5-fold on Dom-bound GluR6 receptors and approximately 6- to 7-fold when the agonist was absent (cf. Figs 3*D* and 5*D*). It is unlikely that these observations reflect Con-A modulating channels recycling through the open state since this mechanism would predict a greater effect on L-Glu responses than on KA or Dom responses. Indeed, our observations report the opposite effect where Con-A has a greater effect on equilibrium responses elicited by Dom or KA when compared to L-Glu (cf. Figs 2 and 5). It is also improbable that Con-A binds to resting channels since GluR6 receptors would be fully bound due to the saturating agonist concentrations used in these experiments. Taken together, these observations are in agreement with recent crystallographic data (Mayer, 2005) showing that partial agonists promote less closure of the agonist-binding domain than full agonists.

Figure 6 summarizes our results with Con-A in the presence and absence of full and partial agonists. Using the full agonist, L-Glu, Con-A's effect was strictly state dependent since the degree of modulation of the equilibrium response was dependent on whether GluR6 receptors adopted an agonist-bound (Co-Application: (0.92 ± 0.3)-fold increase) or unbound conformation (Pre-Incubation: (29.49 ± 3.9)-fold increase) (Fig. 6*A*). These two measurements were used to calculate an accessibility index ratio of 0.03 for L-Glu (Fig. 6*B*) which is consistent with crystallographic data (Mayer, 2005; Nanao *et al.* 2005). This finding also demonstrates that the small equilibrium response elicited by L-Glu at equilibrium (Fig. 3*A*) is associated with substantial conformational changes in the agonist-binding domain. In contrast, with the weak partial agonist Dom, the degree of Con-A modulation was similar whether lectin treatment occurred in the presence (Co-Application: (4.2 ± 1.0)-fold increase) or absence of agonist (Pre-Incubation: (5.62 ± 0.2)-fold increase) (Fig. 6*A*). In this case, the accessibility index ratio of 0.75 indicates that partial agonists promote weaker conformational changes upon binding which is associated with larger equilibrium responses (Fig. 3*A* and *B*). As expected, the accessibility index for KA (0.09) is consistent with its intermediary behaviour compared to full and weaker partial agonists (Fig. 6*B*). Interestingly, structural comparison revealed that Dom was the most bulky and L-Glu the most compact (Fig. 6*C*) suggesting that the physical nature of the agonist molecule may place constraints on the extent of domain closure. Taken together, these results suggest that the efficacy of full and partial agonists at equilibrium (Fig. 3) reflect distinct conformational changes in the agonist-binding domain of intact GluR6 KARs.

Discussion

We show that Con-A can be employed to report agonist-induced conformational changes in the extracellular portion of *intact* GluR6 KARs. As discussed

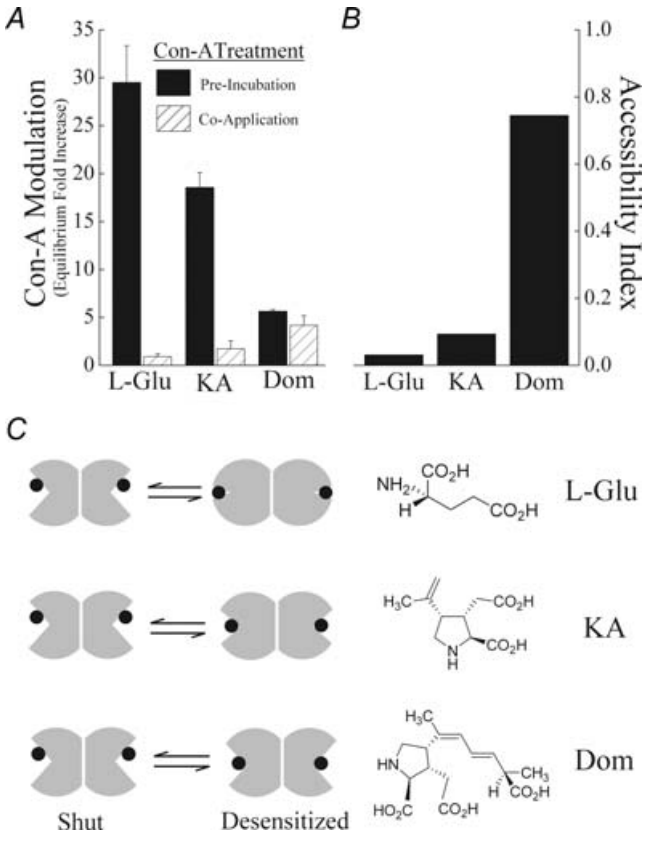

Figure 6. Multi-state model accounts for agonist behaviour at GluR6 receptors
A, summary plot comparing the effect of Con-A on the equilibrium response evoked by each agonist, either following a period of treatment with Con-A alone (filled bars) or in the presence of agonist (hatched bars). Note that although modulation by Con-A is state dependent with L-Glu, Con-A discriminates poorly between ligand-bound or ligand-free states with Dom. The plot is constructed using data in Figs 2, 3 and 5. All data are expressed as the mean ± S.E.M. *B*, data from *A* were used to determine an accessibility index as described in Results. *C*, schematic diagram illustrating that full and partial agonists promote distinct conformational changes in the agonist-binding domain of GluR6 receptors. The extended molecular structure of each agonist is shown opposite revealing that L-Glu is the most compact, and domoate is the most bulky in nature.

below, the most parsimonious explanation for our observations is that Con-A reports structural alterations in the agonist-binding domain. Crystallographical studies have not been able to provide structural information on the entire KAR due to technical considerations. Therefore, agonist behaviour has been examined by reconstituting the agonist-binding domain as two polypeptide chains with a linker domain. In agreement with reports describing KAR crystal structures, we show that GluR6 receptors adopt distinct conformations in the ligand-bound and unbound states. Moreover, the state-dependence of Con-A modulation is agonist-specific suggesting that full and partial agonists elicit distinct conformational changes in the agonist-binding domain during desensitization. Crystal structures of AMPA and KA receptors are thought to represent the activated state of the receptor since the extent of closure in the isolated ligand-binding core correlates with agonist efficacy. However, as addressed below, correlating conformational changes in this structure to functional properties of intact iGluRs remains an unresolved issue.

Comparison with previous studies

Although Con-A has been employed extensively as a pharmacological tool (Mayer & Vyklicky, 1989; Huettner, 1990; Wong & Mayer, 1993; Yue *et al.* 1995; Everts *et al.* 1997, 1999; Paternain *et al.* 1998), the state-dependence of its effects have not been examined in detail. State-dependent binding of Con-A was first observed in invertebrate iGluRs where Con-A-mediated effects (Mathers & Usherwood, 1976) were ineffective on desensitized channels (Evans & Usherwood, 1985). The authors concluded that structural rearrangements during desensitization masked carbohydrate moieties essential for Con-A binding (Evans & Usherwood, 1985). Since then, Con-A effects on mammalian GluR6 KARs have been documented (Yue *et al.* 1995; Everts *et al.* 1997; Paternain *et al.* 1998; Lerma *et al.* 2001) although state-dependent modulation has been described to a much lesser extent (Everts *et al.* 1999). This may reflect the difficulty in comparing observations with Con-A between different laboratories. For example, a significant variability in the potentiation of GluR6 KARs by Con-A has been reported in the literature ranging from 30- to 150-fold (Partin *et al.* 1993; Yue *et al.* 1995; Bowie *et al.* 2003) to 5000- to 6000-fold change (Everts *et al.* 1997, 1999). The reason for these differences is not clear but it does not reflect the electrophysiological recording techniques used (e.g. whole-cell *versus* patch) or the surrogate expression system (e.g. oocyte *versus* mammalian cell) chosen to study recombinant GluR6 receptors. In support of this, in separate experiments where we treated KARs with Con-A before or after excising patches, the degree of Con-A modulation was indistinguishable (data not shown).

Based on previous work, there are two possible explanations to account for Con-A's modulatory effect on equilibrium responses evoked by full and partial agonists (cf. Fig. 3). The first possibility is that Con-A blocks the onset of desensitization (Huettner, 1990; Partin *et al.* 1993; Wong & Mayer, 1993; Yue *et al.* 1995; Everts *et al.* 1997, 1999; Wilding & Huettner, 1997; Paternain *et al.* 1998). As a result, the potentiation of equilibrium responses evoked by strongly desensitizing agonists (e.g. L-Glu) would be expected to be greater than weakly desensitizing agonists (e.g. Dom). This explanation is unlikely, however, as there is no direct experimental evidence to support a mechanism whereby Con-A blocks entry into the desensitized state (Bowie *et al.* 2003). Previous studies had reached the conclusion that Con-A blocked desensitization based on the finding that lectin treatment eliminated the desensitization observed in whole-cell recordings. However, an important caveat in all of this work was that the rate of agonist perfusion used was too slow to accurately resolve the gating properties of GluR6 KARs (Bowie *et al.* 2003). Consequently, peak agonist responses were significantly underestimated in these studies. When experiments are performed in faster perfusion conditions, rates into and out of the desensitized state are unaffected by lectin binding (Bowie *et al.* 2003) demonstrating that Con-A does not block desensitization. The second possibility is based on the mechanism proposed by Bowie *et al.* (2003) whereby ion-conducting, desensitized states (Bowie & Lange, 2002) are up-regulated by lectin treatment. Here, the agonist-dependent nature of Con-A modulation is explained if, as proposed at AMPA receptors (Jin *et al.* 2003), full and partial KAR agonists activate different relative proportions of subconductance levels. As yet, analysis of single-channel currents activated by different GluR6 agonists has not been performed but would be necessary to delineate between an effect of Con-A on open-channel probability and/or unitary conductance (Bowie & Lange, 2002).

State-dependent modulation of KARs by Con-A

Although GluR6 subunits contain 10 *N*-glycosylated residues only nine are exposed to the extracellular surface and accessible to Con-A (Everts *et al.* 1999). The *N*-linked residue that does not bind Con-A is located in the pore region (Everts *et al.* 1999). All nine residues are positioned within or in close proximity to the agonist-binding domain of each GluR6 receptor subunit. Everts *et al.* (1999) have concluded that no single *N*-linked carbohydrate side chain is an absolute requirement for Con-A's effect, although the degree of modulation is significantly less with fewer residues present. Moreover, ectopic *N*-glycosylated sites introduced into the agonist-binding domain also impart sensitivity to Con-A and, as predicted, have a weaker effect compared to the greater number present on wild-type

GluR6 receptors (Everts *et al.* 1999). This observation supports the hypothesis developed here that Con-A binds to different residues in agonist bound or unbound conformations determining the degree of modulation. We further qualify these observations by showing that removal of three amino acid residues (i.e. GluR6(Q)ΔNG5,6,7) is sufficient to abolish the modulation of Dom responses with only a partial effect on L-Glu and KA.

Although used extensively to study invertebrate and mammalian iGluRs, state-dependent binding and modulation by Con-A has been described in only a few studies (Evans & Usherwood, 1985; Everts *et al.* 1999). As discussed above, Con-A modulates GluR6 receptors by binding to residues in close proximity to the agonist-binding domain (Everts *et al.* 1997, 1999) and we show here that this property permits inferences to be made about conformations adopted by this structure. We propose that modulation of GluR6 KARs involves two distinct molecular events. Initially, Con-A binds to either agonist-bound, desensitized channels or GluR6 channels in the closed, unbound state. Due to architectural rearrangements that accompany agonist binding (Armstrong *et al.* 1998; Armstrong & Gouaux, 2000), the number of *N*-glycosylated residues accessible to Con-A (Everts *et al.* 1999; Fig. 2*B*) is different for desensitized and unbound channel conformations. At desensitized receptors, bound Con-A molecules do not affect receptor function with full agonists such as L-Glu (Fig. 2). However, subsequent agonist dissociation sets off changes in protein structure that promote cross-linking of bound Con-A molecules or adjacent amino acid residues to regulate gating behaviour. This process will be different if Con-A has initially bound to GluR6 receptors in the desensitized or closed, unbound state. We propose that this cross-linking event, in both cases, restricts allosteric movement(s) of the external surface of GluR6 receptors affecting gating behaviour.

Correlating Con-A modulation to conformational changes in GluR6 receptors

In principle, the state-dependence of Con-A modulation may reflect conformational changes to the dimer interface, the pore region or the agonist-binding domain. Although Con-A may affect the dimer–dimer interface, our previously published findings (Bowie *et al.* 2003) provide experimental evidence that does not support this possibility. Specifically, we have shown that Con-A binding to GluR6 KARs does not affect rates into and out of desensitization. Since Horning & Mayer (2004) have argued that the dimer interface of KARs (and AMPARs) determines desensitization kinetics, by implication, our data demonstrate that Con-A does not affect dimer–dimer interactions. Likewise, Con-A's action is unlikely to reflect binding to the pore since amino acid residues critical for lectin binding and modulation are distant from this region. Instead, our experiments on GluR6(Q)ΔNG5,6,7 receptors and work by Everts *et al.* (1999) demonstrate that amino acid residues critical for lectin modulation are located in and around the agonist-binding domain. Other mechanisms may emerge as our understanding of KARs progresses. However, given these limitations, the most straightforward explanation of our data is that Con-A modulation reports conformational changes in the agonist-binding domain. In support of this, recent X-ray analysis of the isolated ligand-binding core of GluR6 KARs (Mayer, 2005; Nanao *et al.* 2005) also reported that full and partial agonists elicit distinct conformational changes in this region of the protein.

A potential caveat amongst these studies is that our experiments have focused on desensitized receptors whereas crystal structures of iGluRs are thought to represent the agonist-binding domain in the activated state of the channel (Jin *et al.* 2003). Three possible explanations may account for this apparent discrepancy. The first possibility is that published structures of the KA (and AMPA) receptor ligand-binding core represents the conformation adopted during ion channel activation (i.e. channel openings) as already proposed (Armstrong *et al.* 1998; Hogner *et al.* 2002; Mayer, 2005) but does not represent the binding cleft during desensitization. However, an important issue is that unitary current measurements indicate that single AMPA or KA receptor activations are very short-lived, lasting only a few milliseconds (Swanson *et al.* 1996, 1997; Howe, 1996). Consequently, it is more likely that X-ray crystal structures of the ligand-binding core represent another conformational state that is more thermodynamically stable.

The second possibility, therefore, is that following agonist binding the ligand-binding core adopts a much more stable conformation such as the desensitized state. To date, the possibility that crystal structures of the iGluR ligand-binding core represent the desensitized state has not been examined experimentally though it has been suggested by some authors (Madden, 2002; Colquhoun & Sivilotti, 2004; Naur *et al.* 2005). In support of this, experimental protocols that require an extended incubation period with the ligand (e.g. radioligand-binding assays) are known to accumulate ligand-gated ion channels into high-affinity desensitized states (Colquhoun, 1998). By analogy, crystallization of the ligand-binding core may also promote formation of the desensitized state. Moreover, estimates of the apparent affinity of L-Glu for desensitized GluR6 receptors ($IC_{50} = 0.44$–$0.5\ \mu M$ (Paternain *et al.* 1998; A.Y.C. Wong, A.-M. L. Fay & D. Bowie, unpublished observations) and the isolated ligand-binding core ($K_i = 1.4\ \mu M$) (Mayer, 2005) are almost identical whereas affinity for the activated

state ($EC_{50} = 694\ \mu M$) (Bowie *et al.* 2003) is more than 1000-fold lower.

