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The Role of the C2 Domain of Protein Kinase C Apl II in the Nervous System of *Aplysia californica*

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Our thoughts are ours,
their ends none of our own

W. Shakespeare

For Mom and Dad

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PREFACE TO THESIS

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ABSTRACT

In the nervous system of the marine mollusk *Aplysia*, protein kinase C plays important roles in plasticity and learning. *Aplysia* PKCs are differentially regulated in sensory neurons of the nervous system. The facilitating neurotransmitter serotonin activates the Ca^{2+}-dependent PKC Apl I but not the Ca^{2+}-independent PKC Apl II. The mechanisms underlying this differential PKC activation still remain unclear. To better understand putative mechanisms regulating PKC activity we sought to characterize *Aplysia* PKC structural domains. This thesis has explored the characteristics of C1 and C2 domains from *Aplysia* PKC Apl I and PKC Apl II and whether differences in PKC activation can be attributed to differences in these domains.

First, we demonstrate that the presence of the C2 domain of PKC Apl II lowers the affinity of the C1 domain for the protein kinase C activator, phorbol ester. This C2 domain mediated inhibition of activator binding can be overcome by elevating phosphatidylserine concentrations. Further, phosphatidic acid is much more potent than phosphatidylserine in reducing C2 domain mediated inhibition.

Second, we present a comparison of the C1 and C2 domains of PKC Apl I and PKC Apl II. The C2 domain of PKC Apl I binds to lipids constitutively, while the C2 domain of PKC Apl II does not. In contrast, the C1 domains of PKC Apl I and PKC Apl II exhibit only small differences in lipid interactions. This suggests that while the C2 domain of PKC Apl I assists lipid mediated activation that of PKC Apl II hinders activation.

Finally, we show that there are two primary autophosphorylation sites in the C2 domain of PKC Apl II. These sites map to residues serine 2 and serine 36 located in loop 1 of the C2 domain. *In vitro*, phosphorylation of serine 36 increased binding of the C2 domain to phosphatidylserine membranes. *In vivo*, the PKC activator phorbol ester stimulated PKC Apl II phosphorylation at serine 36 and PKC phosphorylated at this residue translocated more efficiently to membranes. Moreover, mutation of serine 36 to alanine significantly reduced membrane translocation of PKC Apl II. As a whole, these data suggest a phosphorylation dependent mechanism regulating C2 domain membrane binding of Ca^{2+}-independent PKCs. This mechanism of Ca^{2+}-independent PKC plasma membrane localization may play a role in generating persistent PKC activity and the eventual modulation of synaptic plasticity in the nervous system of *Aplysia*.

RÉSUMÉ

La protéine kinase C joue un rôle important dans la plasticité et l'apprentissage du système nerveux du mollusque marin l'Aplysia. Les protéines kinases C de l'Aplysia sont régulées différemment dans les neurones sensoriels du système nerveux. Le neuromédiateur sérotonine active la PKC Apl I mais pas la nouvelle PKC Apl II. Les mécanismes à l'origine de cette activation différentielle restent encore inconnus. Afin de mieux comprendre les mécanismes d'activation des PKC, nous avons entrepris de caractériser les domaines structuraux des PKC de l'Aplysia. Cette thèse a permis d'explorer les caractéristiques des domaines C1 et C2 de la PKC Apl I et de la PKC Apl II et d'étudier si leurs différents mécanismes d'activation sont dus à des différences dans ces domaines.

Nous avons démontré dans un premier temps, que la présence du domaine C2 de la PKC Apl II réduit l'affinité du domaine C1 pour les activateurs de la PKC, les phorbol esters. L'inhibition de la liaison des activateurs de la PKC, liée à la présence du domaine C2 peut être surmontée en augmentant la concentration de phosphatidylserine. L'acide phosphatidique est toutefois beaucoup plus efficace que la phosphatidylserine pour réduire l'inhibition due à la présence du domaine C2.

La section suivante est centrée sur la comparaison des domaines C1 et C2 des PKC Apl I et PKC Apl II. Le domaine C2 de la PKC Apl I se lie constitutivement aux lipides, alors que ce n'est pas le cas du domaine C2 de la PKC Apl II. Par contre, les domaines C1 de la PKC Apl I et de la PKC Apl II ne présentent que de faibles différences quant aux interactions avec les

lipides. Ceci suggère que le domaine C2 de la PKC Apl I favorise l'activation médiée par les lipides tandis que celui de la PKC Apl II l'inhibe.

Finalement, nous avons démontré qu'il existe 2 sites primaires d'autophosphorylation dans le domaine C2 de la PKC Apl II. Ces sites correspondent aux résidus serine 2 et 36 localisés dans la boucle 1 du domaine C2. La phosphorylation *in vitro* de la serine 36 augmente la liaison du domiane C2 aux membranes phosphatidylserine. *In vivo*, les activateurs de la PKC, les phorbol esters, stimulent la phosphorylation de la serine 36 de la PKC Apl II. La translocation vers la membrane de la PKC ainsi phosphorylée, est plus efficace. De plus, la mutation de la serine 36 en alanine réduit significativement la translocation de la PKC Apl II vers la membrane. Ces résultats, pris dans leur ensemble, suggèrent que la liaison du domaine C2 à la membrane est régulée par un mécanisme dépendant de la phosphorylation.

LIST OF ABBREVIATIONS

A	Angstrom
AMPA	α-amino-3-hydroxy-5-methyl-4 isoxazole proprionic acid
AP5	2-amino-5-phosphonopentanoic acid
ARF	ADP ribosylation factor
ATP	adenosine triphospate
B_{max}	maximal binding
Ca^{2+}	calcium
$CaCl_2$	calcium chloride
CaLB	calcium dependent lipid binding domain
CaMKII	calcium calmodulin dependent protein kinase II
cAMP	cyclic adenosine monophosphate
CBR	calcium-binding region
cPKC	conventional calcium-activated PKC
CREB	cAMP response element binding protein
COPI	coat protein I (β isoform)
DAG	diacylglycerol
DNA	deoxyribonucleic acid
DOG	dioctylglycerol
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetate
EGF	epidermal growth factor
EGTA	ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetate

ELH	egg-laying hormone
EPSP	excitatory post-synaptic potential
FGF	fibroblast growth factor
GAP	GTPase activating protein
GAP-43	growth-associated protein 43
GFP	green fluorescent protein
GST	glutathione S-transferase
HCl	hydrochloric acid
I_{Ca}	calcium current
$I_{K,Ca}$	calcium-mediated potassium current
I_{KS}	serotonin-mediated potassium current
I_{KV}	voltage-dependent potassium current
IPTG	isopropyl 1-thio-β-D-galactopyranoside
KCl	potassium chloride
kDa	kilodalton
LB	Luria broth
LPA	lysophosphatidic acid
LTD	long-term depression
LTP	long-term potentiation
MBP	maltose binding protein
$MgCl_2$	magnesium chloride
Min	minute
MOI	multiplicity of infection

NaCl	sodium chloride
NMDA	*N*-methyl-D-aspartate
nPKC	novel calcium-independent PKC
OD	optical density
PA	phosphatidic acid
PAP	phosphatidic acid phosphohydrolase
PBS	phosphate buffered saline
PC	phosphatidylcholine
PCR	polymerase chain reaction
PDBu	phorbol dibutyrate
PDGF	platelet-derived growth factor
PDK	phosphoinositide-dependent protein kinase
PE	phosphatidylethanolamine
PFU	plaque forming unit
PG	phosphatidylglycerol
PH	pleckstrin homology
PI	phosphatidylinositol
PI 3-kinase	phosphoinositide 3-kinase
PI 4-kinase	phosphoinositide 4-kinase
PI 5-kinase	phosphoinositide 5-kinase
PIP_2	phosphatidyl inositol 4,5-bisphosphate
PIP_3	phosphatidyl inositol 3,4,5-triphosphate
PKA	cAMP-dependent protein kinase

PKC	protein kinase C
PKM	Magnesium-dependent catalytic domain fragment of PKC
PLA_2	phospholipase A_2
PLCγ	phospholipase C gamma
PLCβ	phospholipasee C beta
PLD	phospholipase D
PRK	protein kinase C related kinase
PS	phosphatidylserine
PVP	polyvinyl pyrolidone
RACK	receptor for activated C kinase
RNA	ribonucleic acid
Rp cAMP	Rp diastereoisomer of the cyclic adenosine 3', 5'-monophosphorothioate
RT	room temperature
RTK	receptor tyrosine kinase
SD	standard deviation
SDS-PAGE	sodium-dodecyl-sulfate polyacrylamide gel electrophoresis
Sec	second
SEM	standard error of the mean
SOD	superoxide dismutase
TBS	theta burst stimulation
TLC	thin layer chromatography
TPA	12-O-tetradecanoyl phorbol-13-acetate
TPCK	N-tosyl-L-phenylalanine chloromethyl ketone

TTX-R I_{Na}	tetrodotoxin-resistant sodium current
$ZnSO_4$	zinc sulfate

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CONTRIBUTION TO ARTICLES

Chapter 2

Pepio AM and Sossin WS (1998) The C2 domain of the Ca^{2+}-independent protein kinase C Apl II inhibits phorbol ester binding to the C1 domain in a phosphatidic acid-sensitive manner. Biochemistry 37: 1256-1263.

My contribution to this paper included all the experiments shown, including fusion protein generation and phorbol ester binding assays. Dr. Wayne Sossin provided guidance when establishing the phorbol ester binding protocol used in this and subsequent papers. I wrote the paper with the help of Dr. Wayne S. Sossin.

Chapter 3

Pepio AM, Fan XT, and Sossin WS (1998) The role of C2 domains in Ca^{2+}-activated and Ca^{2+}-independent protein kinase Cs in Aplysia. J Biol Chem 273: 19040-19048.

My contribution to this paper included all fusion protein production, lipid binding assays, tryptic protein digests, antibody production, immunoblotting and table generation. In addition, I performed the biochemial purification of all enzymes used in Figure 3.1 and Figures 3.6 - 3.8 with the assistance of Mr. Arash Nakhost. Specific figures generated by me include all of Figures 3.2 - 3.5 and Figure 3.9. X.T. Fan performed the PKC activity assays in Figure 3.1 and Figures 3.6 - 3.8 and instructed me for subsequent studies. Dr. Wayne Sossin produced Figure 3.10. I wrote this paper with the assistance of Dr. Wayne S. Sossin.

Chapter 4

Pepio AM and Sossin WS (2001) Regulation of C2 domain membrane binding by phosphorylation in novel PKCs. J Biol Chem 276: e-published Nov 9, 2000.

My contribution to this paper included all experiments and figures shown, including fusion protein production, PKC purification, antibody production and immunoblotting, PKC activity assays, lipid binding assays, *in vivo* membrane translocation assays, and phosphopeptide and phosphoamino acid analyses. The laboratory technicians X.T. Fan and T. Hueftlein helped me to generate several of the DNA and viral constructs. I wrote this paper in conjunction with Dr. Wayne S. Sossin.

RATIONALE

In the nervous system of the marine mollusk *Aplysia californica*, PKCs play important roles in synaptic plasticity and learning. The Ca^{2+}-dependent PKC Apl I but not the Ca^{2+}-independent PKC Apl II is activated by the facilitating neurotransmitter serotonin in sensory neurons. Unlike the well characterized mechanism of conventional PKC activation, a detailed analysis of Ca^{2+}-independent PKC activation has not been presented. One approach to understanding the differential regulation of these PKC isoforms in the nervous system is to characterize the individual protein domains that comprise the kinase regulatory regions. Both PKC Apl I and PKC Apl II contain similar C1 and C2 domains, raising pertinent questions: Do the C1 and C2 domains of PKC Apl I and PKC Apl II bind lipid activators in similar fashions? Can differences in PKC activation be attributed to differences in a particular protein domain? Finally, can these domains be modulated to regulate the activity of PKC in *Aplysia*? Acquiring knowledge of the biochemical properties of PKC regulatory domains may provide insight to the complex role PKC plays in synaptic plasticity.

OBJECTIVES

There were four main objectives in this thesis. The first objective was to determine whether the C2 domain of PKC Apl II inhibited activator binding to the C1 domain, thus hindering kinase activity. The second was to compare the C1 and C2 domains of PKC Apl I and PKC Apl II to determine which domains accounted for functional differences between the enzymes. The data produced from the first two objectives was consistent with the idea that the primary difference between the two PKCs resided within their C2 domains. While the C2 domain of PKC Apl I assisted kinase activation and membrane binding, that of PKC Apl II did not. The third objective was to identify a potential modulatory mechanism of the novel C2 domain of PKC Apl II. The fourth objective was to determine if this modulatory mechanism could change the inhibitory properties of the C2 domain so that it would assist in kinase translocation or activation.

CHAPTER 1

CHAPTER 1: Literature Review

The goal of this chapter is to introduce the current understanding of the role protein kinase C (PKC) plays in synaptic plasticity, with an emphasis on C1 and C2 domains and their role(s) in regulating protein kinase C activity. First, synaptic plasticity is introduced as a cellular correlate of learning. The role of PKC in cellular learning models is presented. In particular, the *Aplysia* gill-withdrawal reflex, its behavioral sensitization, and facilitation of the corresponding sensory-motor neural connections are described in detail. Second, the role of protein kinase C in the *Aplysia* nervous system and synaptic plasticity is discussed. Third, the protein kinase C family of enzymes, including their cellular distribution, structure, and function are reviewed. As well, a detailed review of C1 and C2 domains is presented. Finally, mechanisms of PKC activation, including data describing the formation of persistently active PKC and substrates involved in plasticity are discussed. As a whole, this review and the studies that follow serve as a framework for further investigation.

I. Cellular Basis of Synaptic Plasticity

A) Roles of Protein Kinase C in Cellular Learning

Protein kinase C has been implicated in many different learning paradigms. Several of these cellular correlates of learning have produced revealing information about the molecular mechanisms involved in the learning process itself. Avoidance learning in chickens, classical conditioning in *Hermissenda*, long-term potentiation in the hippocampus, and reflex learning in *Aplysia* have all demonstrated an important role for PKC in synaptic plasticity. PKC is

involved in the short-term modification of synaptic strength and the persistent activation of PKC is implicated in memory retention.

The passive avoidance model in chicks is one of the earliest models exploring cellular correlates of learning (Gibbs and Ng, 1977, 1979). The learning paradigm in this model uses day-old chickens that are trained in a simple one-trial task to peck a small metallic bead. Beads that have been coated with an aversive compound cause the birds to avoid them on subsequent trials and this memory persists for hours to days (Gibbs, 1991). This memory results in a series of biochemical changes in various forebrain regions of the animal. Initially, the biochemical changes consist of glycoprotein modifications (Sukumar et al, 1980; Burgoyne and Rose, 1980) and increases in kinase activity (McCabe and Rose, 1987). Specifically, memory formation increases membrane bound PKC activity (Burchuladze et al., 1990) and inhibitors of PKC can block memory formation (Serrano et al., 1994; Burchuladze et al., 1990). As well, learning induces a specific change in the phosphorylation of the PKC substrate GAP-43 in the forebrain (Sheu et al., 1993; Ali et al., 1988), which only occurs with memory for the coated beads (Rose and Harding, 1984). These biochemical modifications are associated with a regional increase in presynaptic terminal vesicular content in the chick forebrain (Horn, 1985; Rose, 1981). Following learning, the neuronal activity of these forebrain regions also increases, as a result of increased neuronal excitability (Horn, 1985; Rose, 1981). Thus, there are discrete links between PKC activation and biochemical changes in neuronal properties associated with learning in the chick.

One invertebrate learning model that has demonstrated an important role for PKC is the sea snail *Hermissenda*. The associative learning paradigm in *Hermissenda* involves paring of the conditioned stimulus, light, with the unconditioned stimulus, rotational movement (Alkon, 1984). Light normally induces the extension of the snail's locomotor muscle, and rotation produces a contraction of this muscle. Once the light is paired with rotation, the light alone evokes locomotor muscle contractions (Lederhendler and Alkon, 1986). *Hermissenda* B photoreceptor cells display a reduction in two potassium currents ($I_{K,Ca}$ and I_A) after conditioning that persists for days (Alkon, 1984). PKC is known to play a role in modulation of these photoreceptor cells during learning (Bank et al., 1989). Activation of PKC in B cell photoreceptors produces reductions in both the $I_{K,Ca}$ and I_A potassium currents similar to those occurring after conditioning (Alkon et al., 1986; Farley and Auerbach, 1986). Importantly, inhibitors of PKC activity blocked both the induction and long-term expression of the PKC-mediated reduction in K^+ currents (Farley and Schuman, 1991). As well, a calcium current in B photoreceptors that is reduced by serotonin (5-HT) application is also reduced by PKC activation (Yamoah and Crow, 1996). These findings illustrate the importance of PKC in induction and maintenance of neuronal excitability by modulation of ion channels.

The search for a synaptic correlate of learning in the vertebrate system has focused on long-term potentiation (LTP) in hippocampal brain slices. LTP, the sustained increase in synaptic strength of excitatory afferent connections following brief high frequency theta burst stimulation (TBS) (Bliss and Lomo, 1973), may play a role in plasticity and memory storage. This belief is based on the ability of pharmacological agents that block LTP induction to also

block learning (Bliss and Collingridge, 1993). It has been shown that PKC is activated during the induction of LTP (Klann et al., 1993; Sacktor et al., 1993) and the selective inhibition of regulated PKC activity blocks LTP induction (Malenka et al., 1989; Malinow et al., 1988,1989; Wang and Feng, 1992). In contrast, selective inhibition of the PKC kinase domain blocks the maintenance of LTP (Hrabetova and Sacktor, 1996; Malinow et al., 1988). These findings suggest that constitutively active, non-regulated, PKC activity modulates LTP maintenance (Hrabetova and Sacktor, 1996). PKC phosphorylation of the growth-associated protein-43 (GAP-43) increases during LTP (Pfenninger et al., 1991; De Graan et al., 1990b). As well, LTP-induced increases in channel conductance may be a result of PKC or calcium calmodulin-dependent protein kinase II (CaMKII) phosphorylation of α-amino-3-hydroxy-5-methyl-4 isoxazole proprionic acid (AMPA) and *N*-methyl-D-aspartate (NMDA) receptors (Tingley et al., 1993). The role of PKC in postsynaptic LTP stems from intracellular injection of the PKC pseudosubstrate-like inhibitor. This peptide inhibitor prevented the induction of LTP in postsynaptic neurons (Malinow et al., 1989). Interestingly, in hippocampal slices from PKCγ null mutant mice, the induction of LTP is significantly reduced (Abeliovich et al., 1993). However, after the induction of long-term depression (LTD) by low frequency stimulation, these PKCγ mutants display normal LTP (Abeliovich et al., 1993). The differential expression of LTP suggests that although most hippocampal brain areas display LTP, different stimulation protocols have different signal transduction requirements. Thus, it has been suggested that PKC activation does not directly cause LTP, but rather PKC signaling modulates this form of plasticity (Abeliovich et al., 1993). In support of this idea, it has recently been demonstrated that LTP-induced AMPA receptor phosphorylation is modulated by the prior activity of the synaptic connections (Lee et al.,

2000). LTP and LTD were shown to reversibly modify the phosphorylation state of the AMPA receptor GluR1 subunit. LTP induction in naïve synapses and depressed synapses increases phosphorylation of the GluR1 subunit PKC/CaMKII (serine 831) and the PKA (serine 845) sites, respectively. In contrast, LTD induction in naïve synapses dephosphorylates the major PKA site, whereas in potentiated synapses the major PKC/CaMKII site was dephosphorylated (Lee et al., 2000). Thus, although its precise role still remains unclear, PKC modulates aspects of plasticity in hippocampal connections during LTP.