The third and final possibility is that the conformation adopted by the ligand-binding core is identical whether the pore region is in the activated or desensitized state. This latter possibility would explain our observations on desensitized channels whilst agreeing with recent X-ray crystallographic data. However, this model, would have to reconcile with the fact that L-Glu evokes the largest peak response amongst all the agonists (see Fig. 3*A*) whereas the amplitude of its equilibrium response is the smallest (see Fig. 3*C*). In structural terms, the fact that partial agonists elicit responses of larger amplitude at equilibrium appears at odds with the proposed relationship between closure of the agonist-binding domain and agonist efficacy (Armstrong & Gouaux, 2000; Jin *et al.* 2003; Mayer, 2005). Clearly, further experimentation is required if these issues are to be resolved.

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Functional Characterization and In Silico Docking of Full and Partial GluK2 Kainate Receptor Agonists

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ABSTRACT

Two structural models have been developed to explain how agonist binding leads to ionotropic glutamate receptor (iGluR) activation. At α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) iGluRs, full and partial agonists close the agonist-binding domain (ABD) to different degrees whereas agonist-induced domain closure is apparently fixed at *N*-methyl-D-aspartate receptors. Although kainate (KA) iGluRs are thought to behave like AMPA receptors, the issue has not been formally tested because of the paucity of available receptor agonists. Here we identify a series of structurally related full and partial agonists at GluK2 (formerly GluR6) KARs and predict their docking mode using the in silico ligand-docking program FITTED. As expected, the neurotransmitter L-Glu behaved as a full agonist but modest reduction (e.g., L-serine or L-aspartate) or elongation (e.g., L-α-aminoadipate) in chain length generated weak partial agonists. It is noteworthy that in silico ligand-docking predicted that most partial agonists select for the closed and not, as expected, the open or intermediate conformations of the GluK2 ABD. Experiments using concanavalin-A to directly report conformations in the intact GluK2 receptor support this prediction with the full agonist, L-Glu, indistinguishable in this regard from weak partial agonists, D- and L-Asp. Exceptions to this were KA and domoate, which failed to elicit full closure as a result of steric hindrance by a key tyrosine residue. Our data suggest that alternative structural models need to be considered to describe agonist behavior at KARs. Finally, our study identifies the responsiveness to several neurotransmitter candidates establishing the possibility that endogenous amino acids other than L-Glu may regulate native KARs at central synapses.

iGluRs mediate the vast majority of excitatory neurotransmission in the mammalian brain and have been implicated in numerous CNS disorders (Bowie, 2008). Given this, much research has focused on their structure-function properties because, among other benefits, it provides a rational approach to drug discovery. Insight into their structure was first advanced by homology modeling using the bilobed domain of bacterial amino acid binding proteins as a template (Stern-Bach et al., 1994). Subsequently, the agonist-binding domain (ABD) of the GluA2 (Collingridge et al., 2009) (formerly GluR2 or GluRB) AMPAR was crystallized, revealing the predicted clamshell-like structure of globular domains 1 and 2 that close upon agonist binding (Armstrong and Gouaux, 2000). Since then, a similar approach has permitted the atomic resolution of ABDs of all iGluR family members, including the KAR (Mayer, 2005; Nanao et al., 2005), NMDAR (Inanobe et al., 2005), and, more recently, the δ-2 orphan iGluR (Naur et al., 2007). From these studies, two structural models of agonist behavior have emerged. At the NR1 NMDAR subunit, full and partial agonists differ little in the conformational change they elicit in the ABD (Inanobe et al., 2005). In contrast, at AMPARs, agonist efficacy is thought to reside in the conformations adopted by the ABD, full agonists more effective at promoting domain closure than partial agonists (Armstrong and Gouaux, 2000; Jin et al., 2003).

Although KARs are thought to behave like AMPARs, the structural basis of agonist efficacy of this receptor family has

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ABBREVIATIONS: iGluR, ionotropic glutamate receptor; CNS, central nervous system; ABD, agonist-binding domain; AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; AMPAR, AMPA receptor; KA, kainate; KAR, kainate receptor; Dom, domoate; NMDA, *N*-methyl-D-aspartate; NMDAR, *N*-methyl-D-aspartate receptor; QA, quisqualic acid; SYM 2081, (2*S*,4*R*)-4-methylglutamic acid; BAPTA, 1,2-bis(2-aminophenoxy)-ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid; RMSD, root-mean-square deviation; SOS, L-serine-*O*-sulfate; L-Cys, L-cysteic acid; SSC, *S*-sulfo-L-cysteic acid; HCSA, L-homocysteine sulfinic acid; HC, L-homocysteic acid; Con-A, concanavalin-A; CNQX, 6-cyano-7-nitroquinoxaline-2,3-dione; PhTX, philanthotoxin; FITTED, Flexibility Induced Through Targeted Evolutionary Description.

not been firmly established for several reasons. First and foremost, there are fewer agonist-bound crystal structures available to make the comparison. To date, the ABD of GluK1 and/or GluK2 bound by the full agonist L-Glu and partial agonists KA and domoic acid (Dom) have been resolved at atomic resolution (Mayer, 2005; Nanao et al., 2005). Other structures for quisqualic acid (QA) and SYM 2081 have also been described (Mayer, 2005) but it is not yet clear whether they act as full or partial agonists. Second, the extent of domain closure elicited by the full agonist, L-Glu, differs from partial agonist, KA, by only 3° (Mayer, 2005), which is modest in comparison with the effect of the same agonists at AMPARs (e.g., L-Glu versus KA, 8° difference) (Armstrong and Gouaux, 2000). An added complication is that the apo state of the KAR ABD has yet to be resolved; therefore, the extent of domain closure is given with respect to the GluA2 AMPAR apo state. Third and finally, KARs require external anions and cations as well as the neurotransmitter L-glutamate for activation (Wong et al., 2006), a property not shared by AMPARs (Bowie, 2002). Given this, it is possible that the degree of activation of KARs is shaped not only by the agonist molecule but also by external ions.

Here, we have tested the functionality of a range of L-Glu analogs as a first step in understanding the structural basis of agonist behavior at KARs. To complement this data, we also used the in silico ligand-docking program FITTED to predict the conformation of the ABD preferred by each agonist. It is noteworthy that this combined approach suggests unexpectedly that most partial agonists select for the closed and not the open or intermediate conformation of GluK2 ABD. This finding suggests that agonist efficacy at KARs may not be solely determined by the extent of closure in the GluK2 ABD; therefore, alternative structural models may need to be considered.

Materials and Methods

Cell Culture and Transfection. Techniques used to culture and transfect mammalian cells to express GluR6 KARs have been described in detail elsewhere (Bowie, 2002). In brief, tsA201 cells, a transformed human embryonic kidney 293 cell line stably expressing a simian virus 40 temperature-sensitive T antigen (provided by R. Horn, Jefferson Medical College, Philadelphia, PA) were maintained at a confluence of 70 to 80% in minimal essential medium with Earle's salts, 2 mM glutamine, and 10% fetal bovine serum supplemented with penicillin (100 units/ml) and streptomycin (100 μg/ml). After plating at low density (2×10^4 cells/ml) on plastic dishes, cells were transfected with cDNA encoding unedited rat glutamate receptor subunit 6 using the calcium phosphate technique as described previously (Bowie, 2002). The cDNA for enhanced green fluorescent protein (S65T mutant) was routinely cotransfected to identify transfected cells. In this and all subsequent publications from our laboratory, we adopt the recommended change to iGluR nomenclature (Collingridge et al., 2009). Consequently, GluR6 will be referred to as GluK2 and the GluR-B or GluR2 AMPAR subunit as GluA2.

Electrophysiological Solutions and Techniques. All ligands tested in this study were dissolved in external solutions containing 150 mM NaCl and 5 mM HEPES with low concentrations of $CaCl_2$ and $MgCl_2$ (0.1 mM each) to avoid divalent block. For dose-response relationships to D- and L-Asp (Fig. 3D), however, agonists were applied at concentrations (i.e., >100 mM) that would cause a shift in reversal potential as a result of changes in the driving force for the main permeant ion, Na^+. To avoid this, the ionic strength of all solutions was increased to 200 mM, with the desired agonist concentration balanced by the appropriate amount of NaCl. All concentrated ligand solutions were adjusted to pH 7.3 with NaOH before being stored at −20°C. Saturating agonist concentrations chosen for L-Glu (10 mM), kainate (1 mM), domoate (50 μM) were at least 5-fold higher than published EC_{50} values at GluK2 receptors. We confirmed empirically that these concentrations were saturating by doubling the agonist concentration in each case and observing that peak response amplitudes were unchanged. For sulfur-containing amino acids, QA, SYM 2081, and L-α-aminoadipate, saturating levels were determined empirically by increasing concentrations until a maximal response was observed. In cases in which millimolar concentrations of agonist were required for activation (e.g., 40 mM L-cysteic acid), the reported response amplitudes were corrected for the shift in the reversal potential observed. Internal pipette solution contained 115 mM NaCl, 10 mM NaF, 5 mM HEPES, 5 mM Na_4BAPTA, 0.5 mM $CaCl_2$, 1 mM $MgCl_2$, and 10 mM Na_2ATP to chelate endogenous polyamines. The pH and osmotic pressure of internal and external solutions were adjusted to 7.3 and 295 mOsmol/kg, respectively. Concanavalin-A (Con-A) (Sigma, St. Louis) was prepared in glucose free saline solution and filtered (0.2 μm filter, Corning) immediately before use as described previously (Bowie et al., 2003). All recordings were performed with an Axopatch 200B amplifier (Axon Instruments Inc., CA) using thin-walled borosilicate glass pipettes (2–5 MΩ) coated with dental wax to reduce electrical noise. Control and agonist solutions were rapidly applied to outside-out patches excised from transfected tsA201 cells as described previously (Bowie, 2002). Solution exchange (10–90% rise-time = 25–50 μs) was determined routinely at the end of the experiment by measuring the liquid junction current (or exchange current) between the control and agonist-containing solution in which total Na^+-content was reduced by 5%. Current records were filtered at 5 kHz, digitized at 25 to 50 kHz and series resistances (3–10 MΩ) compensated by 95%. Most recordings were performed at −20 mV membrane potential to ensure adequate voltage clamp control of peak currents. Data acquisition was performed using pClamp9 software (Molecular Devices, Sunnyvale, CA). All experiments were carried out at room temperature (22 to 23°C).

Overview of the Docking Program FITTED. Conformational changes in the ligand-binding domain of iGluRs have been investigated through X-ray crystallography. Previous X-ray data have revealed two fundamental features pertaining to the ligand-binding domain of iGluRs, which has made it difficult to accurately model these proteins. First, the model must allow for protein flexibility, because it is well established that the ligand-binding domain can adopt a range of degree of clamshell closure. Moreover, given that water molecules have been shown to play a key role in stabilizing the ligand in the binding cleft of both AMPA and KARs (Mayer, 2005), the docking program would have to allow for displacement and movement of waters. Until recently, docking software that simultaneously accounted for these features in their search algorithm was not available. However, the development of a genetic algorithm based docking program called FITTED 2.0 (Flexibility Induced Through Targeted Evolutionary Description), which performs all these functions has recently been described previously (Corbeil et al., 2007). This docking tool can uniquely accommodate for displaceable bridging water molecules, whereas treating the ligand/protein as a realistically dynamic system, and therefore provides the most appropriate docking approach to investigate iGluRs. For data shown in this study, we used FITTED version 2.0 using the semiflexible docking option with displaceable waters and, in each case, the pharmacophore-oriented docking function was used (Corbeil et al., 2007).

Protein and Ligand Structure Preparation before Docking. The X-ray structures of GluK2 complexes were retrieved from the Protein Data Bank (codes 1s50, 1s7y, 1sd3, 1s9t, 1tt1, 1yae) and hydrogen atoms were added with their position optimized through energy minimization. The result was visually inspected, as described previously to ensure the optimum hydrogen bond network (Corbeil et al., 2007). Six bridging water molecules found to be conserved

throughout most of the ligand-protein complexes were retained for the docking study. All protein structures were prepared using ProCESS (a module of FITTED), and the ligands were fully ionized and prepared with SMART (a module of FITTED) (Corbeil et al., 2007).

Docking Amino Acid Ligands using FITTED. The data obtained from docking experiments are summarized in Table 1. Six protein structures initially resolved with five different agonists were used as input files [i.e., 1s7y (L-Glu), 1s9t (QA), 1sd3 (SYM 2081), 1tt1 (kainate), 1yae_a (domoate, conformation 1), and 1yae_b (domoate, conformation 2)]. All the original Protein Data Bank files pertain to KAR dimer structures solved with different ligands (Mayer, 2005) with the exception of 1yae, which was solved as a hexamer (Nanao et al., 2005). To compare GluK2 monomers within a given polymer, protein superimposition was achieved by aligning the α-carbons of the residues found with at least one atom within 10 Å from the ligand. With the exception of 1yae, all the monomers within a given polymer were identical. Therefore, only one of the monomers/dimer was retained for the docking studies. As for 1yae, two monomers (1yae_a and 1yae_b) were retained as input files to allow for greater protein fluctuations within the binding pocket. The five agonists (L-Glu, QA, SYM 2081, KA, and domoate) were docked using six protein structures as input files (1s7y, 1s9t, 1sd3, 1tt1, 1yae_a, and 1yae_b). As previously reported, comparison of the crystal structures reveals three distinct protein conformations that we will refer to as closed, intermediate, and open. Consistent with the identical degree of domain closure observed with the binding of L-Glu (1s7y), SYM 2081 (1sd3), or QA (1s9t) at GluK2 crystals, the computed root-mean-square deviation (RMSD) between the active site of all three protein structures were small (1s7y and 1sd3, 0.24 Å; 1s7y and 1s9t, 0.46 Å; 1sd3 and 1s9t, 0.51 Å). For the remainder of the text, the term "closed" conformation will be used to refer to any of these three protein conformations. In agreement with crystallographic studies (Nanao et al., 2005), the "open" state will denote the conformation observed with domoate-bound crystals (RMSD between 1s7y and 1yae, 1.6 Å). Finally, the conformation adopted by the kainate-bound GluR6 crystal conformation will be termed "intermediate" (RMSD between 1s7y and 1tt1, 0.91 Å).

We assessed the validity of FITTED 2.0 for GluK2 KARs in several ways. First, we performed statistical analysis comparing the ligand bound in the actual crystal structures with the docked ligand predicted by FITTED. A ligand pose was considered successfully docked when the RMSD relative to the ligand bound in the actual crystal structure was below 2.0 Å (Table 1) (Corbeil et al., 2007). Second, the protein structure was considered to be accurately selected when the population favored that specific protein conformation over others (Corbeil et al., 2007). Third, we compared the number and position of water molecules in the crystal structure with that predicted by FITTED. In all cases, FITTED correctly predicted the number and position of water molecules. For each pose, FITTED used the RankScore function to yield a docking score, an estimation of the free energy of binding including entropic contributions (Table 1). It is noteworthy that although the scoring function has been trained to reproduce free energies of binding, the accuracy level is not high enough to make highly accurate predictions within two orders of magnitude in K_i. In addition, the apparent agonist affinity (see Fig. 3) is not governed only by the free energy of agonist binding but also by multiple aspects of ion-channel behavior that include channel gating properties and desensitization. A minimum set of 10 runs was carried out for each ligand (Corbeil et al., 2007). An initial population of 500 was enough for docking of all GluK2 KAR ligands to reach the convergence criterion. Moreover, a maximum of 500 generations was used to reach convergence for each ligand.

Assumptions of Molecular Docking Strategy. To perform molecular ligand docking experiments, four assumptions were made. First, our modeling strategy pertained to transposing the information obtained from resolved crystal with the behavior of the mature receptor under physiological conditions. Our electrophysiological recordings from GluK2 KARs were performed under physiological pH 7.3 to 7.4, whereas most of the GluK2 S1S2 isolated cores were crystallized under conditions that were significantly more acidic (ranging from 4.0 to 6.5). To assess the effect (if any) of these pH fluctuations, we compared the two GluK2 KAR crystals in complex with L-Glu that were cocrystallized at distinct pH (1s50, pH 8.0; 1s7y, pH 4.8) (Mayer, 2005). Visual inspection of the superimposed protein-ligand complexes revealed no significant differences between the two structures. We therefore used the 1s7y structure and did not further consider 1s50 in our analysis. Second, our modeling experiments assumed that all the amino acids tested bind in the same cavity between the S1S2 domains (i.e., the orthosteric site) as previously reported for other ligands cocrystallized with GluK2 KARs. Third, because the apo state of GluK2 KARs has yet to be resolved, the degree of domain closure of the agonist-binding domain was obtained in comparison with the apo state of GluA2 AMPA receptor. Fourth, we have assumed that L-Glu analogs bind to one of the three known GluK2 conformations identified through X-ray crystallography (open, intermediate, and closed). The computed RMSD between the active sites of structures for L-Glu (1s7y), SYM 2081 (1sd3) and QA (1s9t) were small (1s7y and 1sd3, 0.24 Å; 1s7y and 1s9t, 0.46 Å;

TABLE 1

Functional and structural properties of GluK2 KAR agonists

Functional properties of responses elicited by the sixteen GluK2 receptor agonists examined in this study. Structural information obtained with FITTED are also provided. All data are expressed as the mean ± S.E.M.

Agonist, Concentration Range	Peak		Ligand Category	Conformation		RMSD	Docking Score (FITTED)
				Selected (FITTED)	Experimental (Crystallography)		
	%	*n*					
L-Glu,10 mM	100	43	Full	Closed	Closed	0.34	−5.73
SYM 2081, 1–3 mM	102.0 ± 7.7	3	Full	Closed	Closed	0.24	−6.52
QA, 1–3 mM	90.0 ± 1.1	3	Full	Closed	Closed	2.0	−8.70
L-Cys, 10–60 mM	73.6 ± 1.9	4	Partial	Closed			−5.60
SSC, 1–2 mM	64.1 ± 7.6	5	Partial	Closed			−8.06
HCSA, 10–40 mM	58.0 ± 5.4	5	Partial	Closed			−6.84
KA, 1–3 mM	39.1 ± 2.0	10	Partial	Intermediate	Intermediate	0.46	−8.33
HC, 10–40 mM	34.4. ± 3.4	8	Partial	Closed			−6.62
CSA, 10–40 mM	31.72 ± 3.75	3	Partial	Closed			−6.97
Dom, 50–150 μM	15.3 ± 1.9	8	Partial	Open	Open	1.2	−8.88
L-Aminoadipate, 10–40 mM	14.05 ± 0.19	4	Partial	Closed			−6.70
D-Asp, 10 mM	3.6 ± 1.15	6	Partial	Closed			−6.29
L-Asp, 10 mM	0.95 ± 0.39	5	Partial	Closed			−5.13
SOS, 1–20 mM	0.52 ± 0.7	3	Partial	Closed			−7.24
L-Ser, 10 mM	0.26 ± 0.3	3	Partial	Closed			−5.01
D-Ser, 10 mM	0.05 ± 0.5	3	Partial	Closed			−4.96

1sd3 and 1s9t, 0.51 Å), and therefore their domain closure was considered indistinguishable in agreement with previous structural analysis (Mayer, 2005).

Results

L-Glu Analogs Exhibit a Wide Range of Agonist Activity at GluK2 Receptors. In an effort to identify receptor ligands that exhibit the full range of agonist behavior, we studied the kinetic properties of a number of commercially available L-Glu analogs (see *Materials and Methods*). In all cases, agonists were applied at saturating concentrations and at frequencies that permit full recovery from desensitization. Figure 1 shows the extended structure of the selected amino acids all of which possess a common L-Glu backbone. We purposely chose this group of amino acids because they would provide information on how agonist efficacy is shaped by changes in chain length, atom substitution, as well as the addition of side groups and/or sulfur groups. Agonist activity of several of these amino acids have been previously reported at AMPARs, NMDARs, and metabotropic glutamate receptors (Patneau and Mayer, 1990; Kingston et al., 1998) but not yet at KARs.

Almost all amino acids tested elicited membrane currents that consisted of a rapidly rising peak response, which declined in the presence of the agonist to a new equilibrium level (Fig. 2 upper, Table 1). In some cases, as with L-serine-*O*-sulfate (SOS) and stereoisomers of serine (Ser) and aspartate (Asp), responses were difficult to resolve because of their small amplitude (even in high-expressing patches), which made detailed kinetic analysis problematic (Figs. 2 and 3). Nevertheless, a wide range of agonist efficacy was observed among all the amino acids tested (Fig. 2, bottom). For example, five sulfur-containing amino acids exhibited the following rank order of efficacy: L-cysteic acid (L-Cys, 40 mM) > *S*-sulfo-L-cysteic acid (SSC; 20 mM) > L-homocysteine sulfinic acid (HCSA; 40 mM) > L-homocysteic acid (HC; 20 mM) > SOS (1 mM) based on peak response amplitude with saturating agonist concentrations (Fig. 2, top). As mentioned above, SOS evoked barely detectable responses demonstrating that even modest changes to the agonist structure has pronounced effects on agonist efficacy (Fig. 2, top). In this case, replacement of the sulfur atom at the ω-position with an oxygen converted the partial agonist, SSC, into the poorly stimulating SOS. Except for SYM2081 and QA, all other

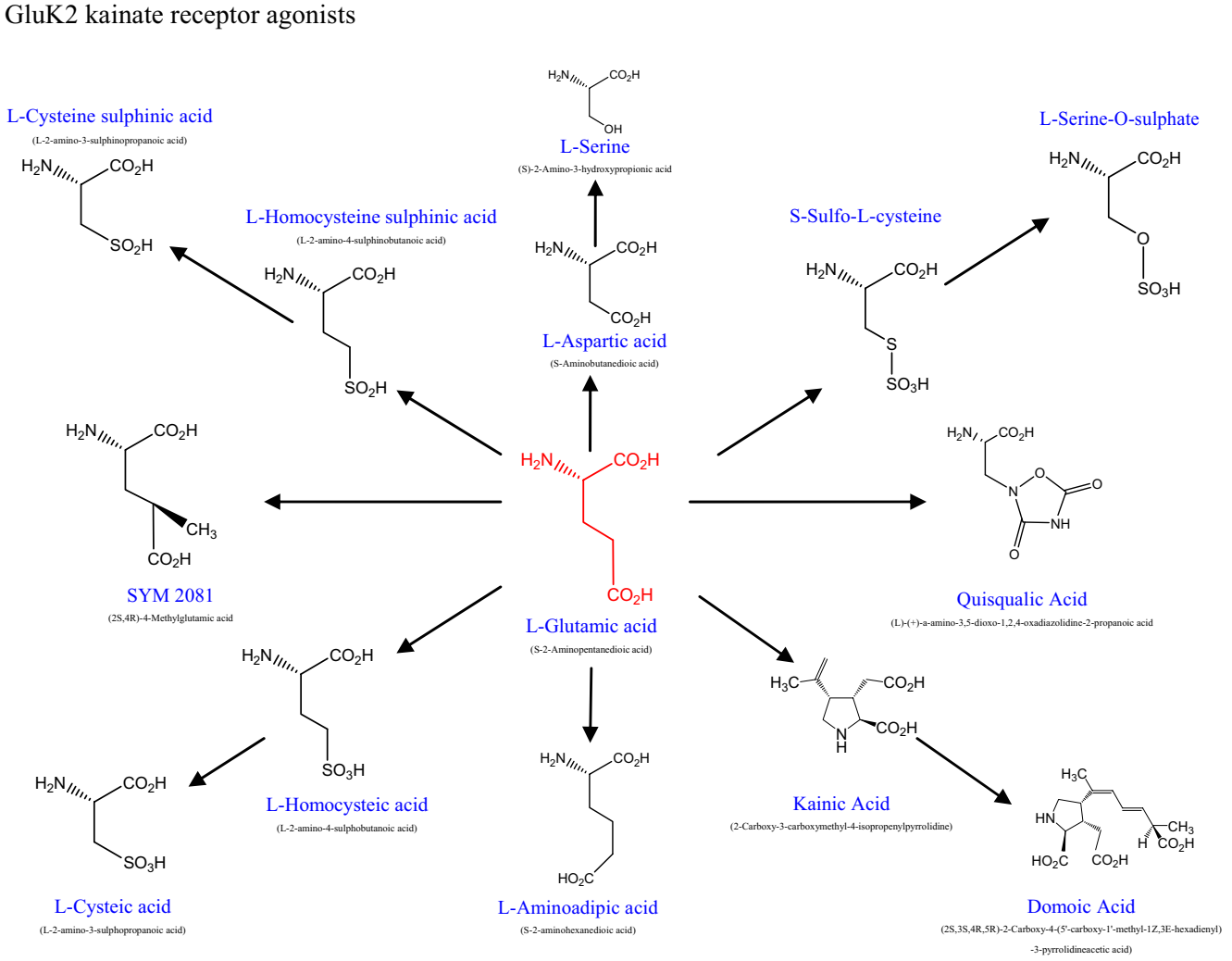

Fig. 1. Extended structure of GluK2 receptor agonists. Schematic diagram showing the extended structure of all the amino acids selected for investigation. All amino acids are structural analogs of L-Glu and thus were chosen purposely to provide information on how changes in chain length, atom substitution, and the addition of side groups and/or sulfur groups affect agonist efficacy. Each structure is identified by both its common and IUPAC nomenclature.

agonists tested were partial agonists because they elicited peak responses smaller than that observed with L-Glu (one sample t test, $p < 0.01$; Fig. 2, bottom). Finally, modest reduction (e.g., L-serine and L-aspartate) or elongation (e.g., L-α-aminoadipate) in chain length of the L-Glu structure generates weak partial agonists suggesting that the KAR ABD is optimized for the binding of this amino acid.

Desensitization Does Not Profoundly Affect Estimates of Peak Response Amplitude. Although solution exchanges performed in this study were rapid, relative rates of activation and desensitization may vary among different agonists. Consequently, agonists designated as poorly conducting (i.e., weak partial agonists) may, in fact, behave as full agonists if studied in the absence of desensitization. To address this issue, we looked more closely at SOS and the stereoisomers (i.e., D and L) of both Asp and Ser, which were ideal for this purpose because these ligands represent the five weakest responding agonists, which, as explained above, may reflect genuine partial agonist activity or result from rapid rates into desensitization. To delineate between these two possibilities, we examined agonist responses after treatment with the plant lectin, concanavalin-A (Con-A).

Although Con-A does not block desensitization or shift apparent agonist affinity, it irreversibly increases current flow through GluK2 KARs (Bowie et al., 2003). We reasoned that this property would permit better resolution of re-

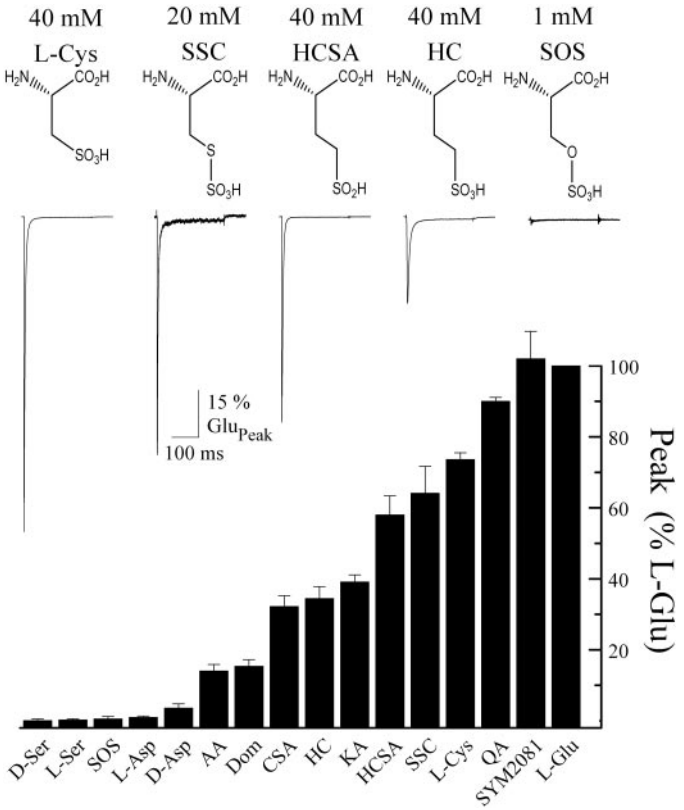

Fig. 2. Response profile of an extended series of GluK2 kainate receptor agonists. Top, structure-function relationship of five sulfur-containing amino acids aligned in order of peak agonist responsiveness. To allow comparison between experiments, membrane currents were normalized to the peak L-Glu response in each recording. Patch numbers were 04622p4 (L-Cys), 04629p2 (HCSA), 060720p1 (HC), 060720p4 (SSC)b, and 04621p1 (SOS). Bottom, summary bar graph comparing the peak response amplitude observed with saturating concentrations of each amino acid (n = 8–43 patch recordings). The data are arranged in increasing order of responsiveness, from very weak partial agonists (stereoisomers of serine and aspartate as well as SOS) to QA, SYM 2081, and L-Glu, which are full agonists. All data are expressed as the mean ± S.E.M.