Based on these models, it is apparent that cellular correlates of learning provide insight to molecular modifications arising from behavioral learning paradigms. Specifically, the importance of PKC activation in modulating neuronal changes during plasticity is also becoming clear.

B) Sensory-Motor Neuron Connections in *Aplysia*

i) The *Aplysia* gill-withdrawal reflex

The marine mollusk *Aplysia californica*, the learning system we study, is well suited to explore changes in synaptic connections. Its simple nervous system is comprised of a relatively small number of neurons (roughly 10^5-10^6) compared to vertebrates (10^{12}) that undergo simple forms of learning (Kandel, 1976). The behavioral withdrawal reflex of the animal's respiratory organs (the gill, siphon, and mantle shelf) has been particularly well studied for its prominent sensory-motor neuron synapses (Pinsker et al., 1970). The behavioral aspects of the reflex consist of the withdrawal of the animal's siphon and gill in

response to light touch stimulation of its siphon. Several studies have identified the neural circuit underlying the gill-withdrawal reflex in *Aplysia* (Byrne et al., 1974; Castellucci et al., 1970; Hawkins et al., 1981a,b). The neural circuit is comprised of roughly 24 mechanosensory neurons which make direct excitatory synapses on the gill and siphon motor neurons (Byrne et al., 1974; Castellucci et al., 1970; Perlman, 1979) and interneurons (Hawkins et al., 1981a,b; Perlman, 1979). These interneurons in turn form inhibitory and excitatory connections with the sensory and motor neurons.

ii) Behavioral sensitization of the gill-withdrawal reflex

Sensitization itself is a simple form of learning, in which the response of an animal's defensive reflex to a previously neutral stimulus is strengthened after exposure to a noxious stimulus (Kupferman et al., 1970; Kandel and Schwartz, 1982; Walters et al., 1983). Sensitization of the *Aplysia* gill-withdrawal reflex can be induced by a strong stimulus, such as a shock, delivered to the animal's head or tail (Kandel and Schwartz, 1982). This noxious stimulation activates facilitatory interneurons that synapse on the mechanosensory neurons (Hawkins et al., 1981b). As a result, the synaptic connection between the sensory neurons and their motor target cells are strengthened (Hawkins et al., 1981b). The presynaptic origin of the facilitation was identified by analysis of neurotransmitter release *in vivo* and in dissociated neurons in culture (Castellucci and Kandel, 1976; Dale et al., 1988). Sensitization of the gill-withdrawal reflex and the associated behavioral memory is proportional to the number of training episodes to which the animal is exposed (Frost et al., 1985). Accordingly, short-term sensitization that can last minutes to hours is produced by a

single tail stimulus, whereas long-term sensitization that persists days to weeks following training can be produced by multiple tail stimuli (Pinsker et al., 1973; Frost et al., 1985).

iii) Morphological changes in the sensory-motor synapse

The application of 5-HT to *Aplysia* sensory-motor neurons in culture evokes functional changes in the synaptic connections that persist for days (Glanzman et al., 1989, 1990; Schacher et al, 1991). This long-term facilitation correlates with the growth of new synaptic connections (Frost et al., 1985; Bailey and Chen, 1988; Lee et al., 1995). These synaptic changes occur with the development of new varicosities on the sensory neuron and additional active zones contacting the axon of the motor neuron (Glanzman et al., 1989, 1990; Schacher et al, 1991). The number, size, and vesicle content of active zones in sensory terminals are also increased in long-term sensitized animals (Bailey and Chen, 1983). Additionally, activation of PKC results in an increase in the number of sensory neuron varicosities (Wu et al., 1995). These PKC-mediated changes in sensory neuron structure occurred rapidly (within 15-30 min of treatment) and are quite susceptible to elimination (Wu et al., 1995). The transient nature of these structural changes led to the suggestion that PKC activation modulates substrates in the sensory neuron growth cones.

iv) Facilitation of the *Aplysia* sensory-motor neuron synapse

The sensory and motor neurons that constitute the *Aplysia* gill-withdrawal reflex circuit have been studied during behavioral sensitization. A single noxious stimulus to the animal's head or tail induces several synapses within the neuronal circuit of the gill-withdrawal reflex to become strengthened (Kandel and Schwartz, 1982). These strengthened synapses include

both the sensory-motor and sensory-interneuron connections and underlie the strengthening of the behavioral response (Kandel and Schwartz, 1982). The sensitizing stimulus activates a group of facilitating interneurons that synapse directly on the mechanosensory neuron terminals (Kistler et al., 1985). A subset of these interneurons are serotonergic and their release of serotonin on the sensory neuron terminal results in an increase of cyclic adenosine mono phosphate (cAMP) within the sensory neuron (Kandel, 1976). This cAMP increase then results in enhanced excitatory neurotransmitter release from the sensory neuron onto the motor neuron following the next sensory neuron firing. Kandel and colleagues have extensively characterized the biochemical events mediated by serotonin in the sensory neuron terminal following the discharge of the facilitating interneurons (Klein and Kandel, 1980; Castellucci et al., 1980, 1982; Siegelbaum et al., 1982).

v) Role of PKA

A model for the biochemical events underlying the facilitating effects of serotonin on the mechanosensory neurons was proposed (Klein and Kandel, 1980). In this model, serotonin released on the sensory neuron terminals activates receptors (as yet these serotonin receptors remain uncloned) that activate heterotrimeric G proteins, which in turn stimulate the activation of adenylyl cyclase (Klein and Kandel, 1980). The increased activation of adenylyl cyclase catalyses the production of cAMP from ATP in the sensory neuron terminals, which then binds to the regulatory subunit of the cAMP-dependent protein kinase (PKA). This binding causes the dissociation of the regulatory subunit of PKA from the catalytic subunit that is then released from kinase inhibition and can phosphorylate its targets. PKA phosphorylates a serotonin-sensitive potassium channel (S-type K^+ channel) or an

associated protein (Klein and Kandel, 1980; Siegelbaum et al., 1982; Dale et al., 1987). Phosphorylation of this channel closes it to reduce the 5-HT-sensitive potassium current (I_{KS}) that normally repolarizes the neuron (Siegelbaum et al., 1982; Shuster et al., 1985; Dale et al., 1987). Reduction of I_{KS} prolongs the action potential by slowing the repolarization rate of the membrane and as a result the open state of the voltage-gated N-type calcium channels is prolonged (Klein and Kandel, 1980; Siegelbaum et al., 1982), allowing more calcium to enter the terminals enhancing transmitter release (Siegelbaum et al., 1982; Scholz and Byrne, 1987). More recently, important roles for PKC have been found during sensory neuron facilitation.

II. Roles of PKC in the *Aplysia* Nervous System

The behavioral sensitization of the *Aplysia* gill-withdrawal reflex was found to be under the molecular control of two different second-messenger pathways. Several studies have demonstrated that in addition to PKA, PKC also contributes to short-term presynaptic facilitation of the sensory-motor connections in *Aplysia* (Ghirardi et al., 1992; Sugita et al., 1992, 1994a,b). Serotonin-induced sensory neuron facilitation is dependent on two physiological mechanisms: i) increased excitability due to the reduction in I_{KS} (Ghirardi et al., 1992; Goldsmith and Abrams, 1992) and ii) increased action potential duration (spike broadening) by modulation of both I_{KS} and the voltage-dependent potassium current (I_{KV}) (White et al., 1994; Sugita et al., 1992, 1994a; Braha et al., 1993). 5-HT-mediated facilitation of the presynaptic sensory neuron can be attenuated by blocking spike broadening (Hochner et al., 1986a,b). Spike broadening itself has been shown to correlate with the increase in presynaptic calcium concentrations (Eliot et al., 1993) and transmitter release

(Sugita et al., 1994a,b; Klein, 1994). As well, 5-HT also mediates facilitation of synapses that have undergone synaptic depression, the reduction in transmitter release with successive action potentials (Hochner et al., 1986a,b).

A) Sensory Neurons

i) Modulation of the action potential: duration and frequency

The 5-HT-mediated increase in sensory neuron transmitter release can be blocked by inhibitors of PKC (Conn et al., 1989; Ghirardi et al., 1992). As well, activation of PKC by the phorbol ester, TPA, produces an increase in synaptic release from these neurons (Hochner et al., 1985). Biochemical evidence has also demonstrated that serotonin induces the membrane translocation and activation of the Ca^{2-}-dependent PKC Apl I in *Aplysia* sensory neurons (Sossin and Schwartz, 1992; Kruger et al., 1991; Sacktor and Schwartz, 1990). Together these data suggest a general role for PKC activation in the modulation of 5-HT induced synaptic release. However, PKC plays an specific role in the slow developing component of spike broadening by modulating I_{KV} (Sugita et al., 1994a). The slow component of spike broadening can be induced by activators of PKC (Marcus and Carew, 1992), and blocked by PKC selective inhibitors (Sugita et al., 1992). Thus, activation of PKC and its modulation of I_{KV} contributes to increased action potential duration underlying late stages of short-term facilitation.

PKC has also been shown to regulate changes in sensory neuron excitability. Activation of PKC causes an increase in sensory neuron excitability in response to injection of depolarizing current pulses both isolated ganglia and dissociated neuron cultures (Manseau et

al., 1998). The PKC inhibitor chelerythrine, blocked this increase in frequency of sensory neuron neuronal firing (Manseau et al., 1998). These findings are supported by other results in which PKC activation produces a slow increase in sensory neuron excitability (Sugita et al., 1992). Additionally, transient PKC activity is capable of producing persistent changes in excitability that do not depend on lasting PKC activation (Conn et al., 1989). These studies suggest that cumulative PKC activity can mediate a large increase in sensory neuron excitability similar to that produced by 5-HT-mediated PKA activation.

ii) Reversal of synaptic depression

Activation of PKC also modulates another form of 5-HT induced facilitation known as reversal of synaptic depression. Repeated activation of the sensory neuron causes synaptic depression, or reduction in synaptic efficacy, of the sensory-motor neuron synapse due to depletion of the presynaptic vesicular pool (Hochner et al., 1986b). Although these "depressed synapses" are no longer facilitated by spike broadening since the vesicular pool is depleted, serotonin still produces facilitation of these synapses through another mechanism that may mobilize synaptic vesicles to the terminal (Hochner et al., 1986a,b; Byrne and Kandel, 1996). Kandel and colleagues demonstrated that PKC activation can facilitate depressed synapses and that this "reversal of synaptic depression" can be blocked by the general protein kinase inhibitor H7 (Braha et al, 1990). Consistent with this role of PKC in reversing synaptic depression, the kinase inhibitor H7 prevented the 5-HT-mediated reversal of depression in highly depressed sensory-motor neuron cultures (depressed to <10% of the control excitatory post-synaptic potential (EPSP)) (Ghirardi et al., 1992). Kandel and colleagues went on to show that in naïve synapses the mechanisms contributing to both

excitability (I_{KS}) and spike broadening (I_{KS} and I_{KV}) are controlled by 5-HT via PKA signal transduction. In contrast, these two mechanisms (excitability and spike broadening) gradually become controlled by 5-HT through PKC in active synapses which undergo progressive synaptic depression (Ghirardi et al., 1992; Byrne and Kandel, 1996). This differential recruitment of the PKA or PKC signaling machinery is also modulated by the duration of 5-HT exposure such that brief 5-HT exposure is modulated by PKA and prolonged 5-HT exposure is modulated by PKC (Byrne and Kandel, 1996). Thus, the quantity of 5-HT released from the facilitating interneurons correlates with the active state of the synapses. This suggests that the extent of "synaptic depression" functions to regulate the 5-HT levels affecting the sensory-motor neuron synapse, effectively determining which signaling pathway (PKA or PKC) will be utilized. Furthermore, the Ca^{2+}-dependent and the Ca^{2+}-independent PKCs in *Aplysia* exhibited differential effects on the reversal of synaptic depression (Manseau et al., 1999). Induction of synaptic depression in sensory neurons after injection with plasmids coding for kinase inactive mutants of the Ca^{2+}-dependent PKC Apl I or the Ca^{2+}-independent PKC Apl II demonstrated kinase selectivity in reversal of synaptic depression. Specifically, the kinase inactive PKC Apl II significantly blocked the 5-HT-mediated "reversal of synaptic depression" EPSP, whereas injection of PKC Apl I did not (Manseau et al., 1999). The weight of this evidence for PKC modulation of sensory neuron plasticity is supported by the known serotonin receptors coupled to phospholipase C (PLC) that produce the PKC activator diacylglycerol (DAG) (Li et al., 1995b; Gerhardt and Van Heerikhuizen, 1997).

iii) Activity-dependent facilitation

Most recently, evidence from Carew and colleagues has demonstrated that 5-HT mediated facilitation of the *Aplysia* sensory-motor neuron synapse is under the control of persistently active PKC pathways (Sutton and Carew, 2000). The induction of activity-dependent facilitation at intermediate timepoints (3 hr) requires coincident sensory neuron activation and brief 5-HT release from the interneurons and is dependent on the persistent activation of PKC. This activity-dependent facilitation could be blocked by the PKC inhibitors H-7 and chelerythrine, but not the PKA inhibitor KT5720 (Sutton and Carew, 2000). These studies reveal the requirement of persistent PKC activation in the plasticity of the sensory-motor neuron synapses of *Aplysia*. However the mechanism by which sensory neuron activity and 5-HT interact to bring about persistent PKC activation remains to be determined.

B) Modulation of Calcium Channels

In addition to the K^+ currents I_{KS} and I_{KV}, serotonin has been shown to increase a voltage-dependent Ca^{2+} current (I_{Ca}) in sensory cells (Braha et al., 1993). PKC can modulate this Ca^{2+} current since the 5-HT-mediated increase in I_{Ca} can be mimicked by the PKC activator, phorbol ester (Braha et al., 1993). This increase in I_{Ca} by PKC activation can be blocked both by the general kinase inhibitor H7 and a PKC pseudosubstrate inhibitor peptide (Braha et al., 1993). The 5-HT-mediated increase in this Ca^{2+} current is specifically mediated by PKC, since the peptide PKC inhibitor also blocked the increase in I_{Ca} while the PKA inhibitor, Rp-cAMP, did not (Braha et al., 1993). Other evidence for the role of PKC modulation of Ca^{2+} channels comes from the peptidergic bag cell neurons of *Aplysia*.

C) Bag Cell Neurons

The peptidergic bag cell neurons of *Aplysia* are responsible for the initiation of egg-laying behavior by secreting egg-laying hormone (ELH) during a prolonged phase of electrical activity known as the afterdischarge (Wilson et al., 1996). These cells contain two physiologically characterized calcium channels distinguished by conductance as well as their sensitivity to PKC (White and Kaczmarek, 1997). One of these calcium channels, BC-α_{A1}, is upregulated by PKC and may underlie increases in action potential height and potentiation of ELH release during the afterdischarge (DeRiemer et al., 1985; Conn et al., 1989; Loechner et al., 1992). Inhibition of PKC during the afterdischarge blocks the action potential enhancement (Conn et al., 1989) and reduces ELH release (Loechner et al., 1992), suggesting BC-α_{A1} modulates these functions. As well, the afterdischarge produces the persistent activation of both Ca^{2+}-dependent and Ca^{2+}-independent PKCs in the bag cell neurons (Wayne et al., 1999). These PKC-sensitive channels are observed in cell bodies of bag cells and, based on calcium imaging studies, are present in growth cones as well (Knox et al., 1992; White and Kaczmarek, 1997). Recently, it has been demonstrated that the PKC-sensitive Ca^{2+} channels are upregulated during development (Nick et al., 1996).

Several physiological lines of evidence have suggested that calcium channel recruitment by PKC in the bag cell neurons may involve translocation of channels from an intracellular pool. First, the BC-α_{A1} channels are PKC-sensitive and are observed in patch-clamped membranes only after PKC stimulation (Strong et al., 1987). Furthermore, use of whole-cell patch-clamping disrupts channel recruitment (De Riemer et al., 1985; Strong et al., 1987). This suggests that recruitment is not mediated simply by phosphorylation of a calcium channel

resident in the membrane (White and Kaczmarek, 1997). Second, calcium imaging studies show that PKC enhances growth cone size concomitantly with electrically stimulated calcium entry, suggesting that calcium entry may be coupled to channel insertion (Knox et al., 1992).

The insulin receptor is a tyrosine kinase receptor found in the mammalian brain and at high concentrations in the bag cell neurons of *Aplysia*. It has been demonstrated that treatment of bag cells with insulin causes an acute rise in intracellular calcium concentrations triggering the release of ELH (Jonas et al., 1997). As well, insulin stimulates an extracellular Ca^{2+} current that can be mimicked by phorbol esters and blocked by PKC inhibitors (Strong et al., 1987; Jonas et al., 1996). Importantly, insulin stimulation of bag cell neurons was shown to induce the persistent activation of PKC Apl II, the Ca^{2+}-independent isoform of PKC in *Aplysia* (Sossin et al., 1996a). Insulin stimulation of PKC Apl II activity was blocked by the phosphoinositide 3-kinase (PI 3-kinase) inhibitor wortmannin, suggesting that PI 3-kinase may mediate insulin-dependent activation of PKC Apl II (Sossin et al., 1996a). Recent studies of the PKC-sensitive Ca^{2+} channel have found a strong correlation between the presence of vesicular BC-α_{A1} channels and the presence of the PKC-sensitive current (White et al., 1998). As well, it has been suggested that calcium entry through these channels may help to sustain ELH release, since ELH release can persist even after the termination of the afterdischarge (Wayne and Wong, 1994). Thus, PKC plays a prominent role in plasticity of both sensory and bag cell neurons in *Aplysia*.

III. Structure and Function of Protein Kinase C

A) The PKC Family of Proteins

i) Protein kinase C isoforms

Protein kinase Cs are a family of Ca^{2+} and phospholipid-dependent serine/threonine protein kinases that are a single polypeptide chain of 77-90 kDa (Parker et al., 1986). The vertebrate PKC family consists of many isoforms with closely related structures and distinct enzyme characteristics that have allowed their division into three sub-classes. The first PKC sub-class is the conventional Ca^{2+}-dependent (cPKCs) (α, β_I, β_{II}, and γ) that are activated by the combination of Ca^{2+}, phosphatidylserine (PS), and DAG (Nishizuka, 1988; Tanaka and Nishizuka, 1994; Newton, 1997). The second sub-class, the novel Ca^{2+}-independent (nPKCs) (δ, ε, η, θ), contain a novel C2 domain and as a result do not require Ca^{2+} in conjunction with PS and DAG for activity (Ono et al., 1988; Newton, 1995a). The third and least well-characterized PKC sub-class is the atypical (aPKCs) (ζ, λ, ι) that lack the C1 and C2 regulatory domains and are not dependent on Ca^{2+}, PS, or DAG for activity (Nakanishi and Exton, 1990; Newton 1997). One of the difficulties in studying the effects of PKC in the vertebrate nervous system is the extensive number of isoforms.