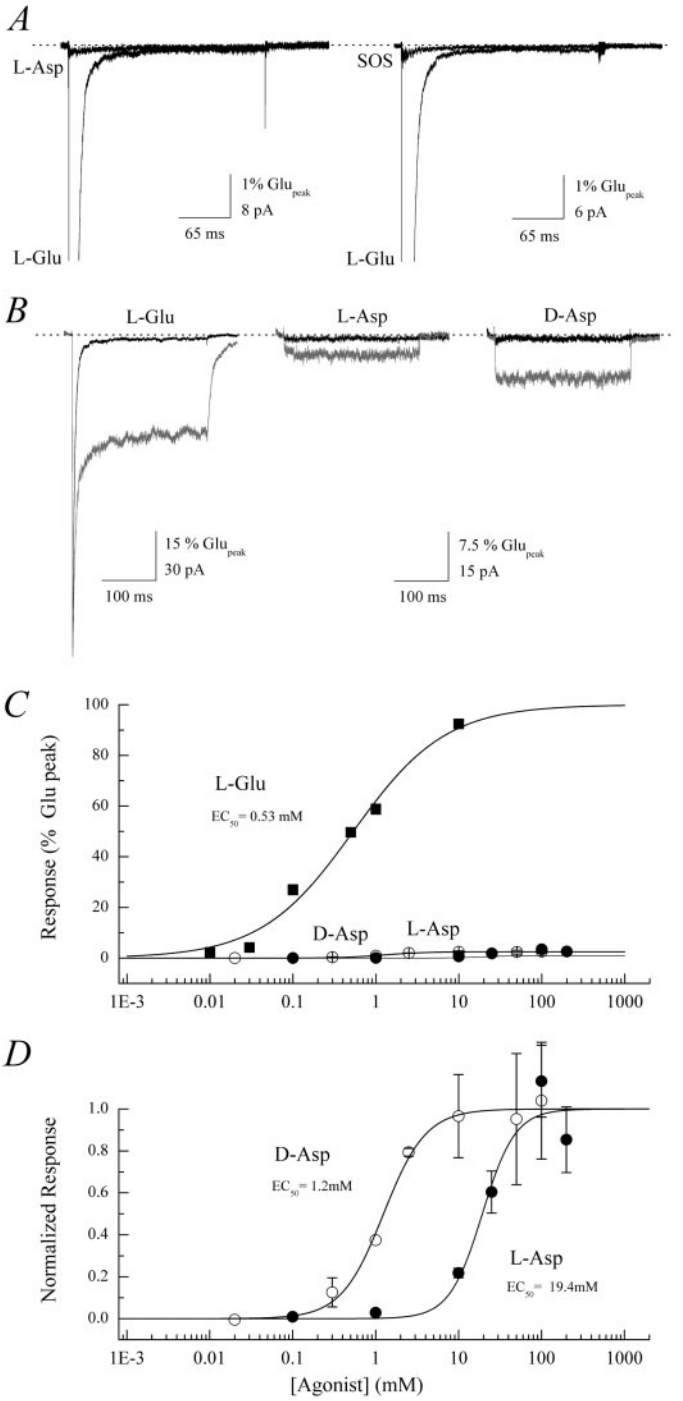

Fig. 3. Stereoisomers of aspartate are partial agonists at GluK2 kainate receptors. A, representative membrane currents elicited by 10 mM L-Glu, 10 mM L-Asp, and 1 mM SOS (patch numbers 080425p2). The dotted line denotes the zero current level. B, typical electrophysiological recordings elicited by 10 mM L-Glu, L-Asp, and D-Asp (10 mM each) before (black line) and after (gray line) Concanavalin-A (3 min) treatment in the same patch (patch number 080425p2). Con-A treatment reveals that both D- and L-Asp elicit rapidly rising, nondesensitizing membrane currents that quickly deactivate upon cessation of the agonist application. C and D, activation curves to L-Glu as well as D- and L-Asp reveal that stereoisomers of Asp are weak partial agonists with significantly lower affinity than with the full agonist L-Glu.

sponses elicited by weakly responding agonists. Before Con-A treatment, typical responses elicited by each of these agonists were small in amplitude, which made accurate analysis of their kinetic properties problematic as shown in Fig. 3A for 10 mM L-Asp and SOS. To allow comparison, membrane currents elicited by the full agonist, L-Glu (10 mM), in the same patch recording are shown superimposed (Fig. 3A). As anticipated, Con-A treatment (10 μM, 3–5 mins) increased current flow through GluK2 receptors activated by SOS and stereoisomers of both Asp and Ser, making it possible to routinely study their peak responses (Fig. 3B). From detailed analysis of the stereoisomers of Asp, two important characteristics of their response were revealed that unequivocally demonstrate that they behave as partial agonists. First, stereoisomers of Asp elicited rapidly rising, nondesensitizing membrane currents showing that these agonists are not weakly responding because of the rapid onset of desensitization (Fig. 3B). Second, construction of activation curves for each agonist revealed that maximal responses in each case were significantly smaller than with L-Glu (Fig. 3C). Compared with the maximal response elicited by L-Glu, responses to saturating concentrations of D- and L-Asp were 2.5 ± 0.1% and 3.0 ± 0.3% (n = 4–6), respectively. In addition, estimated EC_{50} values (Hill coefficient, n_H) for D-Asp and L-Asp were 1.2 ± 0.1 mM (n_H = 1.7 ± 0.3) and 19.4 ± 4.7 mM (n_H = 2.2 ± 1.1), respectively, compared 0.5 ± 0.1 mM (n_H = 0.8 ± 0.1) for L-Glu (Fig. 3, C and D). Taken together, these observations directly demonstrate that D -and L-isomers of Asp elicit responses of small amplitude because they are partial agonists and not due to the rapid onset of desensitization.

In Silico Ligand-Docking Correctly Identifies Conformations Adopted by the Gluk2 Agonist-Binding Domain. To combine this functional data with in silico ligand-docking using FITTED, we first focused on receptor agonists previously cocrystallized with the isolated ligand binding core of GluK2 (Mayer, 2005; Nanao et al., 2005). From electrophysiology recordings, we already identified kainate (1 mM KA) and domoate (50 μM Dom) as partial agonists at GluK2 receptors with L-glutamate (10 mM L-Glu), SYM 2081 (3 mM), and quisqualate (3 mM QA) all behaving as full agonists when applied at saturating concentrations (Fig. 4, A and B). Peak KA and Dom responses were 39.1 ± 2.0% (n = 10) and 15.3 ± 1.9% (n = 8) respectively of the maximal full agonist response (Fig. 4B, Table 1). Previous structural work

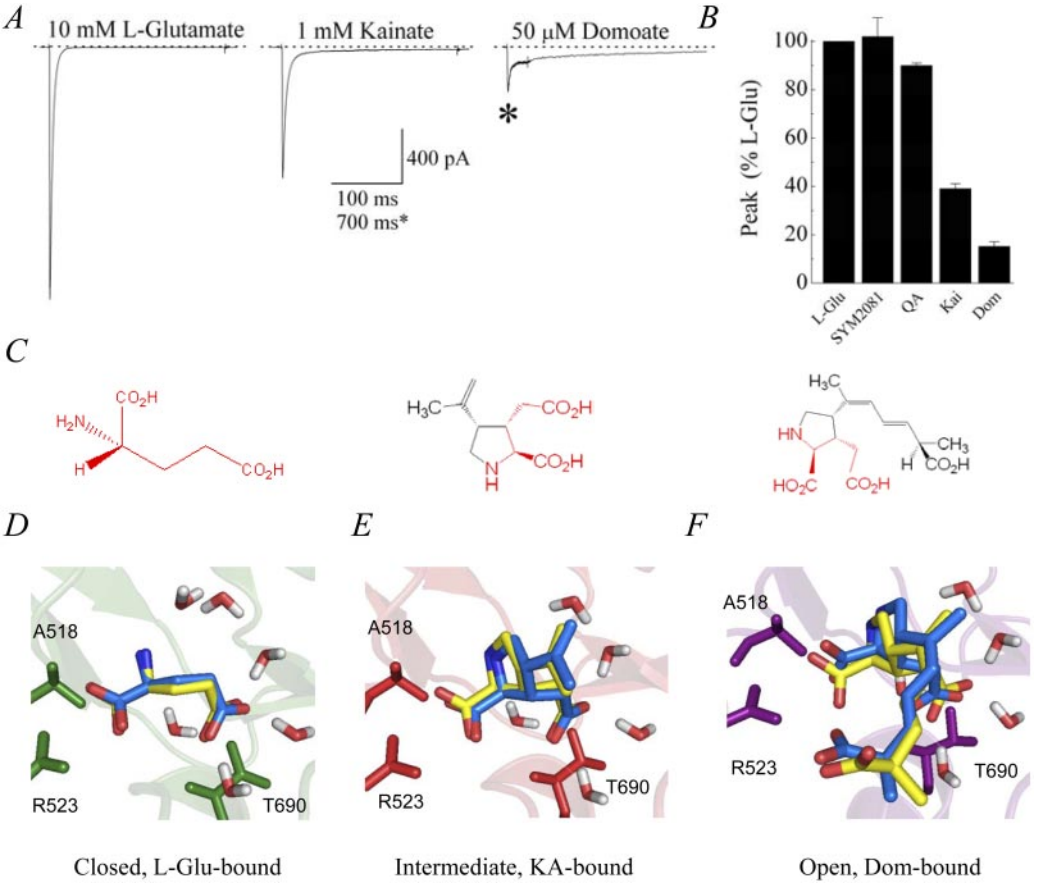

Fig. 4. FITTED accurately predicts conformations adopted by the GluK2 agonist-binding domain. A, membrane currents evoked by L-Glu (10 mM, 250-ms duration, H_p = −20 mV), KA (1 mM), and Dom (50 μM) in the same outside-out patch containing homomeric GluK2 channels (patch number 030724p2). *, Dom response is drawn on a different time base. B, summary plot showing peak responses evoked by five agonists, all of which have been cocrystallized with the GluK2 KAR: L-Glu (n = 13), SYM 2081 (n = 3), QA (n = 3), KA (n = 13), and Dom (n = 8). All data are expressed as the mean ± S.E.M. C, extended molecular structures showing that kainate and domoate have a common L-Glu backbone (red labeling). D to F, superimposition of the GluK2 agonist-binding pocket containing L-Glu, KA, and Dom where the solved crystal structures are compared with that docked by FITTED. In this and subsequent figures, the numbering of amino acid residues begins at the start site of the open reading frame and therefore includes the signal peptide. The solved crystal structures are shown in yellow, whereas the modeled structures are in blue. Key residues as well as agonist and water molecules are shown as sticks. L-Glu, KA, and Dom selected the closed (green), intermediate (red), and the open conformations (purple), respectively. Note that binding of KA and Dom displaces one of the key surrogate water molecules, which are present in the L-Glu-bound crystal. Nonpolar hydrogens are omitted for clarity.

has shown that Dom induces domain closure of 12.3°, KA elicits an intermediate closure of 23.3°, whereas the degree of domain closure with SYM 2081, QA, and L-Glu are between 26.2° to 26.6° (Mayer, 2005; Nanao et al., 2005). Consequently, our electrophysiological data support the current view that agonist efficacy is determined by the degree of closure in the GluK2 ABD (Mayer, 2005; Nanao et al., 2005).

To look at domain closure and binding mode, we performed in silico ligand-docking with the same series of receptor agonists using FITTED (Fig. 4, D–F). FITTED is a suite of programs that is unique in that the fitting process permits flexibility in macromolecules (side chains and main chains) and the presence of bridging water molecules while treating protein/ligand complexes as realistic dynamic systems (Corbeil et al., 2007). These characteristics are particularly relevant to the iGluR ABD because ligand and protein flexibility as well as water molecule mobility are critical determinants of agonist behavior (Arinaminpathy et al., 2006). In practical terms, agonists were docked to previously published structures of GluK2 that together represent the closed, intermediate or open conformation of the ABD (see *Materials and Methods* for details). It is important to emphasize that the final structure only ever represents a composite of these input structures and that FITTED cannot predict a completely novel structure. Upon convergence of the fitting process, we were able to assign a preferred conformation of the GluK2 ABD to each agonist.

In agreement with published X-ray crystal structures (Mayer, 2005; Nanao et al., 2005), the full agonist, L-Glu, selected the closed conformation (Fig. 4D), whereas the partial agonists, KA and Dom, selected intermediate and open conformations, respectively. Superimposition of the agonist-receptor complexes observed with FITTED and published X-ray crystal structures reveal that the structures obtained by each approach were indistinguishable (Fig. 4, D–F). In support of this, comparison of the computed RMSDs between the crystal and docked structures for L-Glu, KA and Dom were 0.34, 0.46, and 1.2 Å, respectively (Table 1) indicating that the ligand pose was accurately selected for each agonist. A closer view of the GluK2 ligand-binding pocket (Fig. 4, D–F) reveals key water molecules and selected amino acid residues involved in ligand recognition. For example, Arg523 and Ala518 are involved in H-bonding with the α-carboxyl group of all ligands. In contrast, Thr690 is involved in both direct hydrogen bonding with the γ-carboxyl group and indirect interactions through surrogate water molecules. Two other full agonists previously crystallized, SYM 2081 and QA, also selected the closed clamshell conformation with small computed RMSDs (SYM 2081, 0.24 Å; QA, 2.0 Å; Table 1). Taken together, our findings validate the use of FITTED in providing information on the conformational state adopted by the GluK2 ABD bound by different receptor agonists.

Agonist Efficacy and Predictions of Domain Closure Do Not Correlate. We next broadened our analysis to include all L-Glu analogs. With the exception of KA and Dom, FITTED predicted that all amino acids bind preferentially to the closed conformation, suggesting that agonist efficacy and the degree of closure in the GluK2 ABD are apparently not correlated (Fig. 5). At first glance, this result was perplexing, because it suggests that weak partial agonists, such as stereoisomers of Asp or Ser, elicit similar degrees of conformational change as L-Glu (Fig. 5B). Our immediate concern was

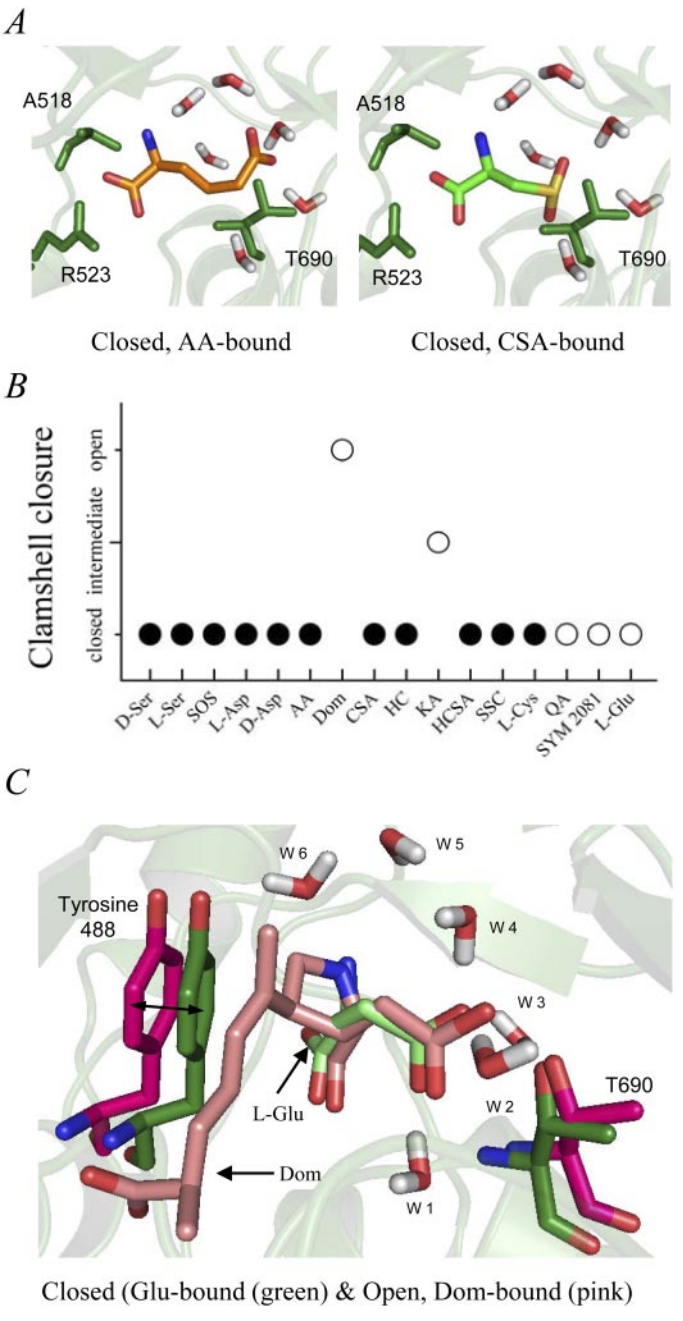

Fig. 5. Tyrosine 488 prevents full cleft closure with domoate and kainate. A, docking of L-aminoadipate (AA; left) and CSA (right) to GluK2 KARs using FITTED selects the closed conformation (green) in each case. The modeled structures are shown in orange and green, respectively. B, summary plot showing the conformation selected by each L-Glu analog using FITTED. Agonists previously cocrystallized with GluK2 are labeled as open circles, whereas the conformation selected by newly identified ligands is denoted by a filled circle. C, superimposition of the GluK2 ABD in complex with L-Glu (green) and Dom (pink). Note that different shading intensities have been used to distinguish between amino acid residues in the GluK2 ABD from the agonist molecule. In addition, only the protein backbone of the closed conformation is illustrated. Note that the pyrrolidine ring of Dom elicits a displacement of the Tyr488 residue as well as a water molecule (W6) normally found in the L-Glu-bound crystal structure. It is noteworthy that KA has the same effect though to a lesser extent because of to its smaller side-chain that extends from the pyrrolidine ring. Agonists, water molecules, and selected key residues are shown as sticks. Nonpolar hydrogens have been omitted for clarity.

that the outcome of the modeling represented a local minimum in the fitting process that is nonsensical from a biological perspective. However, we excluded this on two counts. First, FITTED already predicted the correct docking orientation of ligands previously crystallized with the GluK2 ABD (see Figure 4). Second, the binding mode of all other docked agonists was comparable with the binding orientation observed with L-Glu as would be expected. Typical binding orientation is illustrated by a visual inspection of the GluK2 ligand-binding pocket docked with L-α-aminoadipate and L-homocysteine sulfinic (Fig. 5A). In each case, the α-carboxyl groups of both partial agonists are predicted to form H-bonds with Ala518, Arg523, and AlaA689 (Fig. 5A), whereas the α-amino group is predicted to interact with Pro516 and Glu738 (data not shown). As expected, FITTED predicts that the terminal-carbon interacts with Thr690 via direct H-bonding and surrogate water molecules.

An additional concern was that the limited number of structures of the KAR ABD may bias the outcome of our analysis with FITTED. Although important to consider, we feel that this issue is not critical in our case, because the structures we have used cover an appreciable range of cleft closure in the GluK2 ABD from 12.3° for Dom to 26.2° to 26.6° for L-Glu (Mayer, 2005; Nanao et al., 2005). Furthermore, these structures represent the preferred conformations of full agonists (i.e., L-Glu, SYM 2081, and QA) to moderate and weak partial agonists (e.g., KA, Dom). FITTED does not provide information on whether the ABD adopts discrete or a limitless range of conformations after agonist binding. Nor does it identify any putative twist motion proposed from molecular dynamics to occur with partial agonists acting on GluA2 AMPARs (Bjerrum and Biggin, 2008). However, given these limitations, FITTED still permits us to examine the more general issue of whether there is any proposed relationship between cleft closure and agonist efficacy.

It is not wholly surprising that FITTED predicts that weak partial agonists, such as Asp or Ser, elicit the same degree of domain closure as full agonists, such as L-Glu, especially because almost all ligands used in this study have compact structures. Consequently, it is reasonable that most of the agonists we have docked using FITTED prefer the closed rather than the open or intermediate conformation of the KAR ABD. This conclusion is supported by recent work on AMPARs that has established the precedence that agonist efficacy need not be correlated with the degree of cleft closure (Zhang et al., 2008). Specifically, Zhang et al. (2008) found that mutation of the Thr686 residue of the GluA2 AMPAR renders L-Glu a partial agonist but yet structural changes elicited are indistinguishable from wild-type receptors. The exceptions to this at GluK2 receptors are KA and Dom, which prefer the intermediate and open conformations. However, as explained below, this observation can be simply accounted for by steric hindrance within the KAR ABD that limits the closure achieved by more bulky ligands, such as KA and Dom.

Domain Closure Is Determined by Ligand Interaction with Tyrosine 488. If the degree of closure in the ABD is not correlated with agonist efficacy, what is the basis for differences in closure observed with some agonists? Visual inspection of the ligand-bound complexes predicted by FITTED reveals an important property of the GluK2 ABD unique to Dom- and KA-bound structures (Fig. 5C). Specifically, the large side chain that extends from position 4 on the pyrrolidine ring of Dom causes a translational motion of Tyr488 that prevents complete closure of the GluK2 ABD. Likewise, the shorter side chain extending from the pyrrolidine ring of KA also causes steric hindrance but to a lesser extent accounting for the intermediate closure of the ABD. In contrast, all other amino acids tested, including the full agonist L-Glu, do not interact directly with Tyr488 and, because of their compact structure, allow complete closure of the agonist-binding pocket (Fig. 5B). The exception to this is QA, which possesses a bulky oxadiazolidine ring (Fig. 1). In this case, however, the ring structure of QA occupies a different region of the GluK2 ABD from the pyrrolidine ring of Dom and KA. Consequently, QA binds to GluK2 permitting complete closure of the ABD.

Conformational Changes Elicited by D- and L-Asp Are Indistinguishable from L-Glu. Although docking experiments with FITTED predicts that weak partial agonists, such as D- and L-Asp, bind to the closed conformation of the GluK2 ABD, it was nevertheless important to demonstrate this experimentally. To do this, we examined GluK2 responses after pretreatment with Con-A (Fig. 6). Con-A binds to a number of *N*-glycosylated residues in and around the GluK2 ABD (Fay and Bowie, 2006). In the resting or apo state of the GluK2 ABD, access to these sites is unrestricted; as a result, Con-A can bind to the receptor. Con-A binding in turn leads to the up-regulation of GluK2 responses as we have described previously (Bowie et al., 2003; Fay and Bowie, 2006). A typical experiment showing this effect is illustrated in Fig. 6A. Note that the equilibrium/peak response ratio to 10 mM L-Glu increased to 21.8 ± 2.9% after pretreatment with Con-A (10 μM, 3 mins) (Fig. 6, A and C). Conversely, if appreciable conformational changes are induced in the GluK2 ABD, such as occurs after L-Glu binding (Fay and Bowie, 2006), Con-A access to its binding sites are significantly restricted. As a consequence, pretreatment with Con-A has only a modest effect on the GluK2 response. In the example shown in Fig. 6A, the equilibrium/peak response ratio to L-Glu observed after pretreatment with Con-A was increased only to 6.7 ± 1.8% (Fig. 6, A and C).

State-dependent modulation by Con-A was therefore used to report the conformational changes elicited by D- and L-Asp. As positive controls, we compared the amount of modulation observed when GluK2 receptors were preincubated with Con-A and one of three agonists (i.e., 10 mM L-Glu, 1 mM KA, or 50 μM Dom) (Fig. 6, A and C). We have shown previously that Con-A modulation of GluK2 receptors preincubated with Glu, KA, or Dom corresponds to the closed, intermediate, or open states of the ABD, respectively (Fay and Bowie, 2006) (Fig. 6C). As negative controls, we examined pharmacological compounds that would not be expected to induce significant closure of the GluK2 ABD that were the competitive antagonist, 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX), as well as the ion-channel blocker philanthotoxin (PhTX). Although CNQX induces modest closure in the AMPAR ABD by acting as a partial agonist (Menuz et al., 2007), this effect has not been observed at KARs; consequently, we have assumed it behaves as a competitive antagonist.

As expected, preincubation with CNQX or PhTX did not interfere with the degree of modulation of GluK2 receptors by Con-A (Fig. 6, B and C). In support of this, the degree of Con-A modulation observed with CNQX or PhTX was similar

to that observed for the open conformation of the GluK2 ABD but statistically distinct from the closed or intermediate (Table 2). These findings suggest that occupancy of the pore with a channel blocker or the ABD with a competitive antagonist does not evoke appreciable closure of the GluK2 ABD. In contrast, preincubation with either D- or L-Asp significantly reduced the degree of Con-A modulation (Fig. 6, B and C). In support of this, the degree of Con-A modulation observed with D-Asp or L-Asp was similar to that observed for the closed conformation of the GluK2 ABD but statistically distinct from the open or intermediate (Table 2). This finding further supports the central tenet of our study that weak partial agonists, such as D- and L-Asp, elicit conformational changes in the GluK2 ABD that are indistinguishable from conformations elicited by the full agonist, L-Glu.

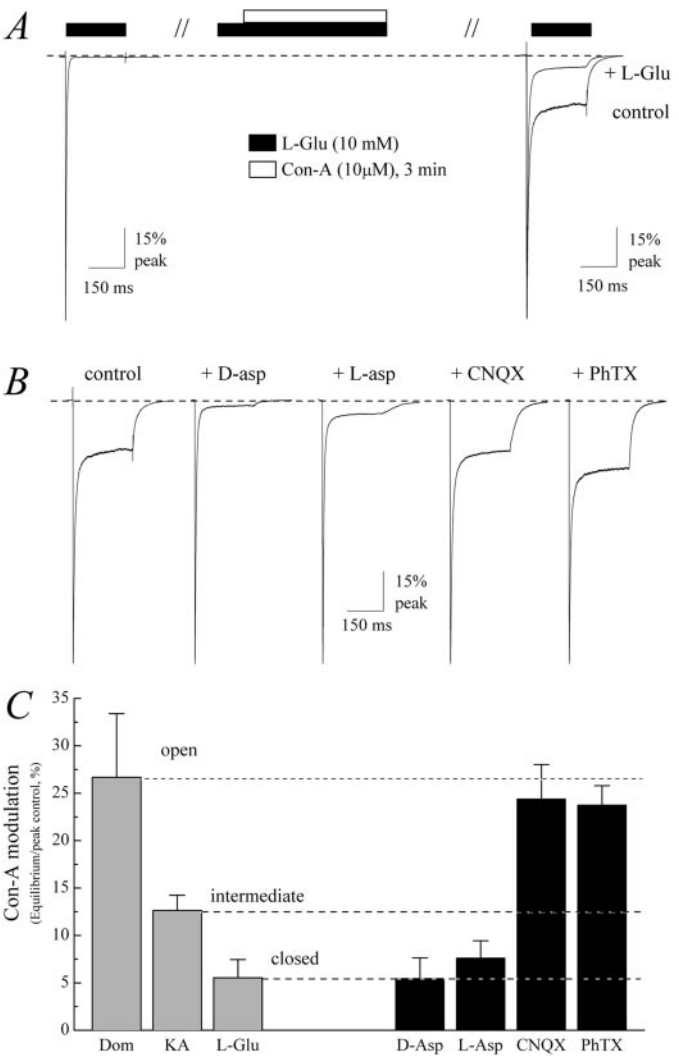

Fig. 6. Conformational changes elicited by L-Glu and stereoisomers of Asp to the GluK2 agonist-binding pocket are indistinguishable. A, typical experiment showing how modulation by Con-A reports conformational changes in the GluK2 ABD (for details, see Fay and Bowie, 2006). Con-A binds to a number of *N*-glycosylated residues in and around the GluK2 ABD. If agonist is bound, access to these sites is restricted; as a result, Con-A has a much weaker effect on the L-Glu equilibrium response. In the example shown, the equilibrium response is much smaller after coapplication of Con-A and L-Glu (10 mM; patch number 030724p2) than when Con-A is applied alone (control; patch number 01817p6). Filled and open bars indicate the application period of 10 mM L-Glu and 10 μM Con-A, respectively. The dotted line denotes the zero current level. The first and third applications of 10 mM Glu had a duration of 250 ms. B, experimental traces showing the extent of Con-A modulation as described in A, with D-Asp (patch number 071018p3), L-Asp (patch number 07906p1), CNQX (patch number 07913p2), and philanthotoxin (PhTX, patch number 07104p1) compared with control. C, Summary bar graphs showing the extent of Con-A modulation after cotreatment with various pharmacological agents (L-Glu, $n = 13$; D-Asp, $n = 4$; L-Asp, $n = 4$; CNQX, $n = 3$; PhTX, $n = 3$). The dotted lines on the graph denote the extent of Con-A modulation observed for the open, intermediate, and closed conformations of the GluK2 ABD, which we have described previously (A.-M.L. Fay and D. Bowie, 2006). CNQX and PhTX adopt the open conformation of the GluK2 ABD because their degree of Con-A modulation exactly matches that observed with Dom. In contrast, both D- and L-Asp adopt the closed conformation because modulation with Con-A is statistically indistinguishable from that observed with L-Glu. All data are expressed as the mean ±S.E.M.

Although these observations are consistent with Con-A reporting conformational changes in the GluK2 ABD, it was nevertheless important to evaluate alternate explanations. For example, it is possible that Con-A modulation reveals that stereoisomers of Asp adopt a desensitized conformation similar to that L-Glu instead of reporting the extent of cleft closure. This possibility, however, is unlikely for three main reasons. First, there is no available evidence to suggest that conformational changes in the dimer interface that accompany the onset of AMPA or KAR desensitization are agonist-dependent (Armstrong et al., 2006; Weston et al., 2006), which would be required to explain Con-A's effects. Second, Con-A binding and consequently modulation of GluK2 is almost entirely eliminated by mutation of three key N-terminal amino acid residues that do not participate in forming the dimer interface (Fay and Bowie, 2006). Although residues distant from the dimer interface may still regulate KAR desensitization, GluK2 receptors that lack the N-terminal desensitize normally (Plested and Mayer, 2007), suggesting that this region of the intact receptor is not functionally coupled to the dimer interface. Third and finally, Con-A does not affect rates into or out of desensitization (Bowie et al., 2003; Fay and Bowie, 2006), which would not be expected if lectin binding reports separation in the dimer interface. Given this, the most parsimonious explanation of our data is that Con-A reports conformational changes in the ABD of GluK2 receptor as discussed in detail elsewhere (Fay and Bowie, 2006).