In contrast, the nervous system of *Aplysia* exhibits only two phorbol ester/diacylglycerol activated PKC isoforms, the Ca^{2+}-activated PKC Apl I with homology to vertebrate PKCα and β, and the Ca^{2+}-independent PKC Apl II with homology to vertebrate PKCε and η (Sossin et al., 1993). As well, there is strong conservation of the N-terminal regulatory C2 domain in several Ca^{2+}-independent PKCs ranging from vertebrate PKCε to its *Aplysia* homologue, PKC Apl II (Schaap and Parker, 1990; Kruger et al., 1991; Sossin et al., 1993;

Land et al., 1994). Since this group of PKCs is enriched in the nervous system, perhaps they serve some common function.

ii) The genes

The genes for the cPKCs (α, β_I, β_{II}, and γ) are located on different chromosomes (Parker et al., 1986). The data from DNA analysis of the PKCβ_I and PKCβ_{II} genes suggests that they are alternative splice forms from a single RNA transcript (Coussens et al., 1986, 1987). The nPKCs have been less well studied at the genomic level. There is, however, some evidence for the alternative splicing of the gene for the novel vertebrate PKCε. Ono and colleagues isolated a rat brain cDNA clone of PKCε that was truncated by 240 amino acids from its N-terminal (Ono et al., 1988). As well, transcripts have been found in rat lung and brain that are shorter than that of PKCε at the 5' end (Ono et al., 1988).

iii) Protein localization

Several different biochemical approaches have been used to study individual patterns of PKC expression in cells and tissues (Nishizuka, 1988). Immunohistochemical techniques examining both the cPKCs (α, β_I, β_{II}, and γ) and nPKCs (δ, ε, η, θ) have shown differential localization of PKC isoforms in the brain (Nishizuka, 1988; Saito, 1994; Tanaka and Nishizuka, 1994). The conventional PKCγ isoform is expressed solely in the central nervous system where it displays differential expression in the brain and absence from all other cell types (Shearman et al., 1987; Nishizuka, 1988, 1992). As well, PKCβ_I and PKCβ_{II} are differentially localized in the brain (Coussens et al., 1987) where they are mainly found in cortical pyramidal cells, cerebellar purkinje cells, and hippocampal pyramidal and granular

cells (Saito et al., 1988). However, PKCβ_I and PKCβ_{II} are also found in a variety of other tissues along side PKCα (Kosaka et al., 1988). Interestingly, there is evidence to support PKCβ_{II} localization at the synapse, since it was found to be associated with cytoskeletal elements in synaptosomes isolated from rat brain (Tanaka et al., 1991).

In contrast, the protein tissue distribution of the nPKC isoforms (δ, ε, η, θ) are not as well characterized but have been found in the brain, heart, lung, liver and kidney (Ono et al., 1988). PKCη displays very low expression levels in the brain, moderate expression in the heart and skin, and fairly prominent expression in the lung (Bacher et al., 1991). The variability in tissue distribution is also present in many cell types, where the expression of PKC isoforms may arise from cell specific functions (Dekker and Parker, 1994).

Similar differences in patterns of subcellular localization have also been found. Conventional PKCβ and PKCγ appear to be exclusively localized to the postsynaptic region of neurons in the rat hippocampus (Kose et al., 1991; Tanaka and Nishizuka, 1994). Specifically, PKCγ has been found in dendrites and dendritic spines by electron microscopy (Saito, 1994). The two types of PKCβ are distributed differently. PKCβ_I is found mainly in the brain stem with subcellular localization in cytoplasmic clusters near the cell membrane (Saito, 1994). In contrast, PKCβ_{II} is localized to pyramidal cells and striatal neurons of the forebrain where it is concentrated around the proximal dendrite and golgi (Saito, 1994). PKCα is associated with presynaptic neurons and neuronal growth cone-structures (Igarashi and Komiya, 1991; Shearman et al., 1991). The novel isoform, PKCε, was found presynaptically in primary sensory neurons, the forebrain, and the spinal cord (Saito, 1994).

Vertebrate PKCε and its *Aplysia* homologue, PKC Apl II, are both enriched in the nervous system, suggesting they mediate similar biological functions. Several biological roles of PKCε have been recently reported.

B) Biological Roles of Vertebrate PKCε

Vertebrate PKCε has been implicated in several cell type-specific physiological roles. Several lines of evidence suggest that neurotransmitter release from presynaptic terminals is modulated by the activation of PKCε (Terrian and Ways, 1995; Prekeris et al., 1996). As well, recent studies of this Ca^{2+}-independent PKC isoform have identified the importance of PKCε in nociceptor function (Khasar et al., 1999a) and cardiac myocyte protection (Liu et al., 1999; Chen et al., 1999).

i) Neurotransmitter release

A detailed study by Terrian and Ways reported that the PKC activator PDBu increases calcium dependent glutamate release during continuous depolarization of synaptosomes, homogenous preparations of nerve terminals obtained when neurons are homogenized under isotonic conditions (Terrian and Ways, 1995). They demonstrated that sustained activation of PKCε increases synaptic vesicle recycling in the presence of saturating concentrations of Ca^{2+}. The persistent effects on glutamate release were directly related to the amount of PDBu, were not observed with ionomycin stimulated glutamate release, and were prevented by I_{CaV} blockers (Terrian, 1995). They suggested that PKC affects the slow phase of glutamate release by increasing the efficiency of vesicle mobilization and recycling. This idea is reminiscent of the potential role for PKC in mediating vesicular mobilization in the

presynaptic terminal of *Aplysia* sensory neurons. The same group went on to show that there is an isoform-specific interaction between PKCε and F-actin in nerve terminals that is PKC activity dependent (Prekeris et al., 1996). The authors suggest that the assembly of this PKCε - F-actin complex plays a primary role in the PKC-dependent facilitation of glutamate release from terminals.

ii) Nociception

A recent role for PKCε in mediating responses to pain and injury has stemmed from studies of PKCε null mutant mice. (Khasar et al., 1999a; 1999b). Activation of PKCε has been shown to sensitize nociceptors (Schepelmann et al., 1993). Recently, PKCε was found to mediate epinephrine-induced mechanical and thermal sensitivity, as PKCε null mutant mice displayed a reduced sensitivity to pain (Khasar et al., 1999b). As well, blocking PKCε translocation and function in wild type animals attenuated the epinephrine-induced pain responses (Khasar et al., 1999b). Epinephrine also enhances a tetrodotoxin-resistant sodium current (TTX-R I_{Na}) important for nociceptor sensitivity in dorsal root ganglion neurons (Khasar et al., 1999a). Importantly, this increase in TTX-R I_{Na} is blocked by PKCε inhibition (Khasar et al., 1999b). These findings indicate that PKCε mediates pain responses by regulating the sodium current TTX-R I_{Na}.

iii) Cardiac protection

Short-term cardiac ischemia has been shown to reduce long-term ischemic injury to cardiac myocytes (Qiu et al., 1998). This cardioprotective effect, known as preconditioning, is selectively blocked by inhibition of PKCε (Liu et al., 1999). Protection from ischemia can

also be induced by PKC activation and, like preconditioning, is reversed by peptide inhibition of PKCε (Liu et al., 1999). Additionally, protection from ischemia can be induced by ethanol exposure and this cardioprotection is also prevented by blocking PKCε function (Chen et al., 1999).

C) PKC Structure

i) Structural topology

Members of the protein kinase C family are a single polypeptide, comprised of an N-terminal regulatory region and a C-terminal catalytic region. The enzymatic structure is of four conserved domains (C1-C4) (Nishizuka, 1992; Newton 1995b, 1997) and five variable regions (V1-V5) (Coussens et al., 1986). Each of the four conserved domains is a functional motif and is contained by many unrelated proteins. The C1 domain contains a cysteine-rich motif, duplicated in most PKC isoforms, which forms DAG and PS binding sites (Newton, 1995b, 1997). The C1 domain is immediately preceded by an autoinhibitory pseudosubstrate sequence, an amino acid region that closely resembles the PKC substrate recognition site (Newton, 1995b). This pseudosubstrate sequence has been demonstrated to inhibit kinase activity by mutation of the PKCα pseudosubstrate (Pears et al., 1990) and by an anti-pseudosubstrate antibody (Makowske and Rosen, 1989), which both led to kinase activation. The C2 domain, in Ca^{2+}-dependent PKC isoforms, contains the Ca^{2+} as well as other phospholipid binding sites. The C3 and C4 domains form the ATP binding and catalytic substrate pocket (Newton, 1995b; Parker et al., 1986).

ii) The catalytic region

The C-terminal half of the kinase, comprised of domains C3 and C4, exhibits significant sequence homology with other protein kinases (Parker et al., 1986) and shares primary structural elements with the catalytic domain of PKA (Nishizuka, 1988). The conserved domain C3 houses the ATP binding site as established by the deletion mutation of C3 (Kaibuchi et al., 1989) and a point mutation in the putative ATP binding site, both of which block enzyme activity (Ohno et al,. 1990). Additionally, it has been shown that the C4 domain plays a role in substrate recognition prior to substrate phosphorylation (Tanaka and Nishizuka, 1994).

iii) The C1 domain

a) Function

The C1 domain of all DAG-dependent PKCs contains tandem repeats of a cysteine-rich sequence, C1A and C1B, that are separated by approximately 25 amino acids and are essential for binding the lipid activators, diacylglycerol, phorbol esters, and phosphatidylserine (Parker et al., 1986; Azzi et al., 1992). Tumor promoting phorbol esters activate PKC by mechanisms similar to that of DAG. Diacylglycerol inhibits phorbol ester binding in a competitive manner, suggesting that their interaction sites are the same (Sharkey et al., 1984; Zhang et al., 1995). Binding of either DAG or phorbol esters, however, causes a conformational change in the enzyme that releases the pseudosubstrate sequence (Newton, 1995a,b, 1997).

The cysteine-rich sequences resemble the zinc-fingers present in DNA binding proteins involved in transcriptional regulation (Berg, 1990). The similarity ends, however, with the fact that both domains coordinate a zinc atom (Hubbard et al., 1991). Each of the two cysteine-rich sequences, C1A and C1B, is capable of binding DAG, although evidence suggests that only one site can be bound to the activator at a time, probably due to steric constraints that do not accommodate two DAG molecules simultaneously (Quest et al., 1994).

b) Structural basis of lipid interactions

The crystal structure of both C1A and C1B is that of two small β sheets and a short C-terminal α helix (Zhang et al., 1995). The 2 β sheets form a pocket that is lined with polar residues providing a binding surface for lipids in the β sheet conformation (Lee and Bell, 1989; Zhang et al., 1995). The β sheet pocket is normally hydrogen bonded to the oxygen atoms of water molecules. When DAG or phorbol esters bind to the C1 domain pocket, they displace the oxygen atoms of the water molecules and replace the lost bridging hydrogen bonds from the water (Zhang et al., 1995). The binding site for phorbol esters was further identified in the second cysteine-rich region, C1B, of PKCδ by site-directed mutagenesis (Kazanietz et al., 1995). Mutation of five of the six cysteines completely blocked phorbol ester binding to the C1 domain (Kazanietz et al., 1995). When activators bind, they cover the polar interior of the β sheet pocket and complete a contiguous hydrophobic surface on a large portion of the C1 domain (Rando and Kishi, 1992; Zhang et al., 1995). Hence, when an activator is bound, the C1 domain can bury its hydrophobic surface 6-8 A° in the membrane without losing its bridging hydrogen bonds in the β sheet pocket (Zhang et al., 1995).

Additionally, when PKC binds to phospholipid monolayers an increase in surface pressure is observed, arguing that critical domains are inserted into the hydrocarbon region (Bazzi and Nelsesstuen, 1988a,b,c). The binding of the C1 domain to membranes through DAG causes the removal of the pseudosubstrate sequence from the catalytic pocket, activating PKC (Newton, 1995a,b, 1997).

iv) The C2 domain

a) Functional diversity

The C2 domain was first identified in the conventional, Ca^{2+}-dependent PKC isoforms (Parker et al., 1986; Knopf et al., 1986; Coussens et al., 1986) and later in novel, Ca^{2+}-independent PKC isoforms (Sossin and Schwartz, 1993). The C2 domain was shown to bind cellular membranes in response to Ca^{2+} fluxes, and as a result it was proposed to be a Ca^{2+}-dependent lipid binding domain (CaLB) (Clark et al., 1991). Although C2 domains were first identified in PKC isoforms similar domains have been found in a variety of other proteins, including neuronal and non-neuronal forms of synaptotagmin (Ullrich et al., 1994; Li et al., 1995a), Ras GTPase-activating proteins (GAPs) (Maekawa et al., 1994; Gaul et al., 1992), phosphatidylinositol 3-kinases α and β (Stephens et al., 1993), phospholipase Cδ, β, and γ (Rhee and Choi, 1992; Kriz et al., 1990; Yagisawa et al., 1994)., and phospholipase D (PLD) (Wang et al., 1994a; Pointing and Parker, 1996).

Much has been learned in recent years about the functional role of the C2 domain in different signaling pathways. Currently, most, if not all, proteins containing C2 domains are thought to interact with cellular membranes, and in several instances, the C2 domain has been shown

to be directly involved in membrane lipid binding (Nalefski et al., 1994). As well, this multi-functional domain can mediate the direct binding of calcium. In synaptotagmin and Ca^{2+}-dependent PKCs, the C2 domain binds PS in a Ca^{2+}-dependent manner (Brose et al., 1992; Davletov and Sudhof, 1993; Fukuda et al., 1994). Interestingly, the C2 domain of phospholipase A_2 (PLA_2) binds phosphatidylcholine (PC), but not PS in a Ca^{2+}-dependent manner suggesting that not all C2 domains have the same lipid specificity (Nalefski et al., 1994).

b) Structure

The solved crystal structures of several C2 domains have been determined, including that of synaptotagmin I (Sutton et al., 1995), PLC-δ1 (Essen et al., 1996), PLA_2 (Perisic et al., 1998), and PKCα and δ (Verdaguer et al., 1999; Pappa et al., 1998). Together these structures identify the C2 domain to be comprised of two four-stranded sheets, each of which is made up of β-strands joined by loop regions. The strands of the β sheets are arranged in an anti-parallel orientation and the sheets themselves are layered against each other (Nalefski and Falke, 1996). The strand order of the domain can be of two different topologies: type I C2 domains, in which the first structural position is occupied by strand 1, and type II, where the first structural position is occupied by strand 8 (Shao et al., 1996; Nalefski and Falke, 1996).

c) Calcium binding

The evidence for calcium coordination by the C2 domain also came from the protein crystal structure. The crystalized structure of the C2 domain bound to calcium ions identified the

"C2 key": 5 aspartate residues important for the interaction with calcium (Shao et al., 1996; Essen et al., 1996; Grobler et al., 1996). These aspartates are not conserved in the novel C2 domains of Ca^{2+}-independent PKCs such as *Aplysia* PKC Apl II or related C2 domain proteins (Sossin, 1997). This suggests that these C2 domains may have some alternate mechanism to promote lipid binding (Sutton et al., 1995; Pepio et al., 1998). These studies revealed that there were 2 distinct calcium binding sites, one high affinity (60 μM K_d) and one low affinity (400 μM K_d) (Shao et al., 1996). The calcium binding sites are formed from the 5 aspartate residues contained in the loop regions connecting strands β2-β3 (loop 1) and β6-β7 (loop 3) in type I C2 domains or strands β1-β2 (loop 1) and β5-β6 (loop 3) in type II C2 domains (Sutton et al., 1995). As a result, the loops between strands β2-β3, β4-β5, and β6-β7 in type I C2 domains (and those of type II) have come to be known as calcium binding regions 1-3 (CBRs 1-3) (Perisic et al., 1998). CBR2 and CBR3 have fairly consistent conformations among most C2 domains (Pointing and Parker, 1996; Perisic et al., 1998). In contrast, CBR1 is the most variable in both length and character often containing an α-helical turn (Nalefski and Falke, 1996; Pointing and Parker, 1996).

d) C2 domain-lipid interactions

The ability of the C2 domain to bind lipid and participate in intramolecular interactions has also been well characterized. Although calcium-binding C2 domains interact with lipid membranes (Keranen and Newton, 1997; Orr and Newton, 1992a,b; Quest and Bell, 1994), binding sites for lipid molecules have not been determined. Mutagenesis of the C2 domain from PKCα identified two pairs of residues directly involved in C2 domain-mediated membrane association (Medkova and Cho, 1998). Two arginine residues in loop 3 of the C2

domain were shown to initiate electrostatic membrane interactions. Subsequently, two adjacent tryptophans also in loop 3 participate in lipid membrane penetration and hydrophobic interactions (Medkova and Cho, 1998). As well, these paired residues displayed similar Ca^{2-}-dependencies for membrane binding (Medkova and Cho, 1998). Recent structural determination of the C2 domain of PKCα bound to phosphatidylserine strongly supports the observations made by Medkova and Cho (Verdaguer et al., 1999). Loop 1 and loop 3 of the C2 domain structure interact with calcium and it is the region between these loops that binds phosphatidylserine (Verdaguer et al., 1999). Superimposition of the C2 domain-PS complex onto a model lipid membrane illustrates that indeed CBR loop 3 penetrates into the lipid bilayer to mediate membrane docking (Verdaguer et al., 1999). Although loops 1 and 3 of Ca^{2-}-independent PKCs do not bind Ca^{2-}, they possess a phosphorylation site in loop 1, supporting an alternate mechanism of lipid interaction. Indeed, differences between C2 domain types have led to the suggestion that there are diverse sub-classes of C2 domains that have evolved equally diverse lipid binding mechanisms (Pappa et al., 1998; Sossin et al., 1996b).

e) C2 domain-protein interactions

Two types of C2 domain interactions with proteins have been demonstrated: intramolecular interactions with PKC itself and interactions with receptors for activated C kinase (RACKs) (Edwards and Newton, 1997; Ron et al., 1994). The C2 domain of $PKC\beta_{II}$ was proposed to interact with the carboxyl-terminal region of the kinase (Keranen and Newton, 1997). Alternatively spliced PKCβ enzymes that varied only in the 26 C-terminal residues displayed different Ca^{2-} requirements for activation and lipid binding (Keranen and Newton, 1997).

Newton and colleagues went on to show that phosphorylation of serine 660 (the hydrophobic site) in PKCβ_{II} caused a ten-fold increase in the enzyme's binding affinity for calcium and phosphatidylserine, functions known to be mediated by the C2 domain (Edwards and Newton, 1997).

Intracellular compartmentalization of various PKC isoforms may be due to the presence of intracellular PKC "receptors" in the cytosol and on the membrane. These proteins have been termed receptors for activated C kinases (Mochly-Rosen et al., 1991). RACKs are proteins isolated from rat brain based upon their interaction and binding of PKCs (Ron et al., 1994). This binding is dependent on the structural conformation of PKC and appears to require PKC activation (Ron et al., 1994). Furthermore, the binding sequences for RACKs on PKC fall within the C2 domain (Ron et al., 1994). RACKs themselves are believed to target activated PKCs to various cellular compartments, since peptides directed to the RACK binding site on PKC inhibit PKCβ translocation in cells (Ron et al., 1995). A recently identified PKCε selective RACK is the COPI (coat protein I) coatomer protein, β-COP (Csukai et al., 1997). β-COP is one of two non-clathrin coats, COPI and COPII, that drive the formation of vesicles mediating transport between the endoplasmic reticulum and the Golgi apparatus, and through the compartments of the Golgi (Salama and Schekman, 1995). Similar to RACK1, β-COP contains seven repeats of the WD40 motif and fulfills the criteria previously established for RACK proteins. Activated PKCε was shown to colocalize with β-COP in cells (Csukai et al., 1997). As well, the binding of PKCε to Golgi membranes was β-COP dependent (Csukai et al., 1997). A role for PKC in control of secretion has been previously

suggested, but this data describes a novel direct protein-protein interaction of PKCε with a protein involved in vesicular trafficking.

Based on these findings the RACK binding site on PKCε was identified in loop 1 of the C2 domain (Johnson et al., 1996). Peptides derived from this binding site, bind to the endogenous RACK, and inhibit PKCε-RACK binding (Johnson et al., 1996; Gray et al., 1997; Yedovitzky et al., 1997). It was later shown that another C2 domain peptide increased the isoform-specific membrane translocation of PKCε (Dorn et al., 1999). The peptide is derived from a conserved sequence around the C2 domain loop 3 similar to the β-COP protein sequence itself, hence it was termed the "pseudo-εRACK site". This site is believed to interact with the RACK binding site in loop 1 to inhibit access of PKCε to RACKS (Dorn et al., 1999). Accordingly, a mechanistic model for the action of this PKCε activating peptide necessarily predicts intra-C2 domain interactions (Dorn et al., 1999).

v) Significance of C1-C2 domain interactions

It has been proposed that C2 domain regulation may be due to C2 and C1 domain interactions for Ca^{2+}-dependent PKCs (Luo and Weinstein, 1993). This interaction may be inhibitory since fusion proteins containing C1 and C2 domains have a lower affinity for phorbol esters than do fusion proteins containing the C1 domain alone (Quest and Bell, 1994).

There is also evidence from studies with the cPKCγ that the C1 and C2 domains sequentially recruit the kinase to the membrane. These studies measured the *in vivo* translocation of green

fluorescent protein (GFP) tagged C1 and C2 domains and PKCγ (Oancea and Meyer, 1998). Repetitive calcium signals, acting through the C2 domain, caused the repetitive translocation of PKCγ to the membrane. When these calcium transients were paired with diacylglycerol signals the C1 domain was also recruited, causing the persistent translocation of PKCγ (Oancea and Meyer, 1998). Based on these results Meyer and colleagues suggested a model in which cPKCs transduce temporal calcium signals. These findings are supported by data using C1 and C2 domains from PKCα and measuring their lipid binding and membrane penetration (Medkova and Cho, 1999). The C2 domain was found to be involved in the initial calcium and PS-dependent electrostatic membrane interaction, while the C1 domain binds to diacylglycerol and penetrates the lipid membrane once the C2 domain is recruited (Medkova and Cho, 1999).

IV. Mechanisms of PKC Activation

A) Process of Kinase Maturation

The post-translational modifications and processing of PKC is relatively complex and not yet completely understood. The current understanding of PKC maturation is that newly translated PKC polypeptides reside in the cytosol. Before PKC can be allosterically regulated, the enzyme must be processed by three sequential "maturation" phosphorylations. At this stage, the pseudosubstrate sequence does not yet occupy the catalytic substrate pocket (Dutil and Newton, 2000). The first phosphorylation is the modification of the activation loop, a segment near the active site (threonine 500 in vertebrate PKCβ_{II}), by the phosphoinositide-dependent protein kinase-1 (PDK-1) (Keranen et al., 1995; Le Good et al., 1998). This phosphorylation is believed to correctly align the residues in the catalytic pocket

for catalysis and initiates the autophosphorylation of two conserved C-terminal residues. The first of these is the turn motif (threonine 641 in PKCβ_{II}), and the second is the hydrophobic site (serine 660 in PKCβ_{II}) that is thought to release the catalytically competent, "mature" PKC to the cytoplasm where it remains until activated by signaling mechanisms (Behn-Krappa and Newton, 1999). PKC that does not undergo these modifications is trapped on the cell membrane, preventing its future activation (Behn-Krappa and Newton, 1999).

B) Lipid Requirements

Lipids play a fundamental role in the activation of PKC. The lipid diacylglycerol is accepted to be the primary activator of protein kinase C activity (Newton, 1995a,b) and the anionic phospholipid phosphatidylserine is an essential cofactor for PKC activity (Hannun et al., 1985; Newton and Koshland, 1989; Orr and Newton, 1992a). However, the absolute requirement of phosphatidylserine for kinase activity suggests that the lipid is also an important PKC activator. The various roles that phosphatidylserine, diacylglycerol, and other phosphoglycerides play in PKC regulation has formed the basis of many studies.

Early studies of the stoichiometry of PKC-phospholipid interactions led Bell and colleagues to develop a detergent-lipid mixed micellar assay for PKC activity that allows for the control of micelle lipid molecular content (Hannun et al., 1985). Using this assay they demonstrated that at saturating calcium concentrations there was a strong dependence on the micelle phosphatidylserine content for diacylglycerol stimulation of PKC from rat brain (Hannun et al., 1985). This dependence was such that at moderate phosphatidylserine concentrations (10 mole %), only one molecule of diacylglycerol was necessary to stimulate maximal PKC

activity. They concluded that PKC was maximally activated by a single molecule of diacylglycerol in the presence of calcium and phosphatidylserine (Hannun et al., 1985).

PKC also displays significant cooperativity in phosphatidylserine binding (Lee and Bell, 1989). This cooperativity of the PKC-phosphatidylserine interaction has been observed by several groups and based on hill coefficients PKC is thought to interact with 8-12 molecules of phosphatidylserine (Lee and Bell, 1989; Newton and Koshland, 1989; Orr and Newton; 1992a). In other words, the binding of several molecules of phosphatidylserine is required to fully activate PKC. These results were supported by similar studies looking at purified PKC activation and phorbol ester binding by detergent-lipid micelles (Newton and Koshland, 1989; Lee and Bell, 1989).

Lee and Bell later demonstrated PS can be replaced by other lipids in some, but not all of the lipid binding sites (Lee and Bell, 1992). Negatively charged lipids, including phosphatidic acid (PA), have been shown to reduce the PS required for maximal kinase activity (Lee and Bell, 1992). There is, however, an apparent distinction between lipid binding to PKC and kinase activation. PKC shows no selectivity for lipid headgroup structure and will bind to monoanionic lipids with approximately equal affinities (Newton and Keranen, 1994). However, the presence of the lipid activator DAG increases the lipid affinity and specificity of PKC for PS by two orders of magnitude (Newton and Keranen, 1994).

The phosphatidylserine dependence of PKC activity was also demonstrated by examining the rate of PKC autophosphorylation and histone substrate phosphorylation (Newton and

Koshland, 1989). The rate of both types of phosphorylation was regulated by the micellar phosphatidylserine content (Newton and Koshland, 1989). Studies of the PS dependence of autophosphorylation showed a similar high degree of cooperativity to that observed with histone substrates (Newton and Koshland, 1990; Hannun and Bell, 1990). There is, however, a differential effect of phosphatidylserine concentrations on PKC substrate phosphorylation and autophosphorylation (Newton and Koshland, 1990). Newton and Koshland found that at higher PS concentrations the enzyme favors substrate phosphorylation and at intermediate PS concentrations PKC preferentially autophosphorylates. In contrast to the differential regulation by PS, DAG is equally effective for substrate and autophosphorylation. These results led Newton and Koshland to suggest that both DAG and PS regulate PKC activity in the membrane (Newton and Koshland, 1990; Hannun and Bell, 1990).

Assays using proteolysis as a measure of kinase conformation found that tryptic cleavage of PKC was also dependent on PS (Newton and Koshland, 1989). Orr and Newton later reported a cooperative effect of the PS dependence of membrane binding as measured by proteolysis (Orr and Newton, 1992a). These results led to model of PKC activation that results in a conformational change of the enzyme to expose multiple phosphatidylserine binding sites (Newton and Koshland, 1989). These sites would account for the high degree of cooperativity and the specific interaction of PKC and PS. Overall the evidence identifies PS as the physiological phospholipid required for PKC activation. In conjunction with DAG, this lipid is necessary to bind at several sites on both the C1 and C2 domains (in conventional PKCs) to maximally activate the enzyme

C) Activation by Signaling Pathways

The activity of PKC is reversibly regulated by its autoinhibitory pseudosubstrate sequence, which blocks the active site of the enzyme in the absence of activators. Once the enzyme has been fully processed by the three "maturation" phosphorylations, PKC activity can then be allosterically regulated. The sustained production of diacylglycerol is necessary for the activation of PKC. Therefore, lipid signaling pathways that produce DAG either as the by-product or end-product of activation, tend to activate PKC.

PKC activation by the phospholipase C family of enzymes is the predominant pathway of PKC activation in the cell (Nishizuka, 1992; Tanaka and Nishizuka, 1994). PLC hydrolyses the phosphoinositide PI-4, 5-bisphosphate (PIP_2) to produce DAG and inositol triphosphate (IP_3) (Berridge, 1984; Nishizuka, 1984). Together these second messengers are sufficient to activate Ca^{2+}-dependent PKC isoforms. This rapid DAG production is only transient, however, and is followed by a slower, persistent phase of DAG production (Pfeffer et al., 1991; Fukami and Takenawa, 1989). This persistent phase of DAG may produce the prolonged activation PKC by sustaining its association with the lipid membrane. As well, this persistent phase of DAG production is thought to be produced from phosphatidylcholine hydrolysis, since the fatty acid composition of this DAG matches that of PC (Holbrook et al., 1992; Qian and Drewes, 1989). The persistent phase of DAG production may be generated by growth factor stimulation of cell receptors (Exton, 1990; Cockcroft, 1992) both of which correlate with PKC activation (Nishizuka, 1995; Olson and Lambeth, 1996). This long-lasting phase of DAG can be produced in the absence of the transient phase of DAG (Exton,

1990; Cockcroft, 1992; Liscovitch, 1992), suggesting that independent mechanisms may underlie rapid and sustained PKC activity.

Alternatively, one of the best-characterized phosphatidylcholine hydrolyzing enzymes is the PC-specific phospholipase D. Many studies have shown that PLD hydrolysis of PC forms phosphatidic acid and choline and has been linked to PKC activation (Cockcroft, 1996; Balsinde et al., 1988; Pai et al., 1988). Phosphatidic acid is then converted to DAG by phosphatidic acid phosphohydrolase (PAP) (Billah et al., 1989). This DAG is not rapidly degraded and as a result prolongs PKC activation (Billah et al., 1989). Subcellular fractionation experiments of whole rat brains demonstrated that the highest specific PLD activity was in synaptic membranes (Kobayashi and Kanfer, 1987), with regional distribution concentrated in the hippocampus and hypothalamus (Kobayashi et al., 1988). Brain-specific PLD preferentially hydrolyses phosphatidylcholine (Horwitz and Davis, 1993), suggesting there may be higher concentrations of persistent DAG levels, and hence PKC activity, in the brain.

D) Persistent Activation of PKC

A probable molecular event underlying the persistence of neuronal changes after an initial stimulus is the lasting phosphorylation of PKC substrates. Accordingly, a requirement in the cascade of events leading to the consolidation of plastic changes would be the persistent activation of PKC. Several mechanisms for the production of persistently active forms of PKC were found in studies of LTP in vertebrates (Malinow et al., 1988; Klann et al., 1991; Sacktor et al., 1993) and facilitation in *Aplysia* (Sossin et al., 1994; Sossin, 1997). The

constitutively active PKMζ produced by proteolytic cleavage of the PKCζ regulatory domain (Suzuki et al., 1992; Sacktor et al., 1993; Hrabetova and Sacktor, 1996), activation of PKC by oxidation (Palumbo et al., 1992; Klann et al., 1998), phosphorylation dependent PKC activation (Klann et al., 1993; Bank et al., 1989; Sweatt et al., 1998), and rapid temporal calcium spikes (Oancea and Meyer, 1998) are all mechanisms of generating persistent PKC activity.

i) Proteolytic cleavage of the regulatory domain

Persistent PKC activity is believed to play a role in the maintenance of LTP. PKC can be converted into a second messenger-independent, constitutively active form, protein kinase M (PKM), by limited proteolysis at the hinge region of the enzyme, separating the regulatory from the catalytic domain (Takai et al., 1977). In the brain, only PKM of the atypical isozyme PKM has been observed consistently (Sacktor et al., 1993). Some of the evidence for this idea comes from the finding that the constitutively active PKMζ is increased during LTP maintenance (Sacktor et al., 1993; Osten et al., 1996). This increase lasts at least 2 hours in the hippocampal slice and linearly correlates with the degree of EPSP potentiation (Osten et al., 1996). As well, they examined the bidirectional regulation of PKMζ in a single synaptic pathway. PKMζ levels were analyzed by immunoblot of hippocampal brain slices in which LTD induction was followed by LTP or test stimulation. They found that the level of PKMζ in slices receiving LTP (100 Hz trains) was higher than in control slices receiving test stimulation (3 Hz) (Hrabetova and Sacktor, 1996). The selective inhibition of regulated PKC activity blocks LTP induction (Malinow et al., 1988,1989; Wang and Feng, 1992). In contrast, selective inhibition of the PKC kinase domain by chelerytherine blocks the

maintenance of LTP (Hrabetova and Sacktor, 1996). These findings suggest that constitutively active, non-regulated, PKC activity modulates LTP maintenance (Hrabetova and Sacktor, 1996). These studies led Sacktor and colleagues to postulate that persistent PKC activity, perhaps PKMζ, underlies the maintenance of synaptic strength in quiescent and potentiated synapses.

ii) Oxidation induced activity

An additional mechanism implicated in prolonged PKC activation is through oxidation by the superoxide anion (O_2^-). It has been shown that NMDA receptor activation during LTP stimulates superoxide production (Bindokus et al., 1996). In cells, superoxide has been shown to stimulate autonomous and regulated PKC activity (Klann et al., 1993). The mechanism by which this is thought to occur is via oxidation of the 6 core cystines in the PKC C1 activator-binding domain. These cystines have been shown to be critical for the direct hydrogen-bonding interaction with the PKC activators DAG and phorbol esters (Kazanietz et al., 1995; Zhang et al., 1995). Oxidation of the C1 domain cystines would competitively inhibit subsequent activator (DAG or phorbol ester) binding and regulation of PKC activity. Sweatt and colleagues recently provided evidence linking superoxide to PKC. They found that the application of the superoxide scavenger, superoxide dismutase (SOD), to hippocampal slices blocked induction of hippocampal LTP (Klann et al., 1998). As well, SOD blocked the increase in regulated PKC activity associated with LTP in this paradigm by catalyzing the removal of superoxide (Klann et al., 1998). These results confirm a role for the superoxide anion in mediating PKC activation during induction of LTP.

iii) Phosphorylation dependent modification

Autophosphorylation has been shown to modulate translocation and reverse translocation of PKC to the plasma membrane, a potential mechanism for persistent activation of PKC. Autophosphorylation at a conserved site in the PKC carboxyl-terminal domain has also been associated with persistent activation of PKC. The expression of LTP is believed to be associated with PKC activity and autophosphorylation (Klann et al., 1993). It was recently demonstrated that NMDA receptor activation is coupled to an increase in PKC phosphorylation during LTP maintenance (Sweatt, et al., 1998). The *in vitro* dephosphorylation of this phosphorylated site on PKC is dependent upon calcium and the PKC activators DAG and PS (Klann et al., 1998). These findings led Sweatt and colleagues to suggest that persistent PKC activation during LTP is regulated by conformation dependent autophosphorylation. Recently, Hannun and coworkers provided evidence for the dephosphorylation dependent persistent membrane association of PKC by imaging the trafficking of wild-type and mutant PKCβII in live cells. They demonstrated that after cell-surface receptor activation, the C1 domain is required but insufficient to recruit the kinase to the plasma membrane (Feng et al., 2000). Moreover, the inability of a kinase-dead PKC to be removed from the membrane was restored by the addition of intracellular calcium chelators, suggesting a role for the C2 region in the persistent phase of translocation (Feng et al., 2000). In contrast, the inability of a C2 domain deletion mutant to translocate was reversed in kinase-dead mutants and by the S660A autophosphorylation site mutation, suggesting that autophosphorylation of this site opposes the action of the C2 domain (Feng et al., 2000). Taken together, these findings indicate that a decrease in PKC

autophosphorylation or an increase in PKC dephosphorylation at serine 660 might produce persistent PKC membrane localization.

iv) Temporal calcium flux mediated activation

A fourth mechanism generating the persistent activation of PKC although categorically different, is that of rapid temporal calcium spikes (Oancea and Meyer, 1998). Each of the previous mechanisms involves direct alteration of protein kinase C itself. This mechanism is noteworthy because it generates prolonged kinase activity solely based upon temporal relationships of the signals converging on PKC. The frequency of calcium spikes or calcium oscillations can be of critical importance for the induction of selective cellular functions. Calcium signals often appear as repetitive calcium spikes that can be induced by electrical or receptor stimuli. These patterns of Ca^{2-} signaling result from the limited range of cytoplasmic Ca^{2-} diffusion and the feedback regulation of pathways responsible for Ca^{2-} mobilization (Thomas et al., 1996). In addition, the spatial organization of $[Ca^{2-}]_i$ changes appears to depend on the strategic distribution of Ca2+ stores within the cell (Thomas et al., 1996). Repetitive calcium spikes typically increase their frequency with the amplitude of the receptor stimuli. In contrast, receptor-induced diacylglycerol increases are typically prolonged and often biphasic and have a slower rate of production (Quest, 1996; Oancea et al., 1998). These studies measured the *in vivo* translocation of GFP tagged PKCγ (Oancea and Meyer, 1998). Meyer and colleagues demonstrated that repetitive calcium transients that were paired with diacylglycerol signals caused the translocation of PKCγ to persist (Oancea and Meyer, 1998). Low-frequency calcium transients do not produce persistent PKC activity because they do not retain PKCγ on the membrane long enough for DAG to stimulate

prolonged kinase activation. However, higher frequency stimulation by calcium causes the retention of PKC on the membrane by DAG after the cessation of the calcium spikes (Oancea and Meyer, 1998). Therefore, the frequency of calcium transients and their temporal relationship may regulate the timing and duration of PKC activity.

In *Aplysia*, behavioral sensitization also induces autonomous activity of PKC Apl II (Sossin, 1997). This activation is specific, as PKC Apl I does not become autonomous under these conditions. The autonomous kinase activity in this system probably results from post-translational modifications in the regulatory domain of PKC Apl II (Sossin, 1997).

E) Plasticity Dependent PKC Substrates

PKC activity plays an important regulatory role in neuronal properties and connections. Therefore, modulation of these neuronal attributes is a function of PKCs various substrates. Changes in PKC activity can elicit changes in synaptic connections by altering synapse morphology and number (Corfas and Dudai, 1991; Schacher et al., 1993), ionic channel conductances and excitability (Kandel and Schwartz, 1982; Miller et al., 1992; Wang et al., 1994b), or synaptic transmission (Kandel and Schwartz, 1982; Malinow et al, 1988; Schumann and Clark 1994). Substrate phosphorylation and regulation is itself dependent on the proximity to PKC and either occurs by direct kinase-substrate interactions or interactions mediated by other associated proteins (Nishizuka, 1995). Several PKC substrates have been shown to be involved in synaptic plasticity by modulating one or more synaptic changes, these include: i) the growth-associated protein-43 (GAP-43/neuromodulin), ii)

RC3/neurogranin, iii) the myristoylated alanine-rich C kinase substrate (MARCKS), iv) the NR1 subunit of the NMDA receptor, and v) the endocytic GTPase dynamin.

i) Growth-associated protein-43 (GAP-43/neuromodulin)

GAP-43 is a 45 kDa nervous tissue-specific protein that is highly expressed in neurons during development and nerve regeneration. GAP-43 has been implicated in neurite outgrowth, long-term potentiation, neuronal signal transduction and neurotransmitter release (Gispen et al., 1991; Liu and Storm, 1990; Pasinelli et al., 1995). GAP-43 expression levels are highest in human associative brain areas and rat hippocampal and olfactory areas. In adult neurons, GAP-43 is localized to presynaptic plasma membranes and excluded from dendrites and postsynaptic structures (Gispen et al., 1991).