Discussion

To our knowledge, this study is the first to identify a series of structurally related amino acids that exhibit the entire range of agonist behavior at KARs. Analysis of their struc-

TABLE 2
Statistical comparisons between the degree of Con-A modulation observed with different GluK2 receptor ligands

The ability of stereoisomers of Asp, CNQX, and PhTX to affect Con-A modulation of GluK2 receptors was compared with the modulation observed with L-Glu, KA, and Dom using Student's *t* test. The modulation observed by preincubating with stereoisomers of Asp was statistically significant from that observed with KA and Dom but indistinguishable from L-Glu. In contrast, the modulation observed by pre-incubating with CNQX or PhTX was statistically significant from that observed with L-Glu and KA but indistinguishable from Dom.

Ligand	L-Glu (Closed)	KA (Intermediate)	Dom (Open)
D-Asp	N.S.	*	*
L-Asp	N.S.	*	*
PhTX	**	**	N.S.
CNQX	**	**	N.S.

N.S., not significant.
* Significant at $P < 0.05$.
** Significant at $P < 0.01$.

ture-function relationship reveals that the agonist binding pocket of KARs is ideally suited to respond to the neurotransmitter, L-Glu, because modest changes in its chain length generates weak partial agonists. Using both in silico docking as well as measurements of conformations in the intact receptor, we show that the majority of full and partial agonists select for the closed conformation of the GluK2 ABD. Although this finding is not wholly surprising given the compact structures of most ligands tested, it is inconsistent with agonist efficacy being solely determined by the extent of closure in the KAR ABD. Exceptions to this were the partial agonists, KA and Dom, which select for the open and intermediate conformations, respectively. However this finding can be simply explained by steric hindrance due to the Tyr 488 residue in domain 1 of the GluK2 ABD. Our findings suggest the value in looking more closely at the relationship between agonist efficacy and the extent of agonist-induced domain closure in KARs.

Can Other Mechanisms Account for Agonist Efficacy at Kainate Receptors? Although the view that agonist efficacy is governed by closure in the ABD has gained much popularity, recent work on AMPARs has identified a different mechanism (though not mutually exclusive) (Robert et al., 2005; Zhang et al., 2008) that may also account for full and partial agonist behavior at KARs. In essence, it is argued that the time the ABD remains in the closed conformation determines several gating properties of AMPARs including agonist efficacy, deactivation rates as well as apparent agonist affinity. For L-Glu, closed-cleft stability is optimized by direct and indirect interactions with domains 1 and 2 of the AMPAR ABD, which permit L-Glu to attain full agonist activity while exhibiting rapid unbinding (Robert et al., 2005; Zhang et al., 2008), essential features for any fast-acting neurotransmitter. In the specific case of AMPARs, mutation of a key threonine (i.e., Thr686) residue in domain 2 of the GluR2 ABD, disrupts the optimization established between the ligand and receptor. As a result, L-Glu is rendered a weak partial agonist with much lower affinity (Robert et al., 2005).

There are several reasons to suggest that basic elements of the mechanism proposed by Zhang et al. (2008) may also account for differences in efficacy between L-Glu and stereoisomers of Asp reported in this study. First, activation curves of partial agonists D- and L-Asp are shifted rightward compared with the full agonist L-Glu (Fig. 3, C and D), suggesting that, in this case, agonist efficacy and affinity may be tightly correlated. Second, deactivation rates for stereoisomers of Asp (e.g., D-Asp, $\tau = 1.2 \pm 0.3$ ms) were faster than with L-Glu ($\tau = 2.6 \pm 0.2$ ms) (Bowie, 2002). Third and finally, the more extended structure of L-Glu permits more contact points (direct and indirect) to be established with the GluK2 ABD than with D- or L-Asp (Fig. 7). This difference in agonist binding would be expected to weaken the stability of the closed GluK2 ABD. It is noteworthy that L-Asp formed considerably less contacts than D-Asp or L-Glu, which may explain its weaker responsiveness based on analysis of activation curves (Fig. 3D). Although more work is required to rigorously test this model, it provides a valuable framework for future work on agonist behavior at KARs.

Are Amino Acids Other Than L-Glu Suitable Neurotransmitter Candidates at Kainate Receptors? Several of the amino acids examined in this study are endogenous to the CNS and have been previously evaluated as neurotrans-

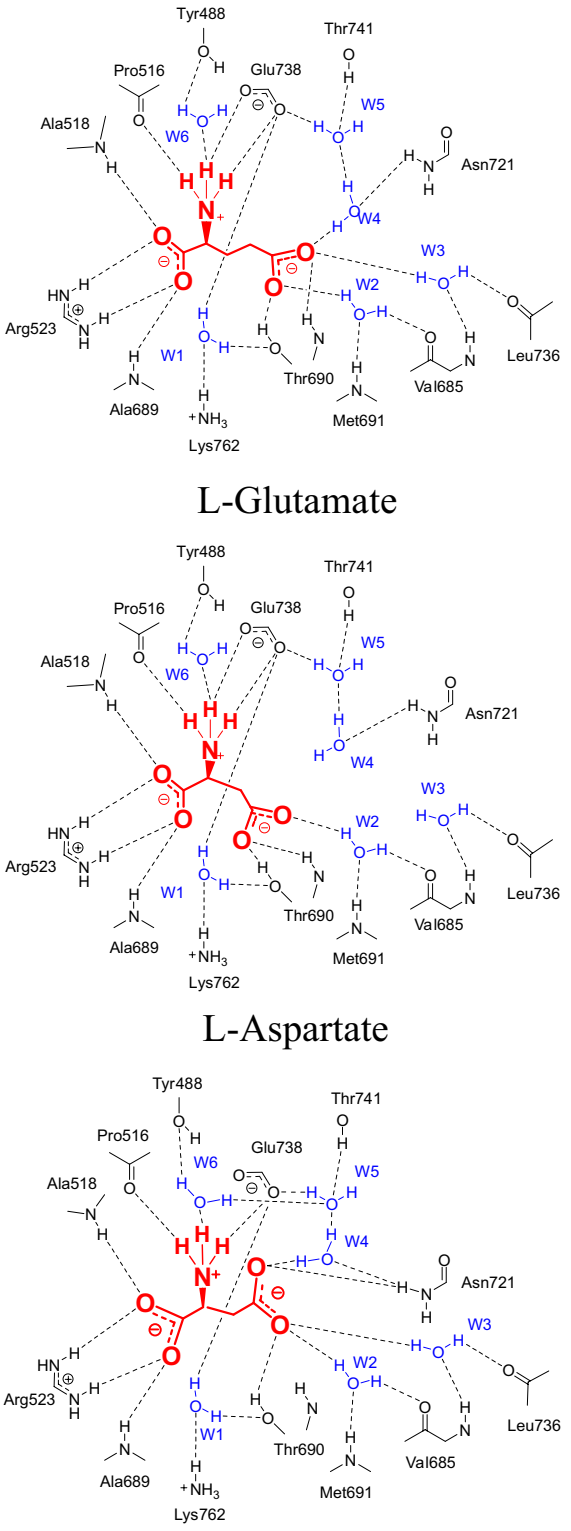

Fig. 7. Stereoisomers of Asp establish fewer contact points with the GluK2 agonist binding pocket than L-Glu. Two-dimensional topographical maps of the GluK2 ABD shows that the number of contact points and the binding orientation of the full agonist, L-Glu, and partial agonists, D- and L-Asp. Topographical maps were deduced from structure complexes obtained with FITTED. Note the number of contact points made by D- and L-Asp was fewer than with L-Glu. In addition, the binding orientation is different between L- and D-Asp, which would be expected for stereoisomers of the same amino acid.

mitter candidates at glutamatergic synapses. The candidature of sulfur-containing amino acids, which include L-Cys, HC, SSC, and HCSA, was considered after mechanisms that lead to their release, uptake, and responsiveness (see below) were identified (Do et al., 1986; Bouvier et al., 1991). Recent attention has focused on their potent activation of metabotropic glutamate receptors (e.g., Kingston et al., 1998). However, earlier work demonstrated that they also activate iGluRs (Thompson and Kilpatrick, 1996). At the time, most investigators argued for their greater ability to activate NMDARs than AMPARs (Patneau and Mayer, 1990); however, their effect on KARs was never tested, because evidence for the existence of this iGluR subclass had yet to emerge (Bowie, 2008). In view of this, our data on homomeric GluK2 receptors suggests the value in testing the responsiveness of native KARs to sulfur-containing amino acids. It is noteworthy that the most potent sulfur-containing amino acid in our experiments, L-Cys, is a very weak partial agonist on homomeric GluR1 AMPARs (A.-M.L. Fay and D. Bowie, unpublished observations). Therefore, it would be interesting in future work to determine whether different non-NMDA receptor subtypes discriminate among sulfur-containing amino acids.

In comparison, there is more compelling evidence linking the stereoisomers of both serine and aspartate to roles in glutamatergic transmission (Boehning and Snyder, 2003). D-Ser was considered in this capacity only after it was shown to act as a coagonist at the glycine binding site of NMDARs (McBain et al., 1989). Because D-serine is expressed in discrete populations of glial cells opposed to NMDARs (Schell et al., 1997a), it has been categorized as a gliotransmitter (Mothet et al., 2000; Panatier et al., 2006). The role of D-Asp is more elusive, although it is found in the developing and adult brain (Schell et al., 1997b). Accumulation of D-Asp in CNS tissue has marked behavioral consequences, such as impaired motor coordination (Weil et al., 2006), which is consistent with its putative role as a transmitter at the climbing fibers of the cerebellum (Wiklund et al., 1982). Likewise, L-Asp's role in neurotransmission has centered on NMDARs (Fleck et al., 1993), although it elicits a high calcium conductance in cerebellar Purkinje cells that apparently involves a novel iGluR (Yuzaki et al., 1996). Our study shows that D- and L-forms of each amino acid are weak partial agonists and, although these properties are not normally expected of a neurotransmitter candidate, it may be interesting to evaluate their roles at native KAR-containing synapses.

Conclusion

It is puzzling that not all iGluR subunits respond to the neurotransmitter L-Glu. In fact, neither the NR1 NMDAR subunit nor the orphan-class δ-2 (δ2) subunit even binds L-Glu. Because the ancestral iGluR, GluR0, possesses an L-Glu binding pocket (Chen et al., 1999), it is conceivable that evolving NR1 and δ2 subunits sacrificed this ability to serve more specialized roles in the mammalian CNS. In this regard, it is interesting that NMDARs (McBain et al., 1989) and orphan-class δ2 iGluR (Naur et al., 2007) retained their ability to bind D-Ser. Likewise, AMPARs (P. Brown and D. Bowie, unpublished observations) and KARs (present study) are also gated by D-Ser (and D-Asp) suggesting that these naturally occurring D-amino acids discriminate little among iGluR families. Whether this observation is a peculiarity of iGluRs that holds little biological significance or hints at a broader role for D-amino acids at glutamatergic synapses awaits future investigation.

Acknowledgments

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Brief Communications

External Ions Are Coactivators of Kainate Receptors

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The activation of ligand-gated ion channels is thought to depend solely on the binding of chemical neurotransmitters. In this study, we demonstrate that kainate (KA) ionotropic glutamate receptors (iGluRs) require not only the neurotransmitter L-glutamate (L-Glu) but also external sodium and chloride ions for activation. Removal of external ions traps KA receptors (KARs) in a novel inactive state that binds L-Glu with picomolar affinity. Moreover, occupancy of KARs by L-Glu precludes external ion binding, demonstrating crosstalk between ligand- and ion-binding sites. AMPA iGluRs function normally in the absence of external ions, revealing that even closely related iGluR subfamilies operate by distinct gating mechanisms. This behavior is interchangeable via a single amino acid residue that operates as a molecular switch to confer AMPA receptor behavior onto KARs. Our findings identify a novel allosteric site that singles out KARs from all other ligand-gated ion channels.

Key words: **agonist; glutamate receptor; desensitization; gating; epilepsy; activation**

Introduction

In the vertebrate brain, ligand-gated ion channels are an important class of signaling protein designed to respond to a specific chemical neurotransmitter such as acetylcholine (ACh) (Colquhoun and Sakmann, 1998), L-glutamate (L-Glu) (Erreger et al., 2004), glycine (Lynch, 2004), or GABA (Maconochie et al., 1994). Although neurotransmitter substances are numerous in number (Krnjevic, 1974), all ligand-gated ion channels are thought to undergo conformations into the activated state by harnessing the energy from neurotransmitter binding (Colquhoun, 1998). There are no exceptions to this rule, although it has been recognized for some time that basal ion-channel activity is regulated by other factors such as phosphorylation and, more recently, by interactions with scaffolding proteins. Previously, we have shown that external anions and cations regulate both the response amplitude and channel kinetics of kainate (KA) ionotropic glutamate receptors (iGluRs) (Bowie, 2002; Bowie and Lange, 2002). Specifically, the rate of channel closure (i.e., deactivation) is ion dependent, suggesting that the stability of the activated/open state of the receptor is regulated by external ions as well as neurotransmitter binding. As yet, the molecular basis of this effect is not understood, although closely related AMPA receptors (AMPARs) are insensitive to external anions and cations (Bowie, 2002; Bowie and Lange, 2002; Paternain et al., 2003).

Here, we tested two opposing mechanisms to account for the effect of external ions on KA receptors (KARs). Experiments in ion-free solutions reveal that external anions and cations do not simply modulate basal receptor activity but instead are an absolute requirement for activation. This observation demonstrates unequivocally that external ions are coactivators of KARs. Furthermore, we identify allosteric cooperativity between ligand- and ion-binding sites and show that KARs enter into a novel inactive state when external sodium and chloride ions are absent.

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Materials and Methods

Cell culture. tsA201 cells were transiently cotransfected with cDNA encoding wild-type (wt) or mutant GluR6 or GluR1 subunits and enhanced green fluorescent protein ($eGFP_{S65T}$) as described previously (Bowie, 2002; Bowie and Lange, 2002). After transfection for 8–10 h (GluR6) or 12 h (GluR1), cells were washed and maintained in fresh medium. Electrophysiological recordings were performed 24–48 h later.

Mutagenesis. Mutation of GluR6(Q) was performed using the Stratagene (La Jolla, CA) Quickchange II XL site-directed mutagenesis kit. Mutant cDNAs were amplified, purified, and initially identified by restriction digest and confirmed by automated DNA sequencing of the entire GluR6 coding region (McGill University and Genome Quebec Innovation Center, Montreal, Québec, Canada).