In vitro, GAP-43 binds calmodulin (Alexander et al., 1988; De Graan et al., 1990a). GAP-43 has an inherently higher affinity for calmodulin in the absence of calcium than in its presence and as such has been classified is an atypical calmodulin binding protein (Baudier et al., 1989; Coggins et al., 1991). *In vivo*, PKC phosphorylates GAP-43 at serine 41 that resides within the putative calmodulin-binding domain (residues 39-51). This phosphorylation is reversible and serine 41 can be dephosphorylated by protein phosphatase (Hens et al., 1995). Calmodulin binding to GAP-43 appears to be negatively regulated by PKC phosphorylation, since phosphorylation of serine 41 reduces its calmodulin affinity (De Graan et al., 1990a; Hens et al., 1995).

It was demonstrated in permeablized rat brain synaptosomes that a GAP-43 antibody, known to interfere with GAP-43 phosphorylation, inhibited calcium induced release of noradrenaline (Dekker et al., 1989a; Hens et al., 1993a). As well, phospho-specific antibodies to the PKC phosphorylation site of GAP-43 also blocked calcium mediated noradrenaline release in permeablized synaptosomes demonstrating that the GAP-43 N-terminal domain appears to play an important role in release (Hens et al., 1993b, 1995). These experiments, as well as GAP-43 presynaptic localization, led to the idea that GAP-43 at the cell membrane regulates the local calmodulin pool and may be important for calcium induced synaptic vesicle release (Dekker et al., 1989b; Ramakers et al., 1995). Changes in the phosphorylation of GAP-43 modulate the available pool of calmodulin and following calcium entry, calmodulin would dissociate from GAP-43. Unbound calmodulin could then activate molecules such as CaMKII that may recruit synaptic vesicles for release (Greengard et al., 1993).

Phosphorylation of GAP-43 was found to occur during paradigms of synaptic plasticity. Induction of LTP in hippocampal slices was found to cause elevated phosphorylation of GAP-43 within 1 hour after induction (Gianotti et al., 1992; De Graan et al., 1990b; Ramakers et al., 1995). Additionally, it has been shown using monoclonal antibodies to the putative PKC phosphorylation site that GAP-43 phosphorylation increases 10 min after LTP.

ii) RC3/neurogranin

Neurogranin is also a neuron-specific substrate of PKC that is expressed in developing postnatal and adult neurons (Represa et al., 1990; Watson et al., 1990). This postsynaptic

protein was isolated from bovine brain and has an apparent molecular weight of 8 kDa. In contrast to the presynaptic localization of GAP-43, neurogranin is found postsynaptically and is localized in dendritic spines (Watson et al., 1992, 1994; Iniguez et al., 1992). Like its presynaptic partner, GAP-43, neurogranin binds calmodulin at low calcium concentrations (Baudier et al., 1989; Huang et al., 1993) and phosphorylation by PKC inhibits its binding to calmodulin (Huang et al., 1993). In the absence of calcium, the structure of neurogranin and GAP-43 is stabilized by calmodulin binding (Gerendasy et al., 1995). As well, PKC mediated phosphorylation of neurogranin expressed in Xenopus oocytes, increases the mobilization of intracellular calcium (Cohen et al., 1993).

Phosphorylation of neurogranin was also found to occur during synaptic plasticity. Induction of LTP in hippocampal slices was found to quantitatively increase the phosphorylation of neurogranin by immunoprecipitation (De Graan et al., 1989; Ramakers et al., 1995). Increased phosphorylation of neurogranin was observed 60 minutes after LTP induction and this was blocked by 2-amino-5-phosphonopentanoic acid (AP5), an NMDA-receptor antagonist (Ramakers et al., 1995). As well, induction of LTP in the CA1 region of the hippocampus was blocked by PKC phosphorylation site specific antibodies (Fedorov et al., 1995).

The increase in the phosphorylation of both GAP-43 and neurogranin during LTP, suggests a physiological role of both proteins in this form of synaptic plasticity. Induction of LTP is accompanied by a remodeling of synaptic contacts that could reflect changes in GAP-43 and

neurogranin phosphorylation (Desmond and Levy, 1990; Geinisman et al., 1991; Greenough and Bailey, 1988; Kandel and O'Dell, 1992).

iii) The myristoylated alanine-rich C kinase substrate (MARCKS)

MARCKS is an 87 kilodalton protein which has been implicated to play roles in secretion, cell motility, and plasticity (Aderem, 1992). MARCKS was first isolated by purification from bovine brain (Albert et al., 1987) and has been shown to bind to calmodulin or actin in the presence or absence of Ca^{2+} ions, respectively (Graff et al., 1989; Hartwig et al., 1992). Both of these two binding interactions are reversably suppressed by PKC phosphorylation (Graff et al., 1989; Hartwig et al., 1992) resulting in the translocation of MARCKS from the plasma membrane to the cytosolic compartment (Wang et al., 1989; Thelen et al., 1991). Recently, both MARCKS and a peptide corresponding to its basic effector domain, MARCKS (residues 151-175), inhibit phosphoinositide-specific PLC hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP_2) in vesicles (Glaser et al., 1996). McLaughlin and coworkers went on to demonstrate that addition of the MARCKS peptide to either PLCδ or PLCβ inhibited hydrolysis of PIP_2 and this inhibition was due to the strong binding of the peptide to PIP_2 (Wang et al., 2000). Electrophoretic mobility measurements and competition experiments suggest that the MARCKS peptide forms an electroneutral complex with approximately 4 PIP_2 molecules (Wang et al., 2000). This suggests that the effector domain of MARCKS can bind a significant fraction of plasma membrane PIP_2, and release the bound PIP_2 upon interaction with Ca^{2+}/calmodulin or phosphorylation by protein kinase C. These membrane dissociative interactions are sufficient to explain why PKC phosphorylation releases MARCKS to the cytosol.

iv) NMDA receptors

NMDA receptors are glutamate-gated ion channels that have important functions in synaptic development and CNS plasticity. NMDA receptors are believed to be heteromeric complexes of three different types of channel subunits (Sheng et al., 1994; Yamakura and Shimoji, 1999). It has been demonstrated that PKC activation increases NMDA receptor evoked currents in hippocampal neurons in culture (Xiong et al., 1998) as well as isolated trigeminal neurons (Chen and Huang, 1992). PKC activity also mediates an increase in NMDA currents from channels expressed in Xenopus oocytes (Kelso et al., 1992; Zukin and Bennett, 1995). One of the receptor subunits, NR1, is known to be directly phosphorylated by PKC (Tingley et al., 1993) and is associated with several molecular modifications. First, phosphorylation of the NR1 subunit by PKC has been shown to cause the redistribution of membrane localized NMDA receptors to the cytosol (Ehlers et al., 1995, 1996). Second, PKC phosphorylation of the NR1 subunit inhibits the binding of the cytoskeletal protein spectrin to the C-terminal of the NR1 subunit (Wechsler and Teichberg, 1998). Most recently, it has been shown that PKC activation in hippocampal neurons enhances Ca^{2+}-dependent inactivation of NMDA receptors that can be reversed by the PKC inhibitor chelerytherine (Lu et al., 2000). The mechanism for this is as follows: the NR1 subunit possesses two binding sites for CaM, a high-affinity site in the C1 cassette and a low-affinity site in the C0 region (Ehlers et al., 1996). This Ca^{2+}-dependent inactivation of the NMDA receptor results from an inhibition of channel gating by the binding of Ca^{2+}/calmodulin (CaM) to the carboxyl tail of the NR1 subunit at the low-affinity (C0 cassette) CaM site (Zhang et al., 1998; Krupp et al., 1999). The role of PKC is to phosphorylate sites in the C1 cassette, displacing CaM binding at this high-affinity site, and allowing it to bind to the C0

site, thereby enhancing channel inactivation (Lu et al., 2000). In this way the NR1 subunit functions as a CaM-binding protein, mimicking proteins such as neurogranin (RC3), GAP-43 (neuromodulin), and MARCKS whose binding of CaM is also inhibited by PKC phosphorylation.

v) Dynamin

Dynamin I is a GTPase enzyme required for the retrieval of synaptic vesicle membranes after exocytosis. Its function in endocytosis after assembly as a helix around the neck of invaginating synaptic vesicles (Koenig and Ikeda, 1989; Stowell et al., 1999). Dynamin can self-assemble as a series of rings in the absence of guanine nucleotide (Hinshaw and Schmid, 1995). These helices form around phospholipid vesicles *in vitro* (Sweitzer and Hinshaw, 1998) or around the neck of invaginating synaptic vesicles (Takei et al., 1995). The helices behave like a spring, with GTP hydrolysis producing an increase in the helix pitch, suggesting that endocytosis might occur by a nucleotide-dependent conformational change in dynamin cleaving vesicles from the plasma membrane (Stowell et al., 1999). Dynamin is also a phosphoprotein found in intact nerve terminals where it is apparently phosphorylated by PKC (Robinson, 1992). It is rapidly dephosphorylated by the phosphatase calcineurin on stimulation of endocytosis by depolarization and calcium influx (Liu et al., 1994) and blocking dephosphorylation prevents endocytosis in nerve terminals (Marks and McMahon, 1998). Dynamin remains dephosphorylated during endocytosis of most vesicles and is rephosphorylated while endocytosis is completing (Robinson et al., 1994). Therefore the phosphorylation of dynamin is not likely to play a role during endocytosis but is probably a priming step prior to endocytosis. One recent study exploring the role of PKC

phosphorylation of dynamin has found that it binds membrane phospholipids in a preferential ratio of PS:PC of 1:3 (Powell et al., 2000). Phospholipid binding was abolished after dynamin I phosphorylation by PKC on serine 795 and was restored after dephosphorylation by calcineurin (Powell et al., 2000). Suggesting that phospho-S795 dynamin can not bind lipids perhaps explaining why the minor pool of phospho-dynamin I that mediates synaptic vesicle retrieval in nerve terminals is localized to the cytosol.

SUMMARY

In the nervous system, PKCs play important roles in synaptic plasticity and learning. Activation of PKC is both necessary and sufficient to produce long-lasting changes in neuronal excitability, morphology, and connectivity. Modulation of neuronal substrates by PKC can elicit changes in synaptic connections by altering synapse morphology, ionic channel conductance, CaM dependent protein kinase activity, and transmitter release. Further, molecular modifications of PKC itself give rise to different properties of the enzyme, such at persistent activity, known to be important during plasticity. Despite the well-characterized mechanism of conventional Ca^{2+}-dependent PKC regulation, a detailed analysis of Ca^{2+}-independent PKC regulation has not been presented. Furthermore, there is little experimental evidence to suggest whether protein domains of Ca^{2+}-independent PKC isoforms function in a similar fashion to those of Ca^{2+}-dependent PKCs. Understanding the role of the C1 and C2 domains comprising the kinase regulatory region of Ca^{2+}-independent PKCs may help to unravel the mechanisms of their regulation in the nervous system.

HYPOTHESIS

The C2 domain of Aplysia PKC Apl II regulates PKC activity in that it can i) prevent the binding of diacylglycerol or analogous activators to the C1 domain, and ii) hinder membrane translocation by its lack of phospholipid binding. Furthermore, the C2 domain of PKC Apl II can be phosphorylated by PKC, and this phosphorylation can modulate the inhibitory effects of the C2 domain.

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PREFACE TO CHAPTER 2

In the nervous system of the marine mollusk *Aplysia californica*, PKCs play important roles in synaptic plasticity and learning. The Ca^{2+}-dependent PKC Apl I but not the Ca^{2+}-independent PKC Apl II is activated by the facilitating neurotransmitter serotonin in sensory neurons. In contrast, the Ca^{2+}-independent PKC Apl II is activated by a insulin-responsive receptor tyrosine kinase in the bag cell neurons of the animal. Unlike the well characterized mechanism of conventional PKC activation, a detailed analysis of Ca^{2+}-independent PKC activation has not been presented. Previous studies using purified *Aplysia* PKC Apl II and the mutant PKC Apl IIΔC2 (lacking the C2 domain) demonstrated that removal of the C2 domain reduced the amount of the phosphatidylserine cofactor necessary for PKC Apl II activation (Sossin et al., 1996). This data led to the formulation of a model whereby the C2 domain acts to negatively regulate PKC Apl II activation by interacting with the C1 domain (Sossin et al., 1996). This model proposes that these C1-C2 domain interactions restrict diacylglycerol binding to the C1 domain thus, preventing maximal activation of *Aplysia* PKC Apl II. As well, at low concentrations of PS, the C2 domain is folded over inhibiting DAG access to the C1 domain, and kinase activity remains low. At higher PS concentrations, however, C2 domain mediated inhibition is removed and the C1 domain binds to PS and DAG, fully activating the kinase. This model makes three predictions: i) that the C1 domain has an inherently high affinity for DAG, ii) that at low phosphatidylserine concentrations the C1 domain has a low affinity for DAG in the presence of the C2 domain, and iii) that high PS concentrations remove C2 domain mediated inhibition and the C1 domain now has a higher affinity for DAG.

To test this model directly we generated fusion proteins of the C1 and C2 domains of PKC Apl II and measured their phosphatidylserine dependence for phorbol ester, the pharmacological analog of DAG, binding. Our results were remarkably consistent with the predictions of the model. Fusion proteins containing only the C1 domain alone had a high affinity for phorbol ester. Proteins containing both the C1 and C2 domains had a variable affinity for phorbol ester that was highly dependent on phosphatidylserine levels. The affinity of these C1-C2 domain proteins for phorbol ester was significantly reduced at lower phosphatidylserine concentrations and when PS concentrations were elevated this could increase the affinity dramatically. Although we found the affinity for phorbol ester to be highly dependent on phosphatidylserine levels, we also found that phosphatidic acid (PA) is much more effective at increasing this phorbol ester affinity. Together, our results confirm the inhibitory model of C2 domain mediated inhibition of the C1 domain. This begins to explain differences in PKC Apl I and PKC Apl II activation in the nervous system of *Aplysia*.

CHAPTER 2

The C2 domain of the Ca^{2+}-independent PKC Apl II inhibits phorbol ester binding to the C1 domain in a phosphatidic acid sensitive manner

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I. ABSTRACT

There are two protein kinase Cs in the *Aplysia* nervous system, PKC Apl I, which is homologous to the Ca^{2+}-activated PKC family, and PKC Apl II, which is homologous to the Ca^{2+}-independent PKCs ε and η. Purified PKC Apl I requires much less phosphatidylserine for activation than does purified PKC Apl II, and this may explain why the neurotransmitter serotonin activates PKC Apl I, but not PKC Apl II in the intact nervous system (Sossin et al., 1996a). PKC Apl II's requirement for high levels of phosphatidylserine may be mediated by its C2 domain, since removing this domain allows PKC Apl II to be activated at lower concentrations of phosphatidylserine. To begin to understand how this inhibition is mediated, we generated fusion proteins containing the C1 and C2 domains from PKC Apl II and determined their lipid dependence for phorbol ester binding. Our results indicate that the presence of the C2 domain lowers the affinity of protein kinase C activators for the C1 domains and this inhibition can be removed by phosphatidylserine. Phosphatidic acid, however, is much more potent than phosphatidylserine in reducing C2 domain-mediated inhibition, suggesting that phosphatidic acid may be a required co-factor for the activation of PKC Apl II.

II. INTRODUCTION

The C1 and C2 domains are conserved amino acid modules first identified in the protein kinase C (PKC) family (Kikkawa et al., 1987; Parker et al., 1986). This family contains both Ca^{2+}-activated and Ca^{2+}-independent members, both of which are activated by the combination of diacylglycerol (DAG) and phosphatidylserine (PS). DAG, and its pharmacological analogs phorbol esters, bind to the C1 domain while PS may act through both C1 and C2 domains (Newton, 1995a). In Ca^{2+}-activated PKCs, C2 is on the carboxyl-terminal side of C1 and confers Ca^{2+}-sensitivity to the enzyme through Ca^{2+}-dependent binding to PS (Brose et al., 1992; Newton, 1995a,b). In some Ca^{2+}-independent PKCs, typified by vertebrate PKCε and invertebrate PKC Apl II, C2 is on the amino-terminal side of C1 and lacks the critical aspartic acids required for Ca^{2+}-binding (Newton, 1995b; Sossin and Schwartz, 1993). It has been suggested that the C2 domain of Ca^{2+}-independent PKCs binds to PS constitutively in the absence of Ca^{2+} to help activate these kinases (Newton, 1995b). However, studies with the *Aplysia* isoform, PKC Apl II, suggest that C2 plays an inhibitory role, since removing this domain lowers the concentration of PS required to activate the kinase (Sossin et al., 1996a).

A common function for the C2 domain has not yet been elucidated despite the large number of identified C2 domains (Nalefski and Falke, 1996), and the considerable amount of structural information about these domains (Grobler et al., 1996; Sutton et al., 1995). Some C2 domains, like those of synaptotagmin, Ca^{2+}-activated PKCs, and phospholipase A_2, bind to lipid in a Ca^{2+}-dependent manner, suggesting that C2 domains help translocate proteins to

an appropriate lipid environment (Brose et al., 1992; Davletov and Sudhof, 1993; Nalefski et al., 1994). However, since not all C2 domains bind to Ca^{2+} ions and the lipid-binding sequence of C2 domains is not well defined, the lipid specificity of most C2 domains is not known. Several C2 domains are involved in protein-protein interactions, but no consensus sequence that these domains bind to has been defined (Mochly-Rosen et al., 1992; Verhage et al., 1997; Shao et al., 1997). Finally, several C2 domains bind to inositol polyphosphates, but only a few C2 domains contain the consensus sequence for inositol polyphosphate binding (Cullen et al., 1995; Fukuda et al., 1994). Elucidating the role of the C2 domain in Ca^{2+}-independent PKCs, like PKC Apl II, should help define the signal transduction pathway that regulates these enzymes.

In the *Aplysia* nervous system PKC Apl II is activated downstream of receptor tyrosine kinase (RTK) activation, but not by stimulation of G protein-activated phospholipase C (Sossin and Schwartz, 1992; Sossin et al., 1994; 1996b). Vertebrate PKCε is also persistently activated by signals acting through RTKs (Ohno et al., 1994; Olivier and Parker, 1994; Ha and Exton, 1993), suggesting that this regulation is due to domains conserved between the two proteins. This activation may be mediated by $PI_{3,4}P_2$ produced by RTK-activated PI-3 kinase (Palmer et al., 1995; Toker et al., 1994) or by DAG produced through RTK-activated phospholipase D (PLD) and phosphatidic acid phosphohydrolase (PAP) (Ha and Exton, 1993; Liscovitch et al., 1993).

In the present study, we constructed fusion proteins containing different domains of the PKC Apl II regulatory region and compared their ability to bind phorbol ester. Our results strongly

confirm the inhibitory model of C2 domain function, since fusion proteins that contain the C2 domain have an affinity for phorbol ester that is highly dependent on PS concentration, while fusion proteins which lack this region do not. Furthermore, phosphatidic acid (PA), the product of PLD, removes inhibition mediated by the C2 domain at concentrations far lower than those of PS, suggesting that Ca^{2+}-independent PKCs containing these C2 domains are stimulated by PA.

III. MATERIALS AND METHODS

Reagents: 4β-Phorbol 12, 13-dibutyrate (LC Services); [^{3}H]4β-phorbol 12, 13-dibutyrate (19.6 Ci/mmol) (New England Nuclear, Boston, MA); (Amersham, Oakville, ON), dioleoyl phosphatidylserine (PS), dioleoyl phosphatidylcholine (PC), distearyl phosphatidylglycerol (PG), dioleoyl phosphatidylethanolamine (PE) and dioleoyl phosphatidic acid (PA) (Avanti Polar Lipids Inc., Alabaster, AL); phosphatidylinositol (PI) (mostly linoleic and palmitic acid) (Sigma, St. Louis MO); Triton X-100 (Avanti); Sephacryl S-100 HR (Pharmacia, Uppsala, Sweden); prestained molecular weight markers (Amersham, Oakville, ON). All other reagents were of the highest grade available.

Construction of plasmids encoding fusion proteins. Apl II cDNA, or Apl IIΔC2 cDNA (Sossin et al., 1996a) was digested with *Sma I/Bgl* II, filled in with Klenow, isolated and ligated into the *Sma* I digested pGEX-5X-1 plasmid (Pharmacia LKB Biotechnologies Inc., Piscataway, NJ) to generate GST-C2-C1 and GST-ΔC2-C1 respectively. Apl II cDNA was digested with either *Xmn I/Bgl* II or *Hinc* II/*Bgl* II, filled in with Klenow, isolated and ligated into the pGEX-3X plasmid cut with *Eco*RI and filled in with Klenow to generate GST-P-C1 and GST-C1 respectively. Apl II cDNA was digested with *Sma/Xmn* and ligated into pGEX-3X cut with *Sma I* to generate GST-C2.

Expression of fusion proteins. DH5 *E. coli* with plasmids encoding the fusion proteins were grown in LB media supplemented with 1 μm $ZnSO_4$ at 27 °C to an A_{600} = 0.3 - 0.4. Isopropyl-1-thio-b-D-galactopyranoside was added to a final concentration of 100 μM to

induce protein expression and grown for an additional 18h. Cells were pelleted and resuspended in 50 ml of ice-cold 1 × PBS with 5 mM 2-mercaptoethanol, 20 μg/ml aprotinin, 5 mM benzamidine, and 0.1 mM leupeptin, sonicated 6 x 10 sec with a probe sonicator (Vibracell, Sonics and Materials, Danbury, CT), incubated in 1% Triton X-100 for 20 min and the debris pelleted by centrifugation in a Sorvall RCB2 centrifuge at 12,000 × g using a SS34 rotor at 4 °C. The supernatant was loaded onto 1 ml glutathione sepharose columns, washed with 20-30 volumes PBS, and eluted with reduced glutathione (100mM Tris, 20 mM Glutathione, pH 8.0, 120 mM NaCl). For each fusion protein preparation, gels were scanned and analysis performed using the public domain NIH Image program (developed at the U.S. National Institutes of Health and available on the Internet at http://rsb.info.nih.gov/nih-image/) to determine the percentage of purified fusion protein at the correct molecular weight (Fig. 1A) and these values were used to calculate the stoichiometries for fusion protein binding.

[^{3}H] PDBu-binding assay. Fusion proteins were assayed for [^{3}H]PDBu binding by the mixed micelle method (Hannun and Bell, 1987; Quest and Bell, 1994). The 50 μl reaction mixture consisted of 20 μM Tris, pH 7.5, 200 μM $CaCl_2$ or $MgCl_2$, mixed micelles (0-40 mol%) of phospholipids in a final concentration of 0.6% Triton X-100, [^{3}H]PDBu from 1 to 250 nM and fusion protein ranging from 0.02 to 1.6 μg. Mixed micelles were prepared by drying the appropriate volume of each lipid under nitrogen and resuspending in 3% Triton X-100, vortexing for 1 min followed by incubation at 30 °C for 10 min. Reactions were started by addition of fusion protein and were allowed to proceed for 10 min at room temperature. Reaction tubes were then placed on ice and bound PDBu was separated from free PDBu on

Sephacryl S-100 HR gel-filtration columns (2-ml column volume) at 4 °C by washing with equilibration buffer (20 μM Tris, pH 7.5, 0.015% Triton X-100, and 200 μM either $CaCl_2$ or $MgCl_2$). The nonspecific binding component for all experiments was measured in the presence of 10 μM unlabeled PDBu and was subtracted from the total binding to yield the specific binding. No differences were seen in experiments using 200 μM $CaCl_2$ or 200 μM $MgCl_2$.

Quantitation of data. The dependence of phorbol ester binding on lipid content of micelles was analyzed by a non-linear least squares fit to a modified Hill equation (Quest and Bell, 1994; Newton and Koshland, 1989) using Systat 5.0 where y is the PDBu binding value, a is the maximum PDBu binding value, x is the concentration of PS, k is the concentration of PS resulting in half-maximal binding and n is the Hill coefficient. $y=a[x^n/(k^n-x^n))$, K_d, and B_{max} values for the Scatchard analysis were calculated using the EBDA binding program (McPherson, 1983).

IV. RESULTS

Characterization of fusion proteins

To determine if the C2 domain of Ca^{2+}-independent PKCs affects phorbol ester binding to the C1 domain we constructed a number of glutathione-S-transferase (GST) fusion proteins (Fig. 1A) containing regulatory regions of PKC Apl II including the C2 domain alone (GST-C2), the C1 domain alone (GST-C1), the pseudosubstrate and the C1 domain (GST-P-C1), the entire regulatory domain (GST-C2-C1), and a deletion of the central core of the C2 domain (GST-C2Δcore-C1). Proteins of the correct molecular weight were observed for all constructs (Fig. 1A and B), along with several degradation products the size of GST-C2 (lane 2, 5) or GST (all lanes) (Fig. 1B). Since neither GST nor GST-C2 bound to PDBu (data not shown), these proteins should not interfere with the PDBu binding assays.

Dependence of PDBu binding on phosphatidylserine concentration

All experiments used the mixed micelle PDBu binding assay, since this assay allows for the measurement of PDBu binding over a wide range of lipid types and concentrations (Hannun and Bell, 1987). In this assay, bound ^{3}H-PDBu is separated from free ^{3}H-PDBu by gel filtration since micelles bound to protein and PDBu are larger than the micelles bound to PDBu alone. Non-specific association of protein and PDBu is determined in the presence of an excess of cold PDBu. This assay will only detect protein bound to PDBu that is also bound to micelles and thus will not detect PDBu binding that is independent of lipids (Kazanietz et al., 1995a). This assay has been used extensively to determine the lipid-dependence for phorbol ester binding of Ca^{2+}-activated PKCs and of GST regulatory domain

fusion proteins (Quest and Bell, 1994; Newton and Koshland, 1989; Lee and Bell, 1989; Quest et al., 1994a; Kazanietz et al., 1995b). Even at saturating concentrations of PS and PDBu, much less than one molecule of PDBu binds to each molecule of fusion protein (Tables 2.1 and 2.2) suggesting that most of the fusion protein is not correctly folded and thus cannot bind PDBu. Similar results are seen with all other PKC regulatory region fusion proteins, and since the unfolded fusion proteins do not bind to PDBu, they should not interfere with this assay (Kazanietz et al., 1995a; Quest et al., 1994a).

As expected from previous studies with Ca^{2+}-activated PKCs, PDBu bound to both GST-C1 and GST-C2-C1 in a PS-dependent manner (Hannun and Bell, 1987; Newton and Koshland, 1989; Lee and Bell, 1989; Quest et al., 1994a,b) (Fig. 2A and B). The $K_{1/2}$ values for PS were similar for PDBu binding to GST-C1 and GST-C2-C1 (Fig. 2A and B and Table 2.1). However, GST-C2-C1 bound less PDBu when the concentration of PDBu was low (20 nM compared to 150 nM) while GST-C1 bound similar amounts of PDBu at the two different concentrations (Fig. 2A and B and Table 2.1). This suggests that the presence of the C2 domain affects the affinity of the fusion protein for PDBu. Moreover, the fusion protein containing the C2 domain showed an increase in the cooperativity of PS needed for PDBu binding, indicating a requirement for more molecules of PS (Fig. 2C and Table 2.1). Both of these findings are consistent with an inhibitory role for the C2 domain (Sossin et al., 1996a). However, since the amount of PDBu bound to GST-C1 alone was also highly dependent on PS, the specific effect of PS on the construct containing the C2 domain was difficult to ascertain in these experiments.

In an effort to determine the specific role of the C2 domain, we performed Scatchard analysis of PDBu binding to GST-C1 and GST-C2-C1 at different concentrations of PS. In these experiments we were able to differentiate the effect of PS on the amount of PDBu bound at saturating concentrations of PDBu (B_{max}) and the effect of PS on the affinity of the fusion protein for PDBu (K_d). The B_{max} for both fusion proteins showed a strong dependence on PS (Table 2.2) suggesting that levels of PS determine the amount of fusion protein available to bind to PDBu independently of the C2 domain. In contrast, the presence of the C2 domain had a significant effect on the affinity for PDBu (K_d). At low concentrations of PS, the construct containing the C2 domain had a much lower affinity than the construct without this domain (Fig. 3A and B and Table 2.2). At higher concentrations of PS this C2-mediated inhibition was reduced, suggesting that the presence of the C2 domain affects K_d in a PS-dependent manner (Fig. 3A and Table 2.2). This was not due to low affinity binding of PDBu to the C2 domain itself, since a fusion protein containing only the C2 domain (GST-C2) bound no PDBu at any concentration tested (Fig. 1). Furthermore, since the pseudosubstrate of Apl II is positioned between the C2 and C1 domain we tested a fusion protein containing only the pseudosubstrate and C1 domain (GST-P-C1) (Fig. 1). This protein behaved similarly to GST-C1 (data not shown), demonstrating that the low binding affinity of GST-C2-C1 for PDBu is not due to the pseudosubstrate. These results indicate that the presence of the C2 domain lowers the affinity of PDBu binding to C1, and that this inhibition can be reduced by high concentrations of PS. However, the level of PS required for this function is quite high, and thus PS may not be the physiological effector of this function. Therefore, we tested the ability of PA, the product of RTK-activated PLD to facilitate PDBu binding.

PA is the most effective lipid for reducing C2 domain-mediated inhibition

PA was more effective than PS in increasing PDBu binding to GST-C1 (Fig. 4A). This effect was specific for PA since other cellular phospholipids were completely ineffective (PC, PE, and PG) or only weakly facilitated PDBu binding (PI) (Fig. 4A). The difference between PA and PS was even greater for GST-C2-C1, suggesting that PA also may be more effective than PS in reducing C2-domain mediated effects on the K_d for PDBu binding (Fig. 4A).

To further characterize the effect of PA, we measured PDBu binding at low concentrations of PS (12 mole %) and PDBu (75 nM). Figure 4B shows that under these conditions, 0.5 mole % PA (or approximately 1 molecule of PA/Triton X-100 micelle) increased PDBu binding to GST-C2-C1 more than to GST-C1, indicating that a single PA molecule in the micelle can effectively reduce C2-mediated inhibition of PDBu binding to C1. To demonstrate that addition of PA affected the K_d and not the B_{max} of the GST-C2-C1 fusion protein, we performed Scatchard analysis under these conditions. Indeed, 0.5 mole % PA increased the affinity of GST-C2-C1 for PDBu from 250 nM to 35 nM but did not significantly change the B_{max} (Fig. 3C and Table 2.2). PC also caused a small increase in PDBu binding to GST-C2-C1 relative to GST-C1 at low concentrations of PDBu, but PE, PG and PI did not (Fig. 4C).

Next, we characterized the ability of PA to directly facilitate PDBu binding to the fusion proteins in the absence of PS. By itself, PA was about twice as potent as PS in stimulating PDBu binding to GST-C1 (Fig. 5A and Table 2.1). In order to compare the relative ability of PA to affect B_{max} and K_d, we measured the affinity of the fusion proteins for PDBu at 5 mole % PA. At this concentration of PA, the B_{max} values for the fusion proteins are similar to those

using 12% PS (Table 2.2). However, while at 12% PS there was a large difference in the affinity for PDBu between GST-C2-C1 and GST-C1 (Fig. 3 and Table 2.2), the two constructs had identical affinities at 5% PA (Fig. 5B and Table 2.2). Thus, in contrast to PS, PA reduced C2 domain-mediated inhibition at a concentration below that required to increase B_{max}.

V. DISCUSSION

Two roles for lipids in PDBu binding to the fusion proteins. In our experiments, both PS and PA play two roles in PDBu binding to the fusion proteins. First, they affect the amount of fusion protein available to bind PDBu as determined by measuring B_{max}. It is likely that lipids increase B_{max} by facilitating binding of fusion proteins to the mixed micelles in a conformation capable of binding PDBu. PA is two-fold more potent than PS at this function and this is consistent with reports that show PA (due to its two negative charges) is also two-fold more potent at binding Ca^{2+}-activated PKCs to mixed micelles (Newton, 1993). Second, PS and PA selectively increase the affinity for PDBu (K_d) in constructs that contain the C2 domain. PA was much more potent (>10 fold) than PS at mediating this effect since one molecule of PA in a TX-100 micelle (0.5 mole %) had a significant effect in the presence of 24 molecules of PS (12 mole %). The level of PS in the nervous system of *Aplysia* and mammals is between 12 and 18 mole % (Piomelli et al., 1987; Sastry, 1985). This is in the range where our data suggests that increases in the levels of PA, which has a basal level of 0 - 1 mole % (Piomelli et al., 1987; Sastry, 1985), would have a significant ability to activate Ca^{2+}-independent PKCs. Thus, PA may be a physiologically significant mediator of this effect.

Our results suggest that the separate effects of lipids on the B_{max} and K_d of PDBu binding are due to separate lipid binding sites. First, the two effects had different lipid specificities. While PS was equally effective at the two functions, PA increases the affinity of C2 domain containing constructs for PDBu (K_d) at a much lower concentration than it increases the

amount of fusion protein available for binding PDBu (B_{max}). Second, more molecules of lipid were required (reflected by increased cooperativity [Table 2.1]) for binding of fusion proteins to PDBu when lipid affected both K_d and B_{max}. Thus, many molecules of PS were required for binding to GST-C2-C1 at 150 nM PDBu (Fig. 2C), since in this case increasing PS increased PDBu binding both by increasing B_{max} and increasing PDBu affinity (K_d). In contrast, fewer molecules of PS were required for binding of 150 nM PDBu to GST-C1 (Fig. 2C) because in this case 150 nM of PDBu was saturating, and thus PS only affected B_{max}. These results suggest that the site where PS or PA binds to the fusion proteins to increase the affinity for PDBu (K_d) is distinct from the site where PS or PA binds to increase B_{max}.

Lipids could increase B_{max} by removing intermolecular interactions that are due to inappropriate folding of the fusion proteins, as opposed to facilitating binding of fusion proteins to the mixed micelles in a conformation capable of binding PDBu. This explanation could account for lipid effects on B_{max}, but cannot account for the effects of 0.5 mole % PA on increasing the affinity of GST-C2-C1 (K_d), since in this case there was no significant difference in the amount of fusion protein available (B_{max}) (Table 2.2), and thus the effect of 0.5 mole % PA was to alter the conformation of fusion proteins that could already bind PDBu. The high levels of lipid required for PDBu binding is also unlikely to be an artifact of fusion protein production in bacteria, as we find similar levels of lipid required when we examine kinase activity of full length PKC Apl II purified from baculovirus (Sossin et al., 1996a; Pepio et al., 1998). Furthermore, we have found that only a small amount of the purified full length kinase can bind PDBu at 12 mole % PS and 75 nM PDBu (0.021 $\pm$ 0.002 mole PDBu/mol PKC, n=5), this amount increases dramatically at 20 mole % PS (0.12 $\pm$ 0.01 mole PDBu/mol

PKC, n=4), or when 0.5 mole % PA is added to 12 mole % PS (0.11 ± 0.01 mole PDBu/mol PKC, n=3). Therefore, if lipids are required to remove intermolecular interactions due to inappropriate folding, this is also true for the full-length protein expressed in an insect cell line.

The C2 domain as a negative regulator of PKC. Other reports also suggest a negative regulatory role for the C2 domain. Addition of the C2 domain of PKCγ □to a single C1 domain fusion protein decreases the affinity of the C1 domain for PDBu (Quest and Bell, 1994). Mochly-Rosen and colleagues have suggested that the C2 domain inhibits PKC activity through intraprotein interactions, which mimic interaction with the receptors for activated C-kinase (RACKs) (Ron and Mochly-Rosen, 1994, 1995). Since RACKs bind to the C2 domain of Ca^{2+}-independent PKCs (Johnson et al., 1996), removing C2-C1 interactions not only frees the activator binding sites in the C1 domain, but may also be important in allowing C2 domains to bind to RACKs. Interestingly, removing the core of the C2 domain from Apl II, but retaining the putative RACK binding site (Johnson et al., 1996), resulted in a kinase that was not activated by phorbol esters (Sossin et al., 1996a). We constructed a C2-C1 fusion protein with an identical mutation (GST-C2Δcore-C1). Despite the stability of the fusion protein (56% in correct molecular weight band) it bound very little PDBu (0.04 mole PDBu/mole protein) even at high concentrations of PS and PDBu. Furthermore, scatchard plots were not informative since increasing PDBu did not increase the amount of PDBu bound, suggesting that the affinity for PDBu was too low for the PDBu to remain bound during gel filtration. One interpretation of these results is that in these constructs inhibition of C1 is intact but the ability of lipid to reduce this inhibition is

impaired, consistent with a model in which the C2 domain contains separate sequences for the effects of lipid and C1/RACK.

The role of PA in PKC regulation. A number of reports have examined the ability of PA to stimulate Ca^{2+}-activated PKCs. PA was less effective than PS in stimulating PDBu binding to Ca^{2+}-activated PKCs using a mixed micelle binding assay similar to the one used in this study (Lee and Bell, 1989), suggesting that the ability of PA to stimulate PDBu binding may be specific for Ca^{2+}-independent PKCs. In some but not all cases PA can assist PS in stimulating kinase activity of Ca^{2+}-activated PKCs (Orr and Newton, 1992a,b; Newton and Keranen, 1994; Lee and Bell, 1992), but this has not been extensively studied in Ca^{2+}-independent PKCs. It will be important to determine how the ability of PA to stimulate PDBu binding to the fusion proteins translates into PA effects on kinase activity. We have found that low levels of PA (0.5 - 2 mole %) can significantly increase PKC Apl II activity in the presence of 12 mole % PS (Pepio et al., 1998).