Electrophysiology. Experiments were performed on outside-out patches, and agonist solutions were applied using a piezo-stack-driven perfusion system (Bowie, 2002; Bowie and Lange, 2002; Bowie et al., 2003). Solution exchange (10–90% rise time, 25–50 μs) was determined at the end of each experiment by measuring the liquid junction current. Recordings were performed with an Axopatch 200B amplifier (Molecular Devices, Palo Alto, CA) using borosilicate glass pipettes (4–6 MΩ) coated with dental wax. Current records were filtered at 10 kHz and digitized at 50–100 kHz, and series resistances (7–12 MΩ) were compensated by 95%. The reference electrode was connected to the bath via a 3 M KCl agar bridge. Data acquisition was performed using pClamp9 (Molecular Devices) and illustrated using Origin 7 (Microcal, Northampton, MA). All experiments were performed at room temperature.

Solutions. External solutions contained the following: 5 mM HEPES, 0.1 mM $CaCl_2$, 0.1 mM $MgCl_2$, and 2% phenol red, to which 1–405 mM NaCl was added as required. For solutions containing 150 mM external NaCl or less, the osmotic pressure was adjusted to 290 mOsm using

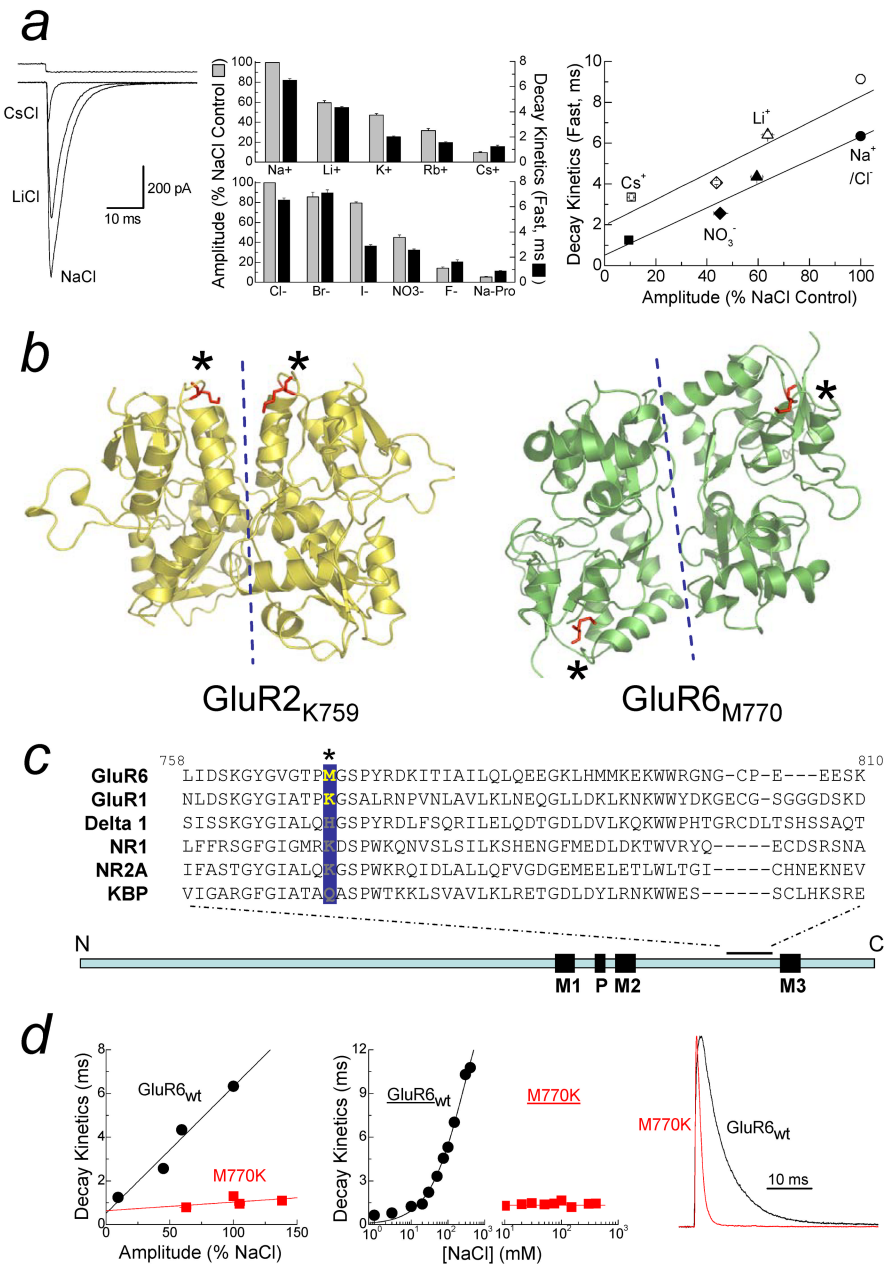

Figure 1. External anions and cations regulate GluR6 KARs. ***a***, Left, Membrane currents elicited by 10 mM L-Glu in 150 mM Na^+, Li^+, or Cs^+, with top trace showing solution exchange (patch 040419p2). Middle, Summary bar graph of external ion effects on GluR6 amplitude (gray bars) and decay kinetics (black bars). Right, Comparison between the amplitude and decay kinetics of GluR6 in different ion concentrations (150 mM, filled symbols; 405 mM, open symbols). Solid lines represent linear regression fits of the data at each ion concentration. ***b***, Crystal dimer structures drawn using Pymol show GluR2 (Protein Data Bank number 1FTJ) and GluR6 (Protein Data Bank number 1S7Y), with M770 and K759 in red and marked by asterisks. Dotted lines show dimer interface. ***c***, Sequence alignment of several iGluRs at the extracellular M2–M3 linker region, with the GluR6 M770 position highlighted in yellow. ***d***, $GluR6_{M770K}$ is unaffected by ion type [left, 150 mM Na^+ (Cl^-), Li^+, NO_3^-, and Cs^+] or concentration (middle, 1–405 mM NaCl) and has faster decay kinetics than $GluR6_{wt}$ (right, 150 mM NaCl).

sucrose. For solutions with higher NaCl (>150 mM), the osmotic pressure was adjusted to 760 mOsm. pH was adjusted to 7.3 using 5N NaOH, with the exception of experiments presented in Figure 1 in which different cations were compared. In this case, pH was adjusted with the corresponding hydroxide solution (e.g., LiOH for LiCl). The internal solution contained the following (in mM): 115 NaCl, 10 NaF, 5 HEPES, 5 Na_4BAPTA, 0.5 $CaCl_2$, 1 $MgCl_2$, and 10 Na_2ATP, pH was adjusted to 7.3 with 5N NaOH, and the osmotic pressure was adjusted with sucrose to correspond with external solutions.

In experiments shown in Figures 2 and 3, solutions lacking external NaCl contained 100 μM each of $CaCl_2$ and $MgCl_2$ to improve patch stability, sucrose to maintain the osmotic pressure at 290 mOsm, and 5 mM Tris or 5 mM ammonium bicarbonate (NH_5CO_3) as pH buffers. With Tris, the pH was adjusted to 7.4 using 5N HCl, whereas the pH of NH_5CO_3-containing solutions was maintained by a gas mixture of 95%O_2/5%CO_2. For agonist solutions, the free acid of L-glutamate was dissolved in NaCl-free solution, and the pH was adjusted using 2.5 M Tris (for Tris buffer) or 2 M NH_5CO_3 (for NH_5CO_3-buffered solution).

Analysis. Concentration–response curve to external NaCl (see Fig. 1*d*, middle) was fit with the following equation: $\tau_{NaCl} = \tau_{max}/1 + (EC_{50}/[NaCl])^N$, where τ_{NaCl} represents the observed decay kinetics at any concentration of NaCl, τ_{max} is the slowest time constant for the fast decay component, which assumes that NaCl has a saturable effect, EC_{50} is the concentration of NaCl that elicits half-maximal decay kinetics, and N is the slope. Inhibition curves shown in Figure 4 were fit with a single- and double-binding site isotherm of the following forms: (for single) $I_{Glu} = I_{max}/1 + ([Glu]/IC_{50})^N$ and (for double) $I_{Glu} = I_{max(High)}/1 + ([Glu]/IC_{50(High)})^N + (1 - I_{max(High)})/1 + ([Glu]/IC_{50(Low)})^N$, where I_{max} is the response to 10 mM L-Glu in the absence of preapplied L-Glu, IC_{50} is the concentration of L-Glu that elicits half-maximal inhibition, N is the slope, and the low-affinity component (I_{Low}) of inhibition by L-Glu is $1 - I_{max(High)}$. Data in all experiments are expressed as mean ± SEM from at least five patches.

Results

External anions and cations modulate KAR amplitude and channel kinetics

Figure 1*a* shows typical effects of external monovalent ions on electrophysiological responses mediated by recombinant GluR6 KARs. Replacement of external Na^+ with an equimolar equivalent of either Li^+ or Cs^+ elicits a reduction in peak response amplitude as well as acceleration in decay (i.e., desensitization) kinetics (Fig. 1*a*, left). Similar findings were observed with an extended series of monovalent cations in which the degree of modulation was dependent on ion species (Fig. 1*a*, middle). Interestingly, substitution of external Cl^- with equimolar concentrations of other anions had a comparable effect to cation replacement (Fig. 1*a*, middle). Together, these data suggest that the chemical nature of the external solution and not its ionic strength regulates KAR gating behavior (Bowie, 2002).

The conventional explanation for these observations as described for other voltage- and ligand-gated ion channels (Yellen, 1997) is that external ions regulate the basal gating behavior of GluR6 KARs but are not an absolute requirement. In this case, the effect of external Cs^+ or propionate ions is to reduce channel activity, whereas it is increased by external Na^+ or Cl^-. An alternative is that GluR6 KARs exhibit an absolute requirement for

external anions and cations; that is, external ions act as coactivators of KARs. In this case, external Na^+ or Cl^- ions are more effective in stabilizing KARs in the open state compared with Cs^+ or propionate ions. We will use the term "coactivator" throughout to include two possible mechanisms: (1) that ions affect KARs simply by binding or (2) ion binding causes conformational changes in the receptor, which affects function. To test whether external ions are coactivators of KARs, we compared GluR6 responses in 150 and 405 mM external ion solutions (Fig. 1*a*, right). Increasing external ion concentration to 405 mM prolonged GluR6 decay kinetics with each external anion (Cl^-, NO_3^-) or cation (Na^+, Li^+, Cs^+) tested (Fig. 1*a*, right). Interestingly, a parallel shift in the relationship between response amplitude and decay kinetics was observed with the rank order of potency for each ion unchanged (Fig. 1*a*, right). This observation is inconsistent with a mechanism whereby external ions modulate the basal gating properties of KARs. In this case, Cs^+ ions would be expected to further accelerate decay kinetics at higher concentrations (i.e., 405 mM). However, the slowing of decay kinetics with all ion species tested supports the hypothesis that external ions are coactivators of KARs.

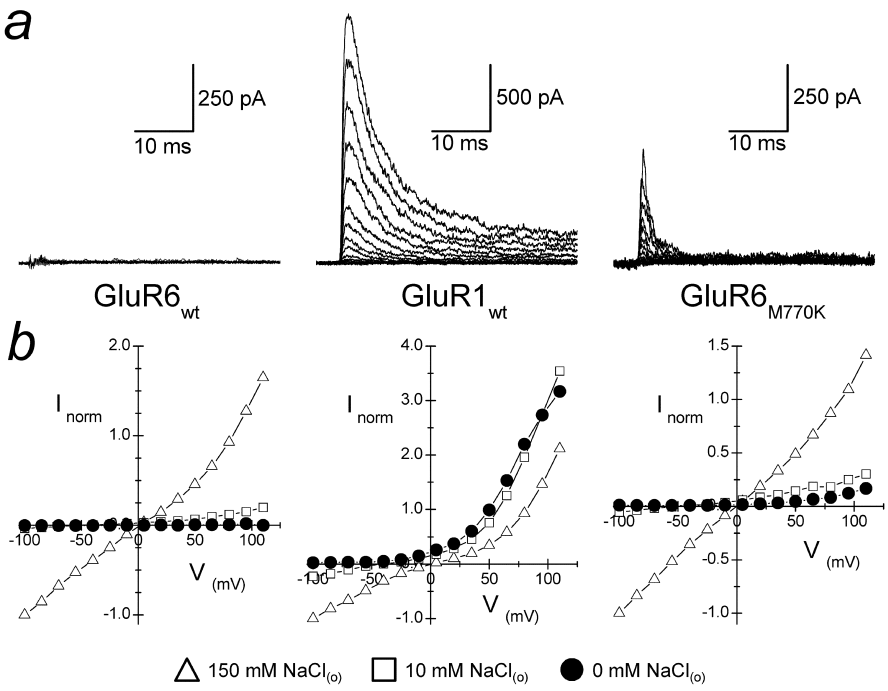

Figure 2. GluR6 KARs have an absolute requirement for external ions. ***a***, Superimposed family of membrane currents evoked by 1 mM L-Glu acting on $GluR6_{wt}$ (patch 050311p1), $GluR1_{wt}$ (patch 050321p2), and $GluR6_{M770K}$ (patch 050405p2) receptors in solutions lacking external NaCl (range, −100 to +110 mV, 15 mV increments). ***b***, Averaged current–voltage plots in 0 mM (filled circles), 10 mM (open squares), and 150 mM (open triangles) NaCl for each iGluR tested.

A single amino acid residue delineates between channel kinetics and response amplitude

It is interesting that KARs are the only iGluR whose response amplitude and decay kinetics is regulated by external anions and cations (Bowie, 2002; Bowie and Lange, 2002) despite structural (Mayer, 2005b) and functional (Dingledine et al., 1999) similarities with other family members, particularly AMPARs. In view of this, we further hypothesized that the gating mechanism of other iGluRs, such as AMPARs, do not have an absolute requirement for external ions; that is, agonist-induced conformational changes into the open state can still occur in the absence of external ions. Furthermore, given their considerable homology, we reasoned that it should be possible to interconvert the gating behavior of KA and AMPARs. In support of this, it has been shown that replacement of methionine-770 (M770) in GluR6 with its equivalent lysine (K_{752} for GluR1) residue in AMPARs (Fig. 1*b*,*c*) blocks ion modulation of the KAR response amplitude (Paternain et al., 2003). Interestingly, recent x-ray diffraction studies of GluR6 KAR (Mayer, 2005a) and GluR2 AMPAR (Armstrong and Gouaux, 2000) dimers places these residues in different locales of the quaternary structure (Fig. 1*b*, red labels and asterisks). An important caveat, however, is that another group has suggested a different dimer organization closer to that of GluR2 AMPARs (Nanao et al., 2005). Although the precise nature of the dimer interface awaits additional study, the structure reported by Mayer (2005) is consistent with KARs possessing a unique ion-binding site(s) that regulates the peak response amplitude. Given the concomitant effect of external ions on amplitude and decay kinetics (Bowie, 2002), we were therefore interested in testing whether M770K also affects GluR6 decay kinetics.

Figure 1*d* summarizes a series of experiments in which desensitization kinetics of $GluR6_{M770K}$ and $GluR6_{wt}$ were compared. In contrast to $GluR6_{wt}$, the decay kinetics of $GluR6_{M770K}$ were almost identical for all external anions (i.e., Cl^- or NO_3^-) and cations (i.e., Na^+, Li^+, and Cs^+) tested (Fig. 1*d*, left), suggesting that inclusion of a positively charged lysine at the 770 site is sufficient in abolishing ion-sensitive effects on channel kinetics. Unexpectedly, external anions and cations continued to regulate the peak response amplitude of $GluR6_{M770K}$, contrary to Paternain et al. (2003). In this case, the rank order of potency was different between $GluR6_{M770K}$ [$NO_3^- > Na^+$ (or Cl^-) ~ $Li^+ > Cs^+$] and $GluR6_{wt}$ [Na^+ (or Cl^-) > $Li^+ > NO_3^- > Cs^+$] (Fig. 1*d*, left). $GluR6_{M770K}$ mutant also blocked the effect of changing the external Na^+ and Cl^- ion concentration on desensitization kinetics (Bowie, 2002; Bowie and Lange, 2002) (Fig. 1*d*, middle), further supporting the pivotal role of M770 in controlling KAR gating behavior. Interestingly, in 150 mM external NaCl, the decay kinetics of $GluR6_{M770K}$ were several-fold faster than $GluR6_{wt}$ (Fig. 1*d*, right) or GluR1 AMPARs (data not shown), suggesting that amino acid residues other than the M/K site may be involved in endowing KARs with ion-dependent gating. As reported by others (Paternain et al., 2003), mutant AMPA receptors containing a Met residue instead of Lys express poorly, and, therefore, we were unable to examine the ion sensitivity of $GluR1_{K752M}$.

External anions and cations are an absolute requirement for KAR activation

To directly test whether KAR activation has an absolute requirement for external ions, we recorded GluR6 responses in the absence of external NaCl at a range of membrane potentials (−100 to +110 mV, 15 mV increments) (Fig. 2). To do this, experiments were performed using the free acid of L-glutamate and either Tris or bicarbonate buffers to maintain an external pH of 7.3 (see Materials and Methods). For comparison, we repeated experiments on GluR1 AMPARs and the KAR mutant $GluR6_{M770K}$.

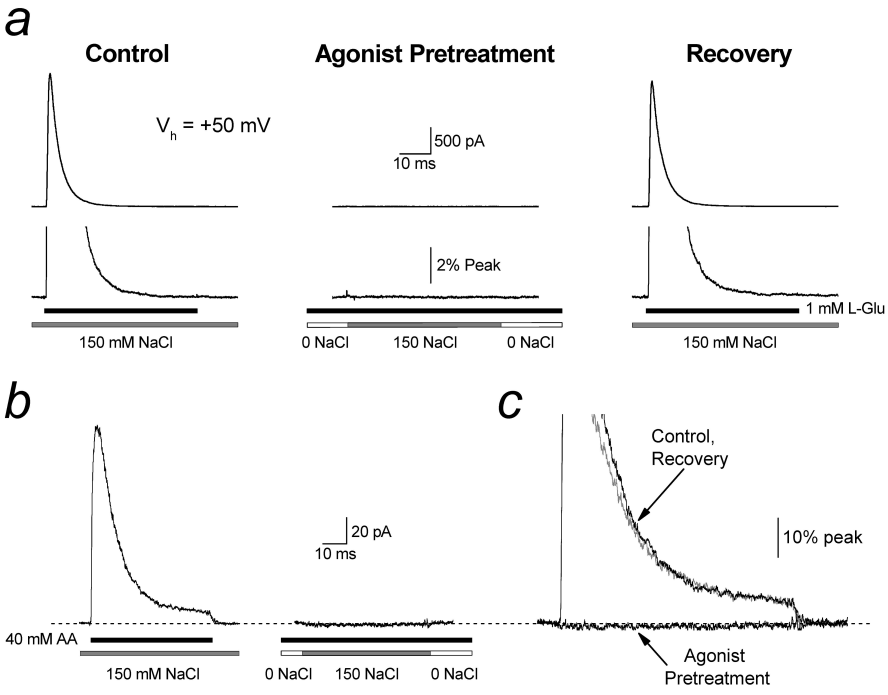

Figure 3. External ions are not a prerequisite for agonist binding to KARs. ***a***, Typical experimental traces (V_h of +50 mV) in which the effect of preincubating GluR6 KARs in NaCl-lacking solutions but containing 1 mM L-Glu was tested (patch 050711p1). Left, GluR6 receptors elicited robust responses to 1 mM L-Glu when preincubated in 150 mM external NaCl. Middle, In contrast, GluR6 receptors were unresponsive to NaCl when pretreated in 0 mm NaCl and 1 mM L-Glu. Right, The response was fully recovered during the addition of 150 mM NaCl to external solutions. ***b***, Same experiment as in ***a*** using 40 mM AA instead of L-Glu (patch 050818p1). ***c***, Experimental traces from ***b*** superimposed for comparison.

$GluR6_{wt}$ were entirely unresponsive in the absence of external NaCl at all membrane potentials tested (Fig. 2, left), consistent with our hypothesis that external ions are coactivators of KARs. Figure 2 (bottom, left) shows the current–voltage (*I–V*) relationships observed in 0 mM NaCl (filled circles) compared with *I–V* plots in 10 mM (open squares) and 150 mM (open triangles) external NaCl. In contrast, GluR1 AMPARs were fully responsive in the absence of external NaCl (Fig. 2, middle), suggesting that external ions are not an absolute requirement for the gating behavior of this iGluR subfamily. The membrane current observed represents the outward movement of permeating ions (i.e., Na^+) from the internal solution of the patch pipette. Interestingly, $GluR6_{M770K}$ was also responsive in solutions lacking external NaCl (Fig. 2, right), supporting the pivotal role of the M/K site in determining KAR gating behavior. Moreover, this observation eliminates the possibility that the functional effects observed in low ionic strength solutions are not attributable to denaturation of the quaternary structure of the intact KARs.

Crosstalk between agonist- and ion-binding sites

Our observations cannot be explained by the failure of agonist binding to KARs in NaCl-free solutions. In support of this, GluR6 KARs failed to respond to both NaCl and L-Glu when preincubated in solutions lacking external ions but containing L-Glu (Fig. 3*a*). This demonstrates that agonist binding (and subsequent receptor desensitization) can occur in the absence of external ions. If agonist binding had not occurred, GluR6 receptors would be expected to respond to the application of 150 mM NaCl (Fig. 3*a*, middle). Unexpectedly, however, we did not observe an equilibrium current typically associated with desensitized GluR6 receptors (Bowie and Lange, 2002; Bowie et al., 2003). We therefore hypothesized that agonist binding precludes the ability of external ions to bind. Because it was difficult to resolve equilibrium responses with L-Glu, we repeated the experiments using L-aminoadipate (AA) (40 mM), which elicits larger equilibrium responses (Fig. 3*b*, left). As with L-Glu, application of NaCl failed to elicit an equilibrium response after pretreatment with AA (Fig. 3*b*, right, *c*). Together, we have shown that, although external ions are required for KAR functionality, GluR6 receptors are able to bind agonists in solutions lacking external ions. Furthermore, conformational changes elicited by agonist binding prevent subsequent ion binding. To explain the failure of GluR6 receptors to respond in solutions lacking external ions, we show below that their removal accumulates KARs in a novel inactive state with high agonist affinity.

Identification of a novel inactive state with picomolar agonist affinity

Figure 4, *a* and *b*, shows a typical experiment in which the occupancy of the desensitized states was determined from inhibition of 10 mM L-Glu responses after incubation in L-Glu (0.1–50 μM). Similar experiments were also performed in 5, 10, 75, 150, and 405 mM external NaCl. A family of curves observed in different concentrations of NaCl were then fit with a single- or double-binding site model of inhibition as shown in Figure 4*c*. The inhibition of L-Glu responses in 150 and 405 mm NaCl (Fig. 4*c*, open and filled circles) were best fit with a single binding site isotherm estimating the IC_{50} to be 0.49 ± 0.04 and 0.56 ± 0.09 μM, respectively, in good agreement with previous studies of GluR6 (Wilding and Huettner, 1997; Paternain et al., 1998). At lower NaCl levels, inhibition plots were biphasic, revealing a high affinity, NaCl-dependent binding site with IC_{50} values of 50 ± 20 pM in 5 mM NaCl and 0.8 ± 0.6 nM in 10 mM NaCl (Fig. 4*c*,*d*). Extending our observations, <5 mM NaCl was not possible because membrane currents were small in amplitude, making measurement and analysis difficult. Moreover, patch stability was compromised in low ionic strength solutions. However, extrapolated fits of occupancy of the high- and low-affinity states (Fig. 4*e*) revealed that GluR6 receptors accumulate into this high-affinity inactive state as external ions are lowered. This finding explains the failure of KARs to gate in the absence of external NaCl (Fig. 2*a*, left).

Discussion

Classically, it has been thought that ligand-gated ion channels depend solely on chemical neurotransmitters for activation. This is exemplified by work on nicotinic ACh receptors in which channel lifetime is solely dependent on the nature of the ligand (Colquhoun and Sakmann, 1985). Here we show that external ions are coactivators of KARs, suggesting, unexpectedly, that channel lifetime is not only controlled by the ligand but also by ions. As yet, it is not clear whether external ions control KARs simply by binding or whether an additional conformational change is required in much the same way that glycine acts as a coagonist at NMDARs (Kleckner and Dingledine, 1988). It is also unclear whether M770 represents the ion-binding site(s) or a

residue critical in the transduction process. Intuitively, it would be expected that anions and cations bind to discrete sites to satisfy electrostatic principles; however, other mechanisms, such as the establishment of a dipole, are possible (Bowie, 2002). Differentiating between these mechanisms can only be resolved through additional structure–function analysis of KARs.

How ligand and external ions determine the stability of the open state is not clear, but two possibilities may be considered. First, if external ions have a lower affinity (i.e., shorter residency time) than ligands, the time the KAR ion channel remains in the open state will be dependent on the rate of ion unbinding. This mechanism, however, fails to account for the slowing of channel kinetics at elevated ion levels (Bowie, 2002). Moreover, because agonist occupancy prevents ion rebinding (Fig. 3), KARs would also be expected to accumulate into the novel inactive state during prolonged agonist application. In this case, a decline in the equilibrium response would occur as ions unbind. However, we observe a well maintained equilibrium response, arguing against this mechanism (Fig. 3*b*). Alternatively, external ions may stabilize the ligand-binding cleft, which would account for the slowing of KAR deactivation and desensitization at high ion concentrations as well as the sustained equilibrium response. However, this mechanism seems inconsistent with the observation that L-Glu activation curves are weakly ion dependent (Bowie, 2002). Clearly, if KAR gating is to be elucidated, an important step will be to resolve the interplay between ligand and external ions.

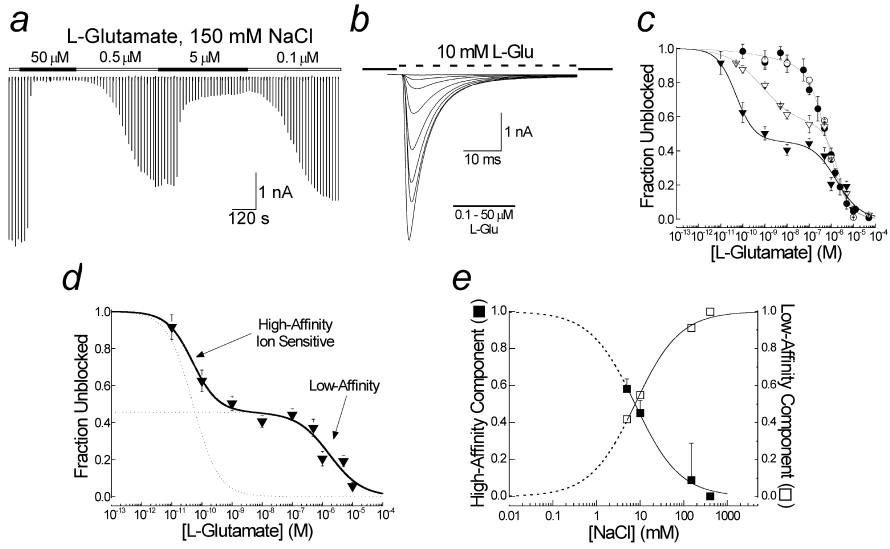

Figure 4. External ions regulate occupancy of a novel, high-affinity inactive state. ***a***, Typical experiment showing the onset of and recovery from inhibition of peak GluR6 responses by L-Glu. ***b***, Individual traces shown in ***a*** superimposed to show response profile in detail (patch 030613p1). ***c***, Family of inhibition curves to L-Glu in 5 mM (filled triangles), 10 mM (open triangles), 150 mM (filled circles), and 405 mM (open circles) NaCl. Solid lines are fits to single- or double-binding site isotherms. The IC_{50} of the low-affinity state was concentration independent (1.5 μM), whereas the Hill coefficient, n_h, was 0.8 in 5 mM NaCl and 1.1 in 10–405 mM NaCl. The IC_{50} for the high-affinity inactive state was concentration dependent, being 70 nM ($n_h = 0.5$) in 10 mM NaCl and 10 pM ($n_h = 1.0$) in 5 mM NaCl. ***d***, Inhibition curve observed in 5 mM external NaCl in more detail showing contribution of high- and low-affinity states (dotted curves). ***e***, Effect of NaCl on the fractional occupancy of high- and low-affinity states. Dotted lines represent fit extrapolations.

Although the M770 residue is restricted to GluR6 and GluR7 subunits, equivalent residues in other KAR subunits also confer sensitivity to external Cs^+ block (Paternain et al., 2003). In contrast, all AMPA and NMDA iGluRs have a conserved lysine residue in the M/K position (Fig. 1*c*), suggesting that only members of the KAR family are coactivated by external ions. Recent crystallographic work has indicated that full and partial agonists elicit different degrees of closure in the ligand-binding core of GluR6 KARs (Mayer, 2005a; Nanao et al., 2005). From work on AMPAR crystal structures, the degree of domain closure has been shown to be directly correlated to agonist efficacy (Jin et al., 2003). Our results, however, suggest that agonist behavior is not governed solely by conformations in the agonist-binding domain but that occupancy of a novel ion-binding site(s) must also be considered.

Finally, KARs are therapeutic targets in the treatment of several neurological diseases, including neuropathic pain (Palecek et al., 2004) and epilepsy (Smolders et al., 2002). Although some success has been achieved in developing selective KAR antagonists, a recurrent obstacle is that most, if not all, strategies rely on exploiting differences between the agonist-binding domain of iGluR subtypes (Bleakman et al., 2002). This is particularly problematic for AMPARs and KARs whose agonist-binding domains have overlapping pharmacology (Dingledine et al., 1999), which is expected given their significant structural homology (Mayer and Armstrong, 2004; Mayer, 2005a; Nanao et al., 2005) (Fig. 1). In principle, drug selectivity is best achieved when a unique pharmacological target can be identified. Our findings point to a mechanism that may be exploited to design drugs with a high selectivity for KARs that are useful in the treatment of neurological conditions.

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