Physiological role for PA in activation of PKC Apl II. The importance of PA for the removal of C2 domain mediated inhibition suggests that stimulation of PLD leads to Ca^{2+}-independent PKC activation by producing a combination of DAG and PA. In support of this, the time course for the activation of PKCε matches the time course of PA production downstream of PLD (Ha and Exton, 1993; Liscovitch et al., 1993). PLD is stimulated by Ca^{2+}-activated PKCs, $PI(4,5)P_2$ and the small G proteins ARF, RHO, and RAC (Kiss, 1996; Liscovitch et al., 1994; Morris et al., 1996; Hammond et al., 1997). Several of these factors are stimulated downstream of RTK activation and provide a pathway underlying activation of

Ca^{2+}-independent PKCs by RTKs through activation of PLD (Yeo and Exton, 1995; Hess et al., 1997; Natarajan et al., 1996). In particular PI 3-kinase has been shown to be important in this pathway (Klarlund et al., 1997; Moriya et al., 1996). Consistent with this, activation of PKC Apl II in bag cell neurons by the insulin responsive RTK is blocked by the PI 3-kinase inhibitor wortmannin (Sossin et al., 1996b). Wortmannin may also block PLD activation through inhibition of PI 4-kinase and the subsequent decrease of the PLD activator $PI(4,5)P_2$ (Nakanishi et al., 1995).

RTK activation is implicated in several forms of synaptic plasticity (Kang and Schuman, 1995; Korte et al., 1995; Figurov et al., 1996; Patterson et al., 1996). Ca^{2+}-independent PKCs are excellent candidates to mediate some of the actions of RTKs since they are specifically activated by RTKs (Sossin et al., 1996b; Ohno et al., 1994; Olivier and Parker, 1994; Ha and Exton, 1993), are enriched in the nervous system throughout phylogeny (Koide et al., 1992; Kruger et al., 1991; Land et al., 1994), and can regulate important synaptic functions (Akita et al., 1994; Hundle et al., 1995; Pasinelli et al., 1995). In the invertebrate *Aplysia*'s nervous system, the Ca^{2+}-independent PKC Apl II is activated downstream of an insulin responsive RTK and may mediate the insulin-stimulated increase in Ca^{2+} current (Sossin et al., 1996b; Jonas et al., 1996). It will be important in the future to determine if RTK-activation of PLD underlies the activation of PKC Apl II by insulin.

We suggest a novel mechanism for the regulation of PKC Apl II. The C2 domain of this kinase inhibits binding of the PKC activator DAG, and this inhibition can be removed by the actions of phosphatidic acid. This suggests that this kinase is regulated by phospholipase D.

Since phospholipase D is activated by RTKs, this suggests a pathway between RTKs and synaptic plasticity.

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Table 2.1 Cooperativity of lipid activation is dependent on the C2 domain

Fusion protein	PDBu (nM)	Lipid	Hill number (n) ± SD	Lipid for half-maximal PDBu binding (mol%) ± SD	Maximal PDBu binding (mol/mol) ± SD	N
GST-C2-C1	20	PS	6.2 ± 2	22 ± 3	.063 ± .005	3
	150	PS	7.0 ± 2	19 ± 1	.089 ± .02	3
	150	PA	2.8 ± .6	10 ± 2	.14 ± .04	2
	500	PS	4.2 ± 2	15 ± 3	.11 ± .05	4
GST-C1	20	PS	5.0 ± .5	20 ± .6	.29 ± .03	3
	150	PS	3.4 ± .6	18 ± 1	.24 ± .07	3
	150	PA	2.8 ± .3	8.5 ± 2	.25 ± .09	2
	500	PS	3.1 ± .2	17 ± 3	.37 ± .07	3

Values for the Hill coefficient and for half-maximal binding were calculated by non-linear regression (see Materials and Methods). N refers to the number of independent experiments.

Table 2.2 The affinity for C1 activators is dependent on the C2 domain

Fusion protein	Lipid	Mole %	Affinity for PDBu K_d (nM) ± SD	Maximal PDBu binding (mol/mol) ± SD	N
GST-C2-C1	PS	30	8.7 ± 4	.053 ± .02	3
	PS	20	27 ± 8	.039 ± .02	3
	PS	12	227 ± 33	.018 ± .008	5
	PA	5	13 ± .6	.014 ± .007	3
	PS / PA	12 / .5	35 ± 4	.02 ± .003	2
GST-C1	PS	20	5.9 ± 3	.23 ± .1	3
	PS	8	14 ± 5	.044 ± .01	3
	PA	5	11 ± 6	.032 ± .02	3

B_{max} and K_d values for PDBu binding to GST-C2-C1 and GST-C1 were calculated with EBDA (McPherson, 1983). N refers to the number of independent experiments.

VIII. FIGURE LEGENDS

Figure 1. *Characterization of GST fusion proteins.* A) Schematic of GST fusion proteins along with the expected molecular weight and the percentage of purified protein migrating at the correct molecular weight. B) GST fusion proteins; GST-C2 (lane 1), GST-C2-C1 (lane 2), GST-P-C1 (lane 3), GST-C1 (lane 4), and GST-C2Δcore-C1 (lane 5) after separation by 12% SDS-PAGE and subsequent staining with Coomassie Blue.

Figure 2. *Dependence of PDBu binding on PS.* The amount of PDBu bound to GST-C1 (A) or GST-C2-C1 (B) was measured at various concentrations of PS using either 20 nM (filled circles; solid line), 150 nM (open circles; large dotted line) or 500 nM (crosses; small dotted line) PDBu. The lines drawn represent fits to a modified version of the Hill equation (see Materials and Methods) using Hill coefficients and $K_{1/2}$ values calculated by non-linear regression using Systat 5.0 (Table 2.2). The moles of PDBu bound was standardized to the moles of fusion protein migrating at the correct molecular weight added to the reaction (see Fig. 1). C) Comparison of PDBu binding to GST-C1 (open circles; dotted line) or GST-C2-C1 (closed circles; solid line) at 150 nM PDBu standardized to 100% binding in order to compare the activation curves between the two constructs.

Figure 3. *PS and PA reduced C2-domain mediated inhibition of PDBu binding to C1.* The scales for all Scatchard plots are standardized so the slopes of the line (or $-1/K_d$) can be directly compared between experiments. Different amounts of protein are used in each experiment and thus the number of fmoles bound does not reflect the B_{max} for each protein.

Values for B_{max} are shown in Table 2.2. Scatchard plots of PDBu binding to (A) GST-C2-C1 at 12 mole % PS (filled squares) and at 20 mole % PS (filled circles), (B) GST-C1 at 8 mole % PS, (C) GST-C2-C1 at 12 mole % PS plus 0.5 mole % PA. In panel A, 0.57 μg (12 mole % PS) and 0.18 μg (20 mole % PS) of GST-C2-C1 (6.4 or 0.9 μg total protein) was used, in panel B, 0.03 μg of GST-C1 (0.15 μg total protein) was used and in panel C, 0.18 μg of GST-C2-C1 (1.8 μg of total protein) was used.

Figure 4. *PA is the most effective phospholipid at removing C2-domain mediated inhibition.* A) Fold increase in PDBu binding to GST-C1 (white bars) or GST-C2-C1 (dark bars) at 20 mole % of various phospholipids and 150 nM of PDBu compared to 20 mole % PS (set to 1). Values are averages ± S.D. n=2 independent experiments. B) Fold increase in PDBu binding to GST-C1 (white bars) or GST-C2-C1 (dark bars) by 0.5 mole % PA or 2 mole % PA in the presence of 12 mole % PS and 75 nM PDBu compared to 12 mole % PS (set to 1). Values are averages ± S.E.M., n=4 independent experiments. C) Fold increase in PDBu binding to GST-C1 (white bars) or GST-C2-C1 (dark bars) at 10 mole % of various phospholipids in the presence of 20 mole % PS and 75 nM of PDBu compared to 20 mole % PS (set to 1). Values are averages ± S.D. n=2 independent experiments.

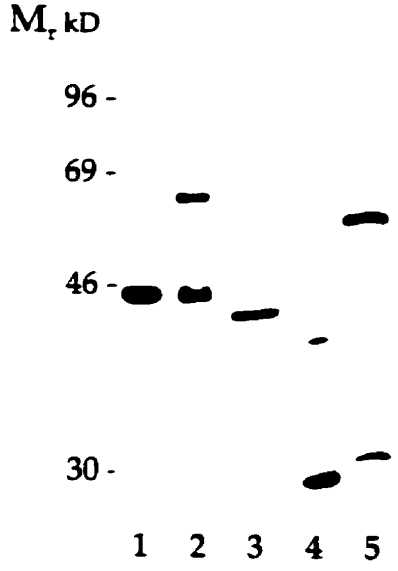
Figure 5. *Dependence of PDBu binding on PA.* A) The amount of PDBu bound to GST-C1 (open circles; dotted line) or GST-C2-C1 (filled circles; solid line) was measured at 150 nM PDBu. The lines drawn represent fits to a modified version of the Hill equation (see Materials and Methods) using Hill coefficients and $K_{1/2}$ values calculated by non-linear regression using Systat 5.0. B) Scatchard plots measuring PDBu binding to GST-C1 (open

circles) or GST-C2-C1 (filled circles) at 5 mole % PA. 0.03 μg of GST-C1 (0.15 μg of total protein) and 0.18 μg of GST-C2-C1 (1.8 μg of total protein) were used in the experiment shown.

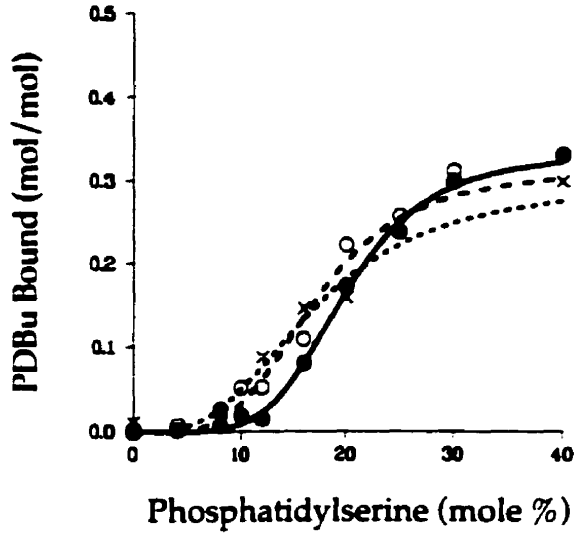
A

Lane	Name	Schematic	Predicted MW (kDa)	% of Protein at Predicted MW
1	GST-C2	GST C2 —c	48	94 ± 2
2	GST-C2-P-C1	GST C2 P C1/C1 —c	65	19 ± 9
3	GST-P-C1	GST P C1/C1 —c	47	60 ± 1
4	GST-C1	GST C1/C1 —c	44	24 ± 12
5	GST- C2-P-C1	GST sgnt C2 P C1/C1 —c	59	56 ± 4

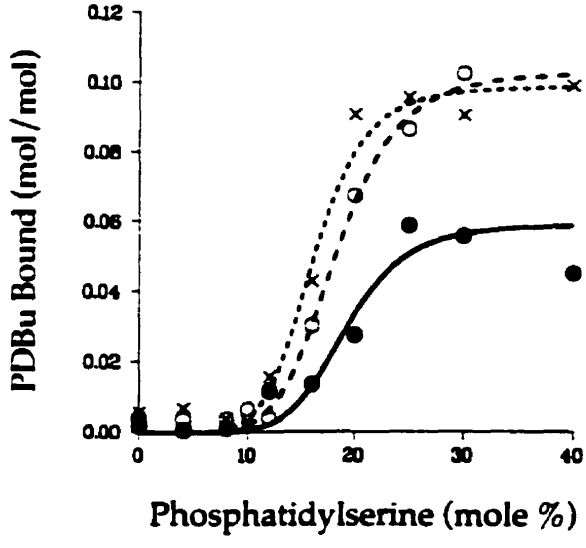
B



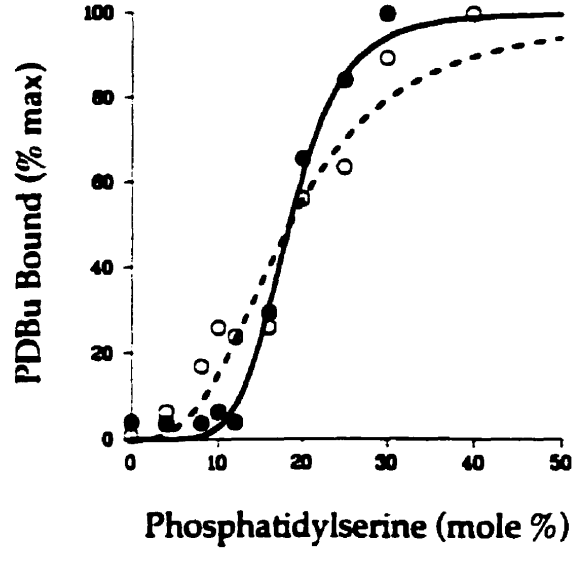
A

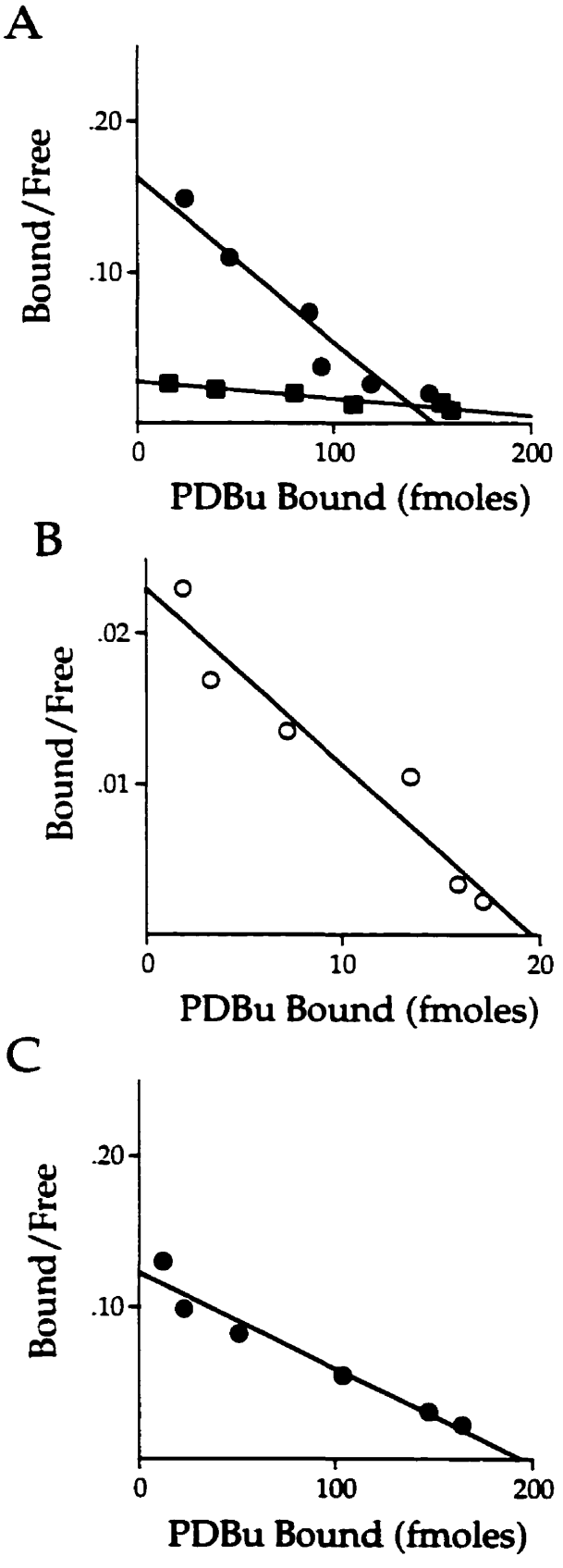

PDBu Bound (mol/mol)
0.5
0.4
0.3
0.2
0.1
0.0
0
10
20
30
40
Phosphatidylserine (mole %)

B

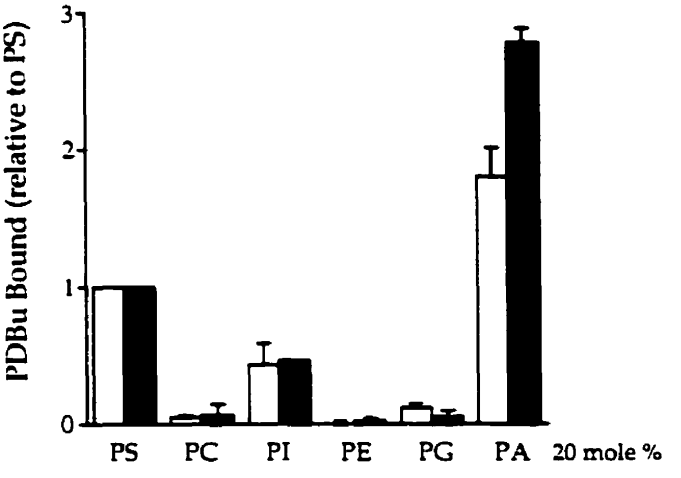

PDBu Bound (mol/mol)
0.12
0.10
0.08
0.06
0.04
0.02
0.00
0
10
20
30
40
Phosphatidylserine (mole %)

C

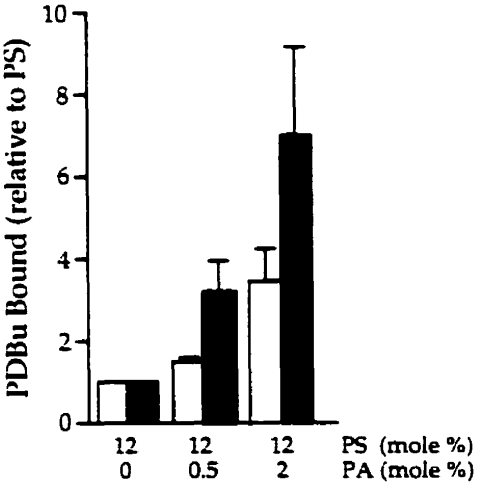

PDBu Bound (% max)
100
80
60
40
20
0
0
10
20
30
40
50
Phosphatidylserine (mole %)


A
Bound/Free
.20
.10
0
100
200
PDBu Bound (fmoles)
B
Bound/Free
.02
.01
0
10
20
PDBu Bound (fmoles)
C
Bound/Free
.20
.10
0
100
200
PDBu Bound (fmoles)

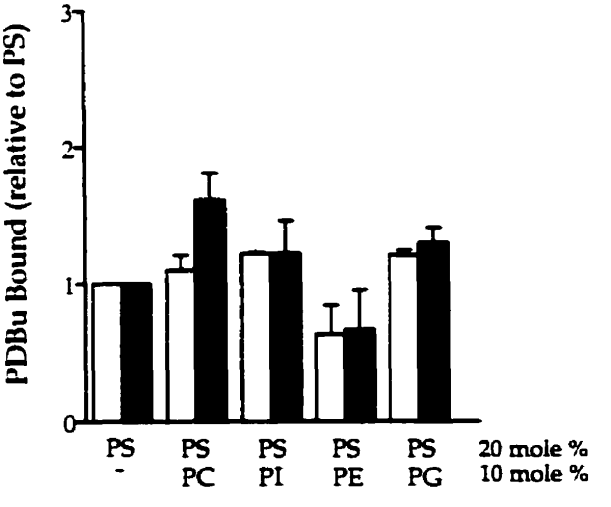
A


PDBu Bound (relative to PS)
0
1
2
3
PS
PC
PI
PE
PG
PA
20 mole %

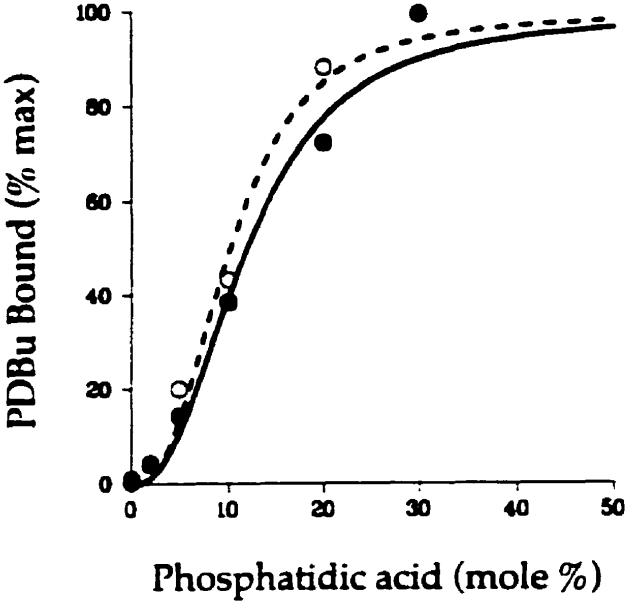
B


PDBu Bound (relative to PS)
0
2
4
6
8
10
12
12
12
PS (mole %)
0
0.5
2
PA (mole %)

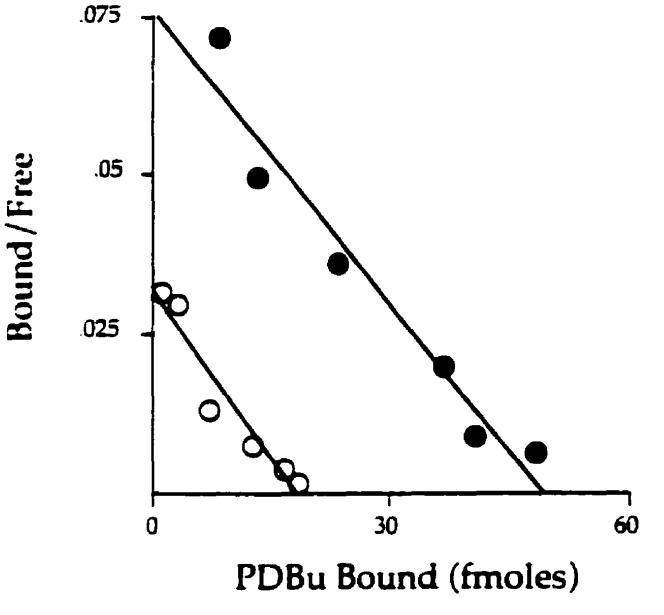
C


PDBu Bound (relative to PS)
0
1
2
3
PS
-
PS
PC
PS
PI
PS
PE
PS
PG
20 mole %
10 mole %

A


PDBu Bound (% max)
100
80
60
40
20
0
0
10
20
30
40
50
Phosphatidic acid (mole %)

B


Bound/Free
.075
.05
.025
0
30
60
PDBu Bound (fmoles)

PREFACE TO CHAPTER 3

Protein kinase C activity is under the regulatory control of its C1 and C2 domains. The biochemical properties of C1 and C2 domains and their interactions with other molecules form the molecular basis for regulation of PKC activity. The aim of the preceeding chapter was to determine whether interactions between C1 and C2 domains could explain decreased activity of the Ca^{2+}-independent PKC Apl II in *Aplysia*. It was demonstrated that indeed, the presence of the C2 domain reduces the ability of the C1 domain to bind PKC activators. Furthermore, primary structural analysis of the C2 domain reveals it to be the major structural difference between the Ca^{2+}-activated and Ca^{2+}-independent PKC isoforms in the *Aplysia* nervous system. This suggests that the molecular difference in C2 domains between PKC Apl I and PKC Apl II may account for the physiological difference in activation of the isoforms.

The following chapter addresses whether the difference in the lipid requirements for kinase activation of PKC Apl I and PKC Apl II stems from differences in the kinases structural domains. Using both a direct liposome binding assay and an assay measuring the ability of lipids to induce phorbol ester binding, we examined lipid interactions of fusion proteins containing C1 or C2 domains of the cPKC Apl I and the nPKC Apl II. We then compared these results to the ability of lipids to stimulate either PDBu binding or kinase activity of purified preparations of PKC Apl I, PKC Apl II and PKC Apl II without its C2 domain (PKC Apl IIΔC2). Our results indicate that the major difference between cPKCs and nPKCs is that lipids act through the C2 domain to enhance activity of cPKCs but not nPKCs.

CHAPTER 3

The role of C2 domains in Ca^{2+}-activated and Ca^{2+}-independent protein kinase Cs in *Aplysia*

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I. ABSTRACT

In the nervous system of the marine mollusk *Aplysia* there are two protein kinase C isoforms, the Ca^{2+}-activated PKC Apl I and the Ca^{2+}-independent PKC Apl II. PKC Apl I, but not PKC Apl II is activated by a short-term application of the neurotransmitter serotonin. This may be explained by the fact that purified PKC Apl II requires a higher mole percentage of phosphatidylserine to stimulate enzyme activity than does PKC Apl I. In order to understand the molecular basis for this difference, we have compared the ability of lipids to interact with the purified kinases and with regulatory domain fusion proteins derived from the kinases using a variety of assays including kinase activity, phorbol dibutyrate binding and liposome binding. We found that a C2 domain fusion protein derived from PKC Apl I binds to lipids constitutively, while a C2 domain fusion protein derived from PKC Apl II does not. In contrast, fusion proteins containing the C1 domains of PKC Apl I and PKC Apl II showed only small differences in lipid interactions. Thus, while the presence of a C2 domain assists lipid-mediated activation of PKC Apl I, it inhibits activation of PKC Apl II.

II. INTRODUCTION

Protein kinase Cs (PKC) are a family of lipid-activated enzymes that play important roles in many cellular processes including regulating the strength of synapses in the nervous system (Nishizuka et al., 1991; Nishizuka, 1992). This role of PKC has been well conserved over evolution with activation of PKC causing similar changes in vertebrate and invertebrate synapses (Nishizuka et al., 1991; Malenka et al., 1987; Parfitt and Madison, 1993; Sugita et al., 1992; Braha et al., 1990). The structure of PKCs has also been well conserved. Ca^{2+}-activated PKCs (cPKCs) in vertebrates (α, β, and γ) and invertebrates (PKC Apl I in *Aplysia*) have two C1 domains and a C2 domain. Ca^{2+}-independent or novel PKCs (nPKCs) in vertebrates (δ, ε, η, and θ) and invertebrates (PKC Apl II in *Aplysia*) also have two C1 domains and a C2 domain. However, the C2 domain in these kinases is at the N-terminal side of the C1 domain and lacks several of the critical aspartic acids that are required for Ca^{2+} binding (Nalefski and Falke, 1996).

In cPKCs, the C2 domain confers the ability to bind to acidic lipids in the presence of Ca^{2+} ions, while the C1 domain confers the ability to bind to lipid in a diacylglycerol (DAG)-dependent manner (Newton, 1995a,b). The DAG-dependent lipid binding to the C1 domain is specific for phosphatidylserine (PS) while binding to other lipids is independent of DAG (Newton and Keranen, 1994). The two lipid binding domains act in concert to activate cPKCs by causing a conformational change leading to the release of kinase inhibition by the pseudosubstrate. In contrast to cPKCs, a detailed model for the activation of nPKCs has not been presented. Removing the C2 domain of PKCη does not affect substrate specificity or

the ability to be activated by PIP_2 or PIP_3 (Dekker et al., 1993; Palmer et al., 1995). However, removing the C2 domain of PKC Apl II reduces the levels of PS required for enzyme activation suggesting that the C2 domain plays an inhibitory role (Sossin et al., 1996a). As well, peptides derived from the C2 domains of both cPKCs and nPKCs act as isoform-specific dominant negative inhibitors in cells suggesting that the physiological function of both kinases requires specific binding of factors to these domains (Johnson et al., 1996; Ron and Mochly-Rosen, 1994).

C2 domains are thought to be important in the translocation of proteins to membranes (Nalefski and Falke, 1996; Newton, 1995a; Nalefski et al., 1994). In *Aplysia*, short-term treatments with the facilitating transmitter 5-HT can translocate PKC Apl I to membranes, but not PKC Apl II (Sossin and Schwartz, 1992). In contrast, insulin can persistently translocate PKC Apl II, but not PKC Apl I to membranes (Sossin et al., 1996b). These results suggest that the regulation of enzyme translocation is different in these two PKC isoforms, perhaps due to their distinct C2 domains.

One way to determine the roles of C1 and C2 domains is to study these domains in isolation using fusion proteins containing one or both of these domains. These studies have illustrated that the C1 domains are sufficient to bind to phorbol dibutyrate (PDBu, a pharmacological analog of DAG) in a lipid dependent manner, and that some C2 domains bind to lipids in a Ca^{2+}-dependent manner (Nalefski et al., 1994; Kazanietz et al., 1995; Quest et al., 1994; Quest and Bell, 1994; Davletov and Sudhof, 1993). Furthermore, there is some evidence that these two domains may interact since addition of a C2 domain to a single C1 domain of

PKCγ leads to a decrease in PDBu affinity (Quest and Bell, 1994). Similarly, a fusion protein containing both the C2 domain and C1 domains of PKC Apl II has a lower affinity for PDBu than a fusion protein containing only the C1 domains (Pepio and Sossin, 1998). Small amounts of phosphatidic acid (PA) increase the affinity of the C2 domain containing construct for PDBu, suggesting that PA might act specifically to remove the effect of the C2 domain of PKC Apl II (Pepio and Sossin, 1998).

In this paper we have examined lipid interactions of fusion proteins containing C1 or C2 domains of the cPKC Apl I and the nPKC Apl II, using either a direct liposome binding assay or measuring the ability of lipids to stimulate PDBu binding to the fusion proteins. We have compared these results to the ability of lipids to stimulate either PDBu binding or kinase activity of purified preparations of PKC Apl I, PKC Apl II and PKC Apl II without its C2 domain. Our results indicate that the major difference between cPKCs and nPKCs is that lipids act through the C2 domain to enhance activity of cPKCs but not nPKCs.

III. MATERIALS AND METHODS

Reagents. 4β-Phorbol 12, 13-dibutyrate (LC Services); [^{3}H]4β-phorbol 12, 13-dibutyrate (19.6 Ci/mmol) (New England Nuclear, Boston, MA); 1,2-dipalmitoy, L-3-phosphatidyl [N-methyl-^{3}H] choline (100 mCi/mg) (Amersham, Oakville, ON), dioleoyl phosphatidylserine (PS), dioleoyl phosphatidylcholine (PC), dioleoyl phosphatidylethanolamine (PE), dioleoyl phosphatidic acid (PA), dioctylglycerol (DOG) (Avanti Polar Lipids Inc., Alabaster, AL); phosphatidylinositol (PI) (mostly linoleic and palmitic acid) (Sigma, St. Louis MO); Triton X-100 (Pierce, Rockford, IL); Sephacryl S-100 HR (Pharmacia, Uppsala, Sweden); prestained molecular weight markers (Amersham Corp), TPCK-treated trypsin (Worthington Biochemicals, Freehold, NJ). All other reagents were of the highest grade available.

Construction of plasmids encoding fusion proteins. The generation of GST-Apl II-C1 and GST-Apl II-C2 has been described (Pepio and Sossin, 1998). GST-Apl I C1 domain was made using PCR with VENT polymerase (New England Biolabs, Beverly, MA) from the plasmid expressing PKC Apl I. The 5' primer was GGGATCCTTATGGAGAAGCGAGTCGCTAGGC and the 3' primer was GGAATTCCTTGACAGCTCCTTTGATGA. The amplified product was cut with BamHI and EcoRI and inserted into pGEX-3X (Pharmacia) which had been cut with the same enzymes. This generated a fusion protein with PKC sequences beginning from the initiator methionine of PKC Apl I Met-Glu-Lys-Arg and ending with Gly-Ala-Val-Lys, which included the pseudosubstrate of PKC Apl I at the 5' terminal and strand 1 of the C2 domain at the 3' terminal. The pseudosubstrate in PKC Apl I is immediately after the initiator

methionine and there is no V1 region in this kinase. Earlier constructs made with shorter boundaries did not produce stable fusion proteins. We had previously made a GST-PS-C1 domain construct from PKC Apl II that included the pseudosubstrate and its behavior was identical to that of the construct without this domain (Pepio and Sossin, 1998). For GST-Apl I-C2 an Eco47III fragment was excised from the PKC Apl I plasmid and inserted into pGEX-3X after cutting the vector with EcoRI and filling in with Klenow. The fusion protein contains PKC sequences beginning with Cys-Glu-Lys-Asn from near the end of the C1 domain to Ser-Ser-Glu-Lys in the hinge region of PKC Apl I, 30 amino acids after the end of strand 8 from the crystal structure. Expression of fusion proteins was as described (Pepio and Sossin, 1998). For each fusion protein preparation, gels were scanned and analysis was performed using the public domain NIH Image program (developed at the U.S. National Institutes of Health and available on the Internet at http://rsb.info.nih.gov/nih-image/) to determine the percentage of purified fusion protein at the correct molecular weight. These values (Apl I-C1, 19 ± 3; Apl I-C2, 81 ± 10; Apl II-C1, 26 ± 11; Apl II-C2, 82 ± 20; errors are S.D., n=3) were used to calculate the stoichiometries for fusion protein binding. Much of this degradation occurred during or after elution of the proteins from the glutathione beads as the percentage of purified fusion protein at the correct molecular weight was higher for experiments which used fusion proteins still attached to beads (Apl I-C1, 63 ± 6; Apl I-C2, 90 ± 2; Apl II-C1, 89 ±10; Apl II-C2, 96 ± 3; errors are S.D., n=2). These values were used to standardize the amount of fusion protein in liposome binding experiments.

Purification of PKC Apl I and PKC Apl II from baculovirus. We purified the kinases after expression in baculovirus as described (Sossin et al., 1996a). The purification was done in

one day leading to less autonomous activity and higher specific activity than previous purifications. Aliquots of kinase were stored in 10% glycerol at –70 °C. Maximal activities of preparations of PKC Apl I ranged from 100-400 pmoles/min/mg and ranged from 20-100 pmoles/min/mg for PKC Apl II. The kinases were both approximately 50% pure based on Coomassie staining of the purified proteins (Sossin et al., 1996a) and this value was used to estimate the stoichiometry of PDBu binding.

[^{3}H] PDBu-binding assay. Assays for [^{3}H]PDBu binding used the mixed micelle method (Hannun and Bell, 1987; Quest et al., 1995). The 50 µl reaction mixture consisted of 20 µM Tris, pH 7.5, 200 µM $CaCl_2$ or $MgCl_2$, mixed micelles (0-40 mol%) of phospholipids in a final concentration of 0.6% Triton X-100, [^{3}H]PDBu from 1 to 200 nM, fusion protein ranging from 0.02 to 1.6 µg, and purified kinase from 0.25 to 0.35 µg. Mixed micelles were prepared by drying the appropriate volume of each lipid under nitrogen and resuspending in 3% Triton X-100, vortexing for 1 min followed by incubation at 30 °C for 10 min. Reactions were started by addition of protein and were allowed to proceed for 10 min at room temperature. Reaction tubes were then placed on ice and bound PDBu was separated from free PDBu on Sephacryl S-100 HR gel-filtration columns (2-ml column volume) at 4 °C by washing with equilibration buffer (20 µM Tris, pH 7.5, 0.015% Triton X-100, and 200 µM either $CaCl_2$ or $MgCl_2$). The nonspecific binding component for all experiments was measured in the presence of 10 µM unlabeled PDBu and was subtracted from the total binding to yield the specific binding. No differences were seen in experiments using 200 µM $CaCl_2$ or $MgCl_2$.

Liposome binding assay. Phospholipids were prepared as described (Davletov and Sudhof, 1993). Liposome mixtures consisted of either 1.75 mg of PC or 1.4 mg of PC and 0.35 mg of either PA, PS, PI, or PE. Phospholipids were mixed in a 15-ml tube and dried under a stream of nitrogen. All experiments measuring phospholipid binding used liposome mixtures containing 5 μCi of 1,2-dipalmitoyl, L-3-phosphatidyl [N-*methyl* -^{3}H] choline (specific activity 108 mCi/mg) as a tracer. Dried phospholipid mixtures were resuspended in 5 ml of binding buffer (50 mM HEPES, pH 7.2, 100 mM NaCl) by vortexing for 3 min. The phospholipid suspensions were then sonicated for 30 s using a probe sonicator (Vibraceil) and centrifuged before use to pellet aggregates. The liposome suspensions were stored at 4 °C and used within 1 month.

Recombinant proteins (5 or 30 μg of protein) bound to glutathione-sepharose beads (standardized to 15 μl wet volume) were prewashed with binding buffer and then added to a reaction mixture containing either 1 mM $CaCl_2$ or 1 mM $MgCl_2$ and 1 mM EGTA, 50 mM HEPES, pH 7.2, 100 mM NaCl and ^{3}H-labeled liposomes (approximately 200,000 cpm) in a total assay volume of 400 μl. The mixture was incubated at room temperature for 15 minutes with vigorous shaking and then briefly centrifuged in a tabletop centrifuge. The bound beads were then washed three times with 1 ml of the binding buffer and liposome binding was quantified by liquid scintillation counting of the beads. We confirmed that equal amounts of fusion proteins were used in the assay by visual inspection of Coomassie stained gels.

Kinase assays. The kinase assays using mixed micelles and an Apl II-derived pseudosubstrate peptide were performed as described (Sossin et al., 1996a).

Trypsin digestion. The 50 µl proteolysis reaction contained 10 µl of either purified PKC Apl II, GST-C2 domain fusion protein derived from PKC Apl II, or GST alone. The protein component was then incubated with 5 µl 1% Triton X-100 micelles and 25 µl 2 × proteolysis buffer (40 mM Tris, pH 7.5, 40 mM $MgCl_2$, 4 mM 2-mercaptoethanol) for 3 min at 30 °C. The digestion was initiated by addition of 10 µl of trypsin diluted in proteolysis buffer and the reaction was allowed to proceed at 30 °C for 5 min. The final reaction contained either 50 ng or 200 ng protein, 0.1% Triton X-100, 20 mM Tris-HCl, pH 7.5, 20 mM $MgCl_2$, 2 mM 2-mercaptoethanol, and either 0, 0.4, 1, 2, 5, or 10 µg/ml TCPK-treated trypsin. Proteolysis was quenched by addition of 20 µl of Laemmli sample buffer. Samples were analyzed by SDS-PAGE.

Antibody production and immunoblotting. The MBP-Apl II-C2 fusion protein was injected intramuscularly into rabbits along with TitreMax (Cedar Lane) three times at three to four week intervals. Serum from the animals was passed over an affinity column consisting of the GST-Apl II-C2 fusion protein (Sossin et al., 1996a) coupled to Affigel (Biorad, Hercules, CA) and specifically retained antibodies were eluted and concentrated in a Centriplus-10 (Amicon Inc, Beverly, MA). Western blots were performed as described (Sossin et al., 1996b) with this antibody at a 1:1250 dilution using a goat anti-rabbit horseradish peroxidase-conjugated secondary antibody (Pierce, Rockford, IL). Results were visualized by enhanced chemiluminescence (NEN-Dupont, Boston, MA).

Quantitation of data. The dependence of phorbol ester binding or kinase activity on the mole % of micelles was analyzed by a non-linear least squares fit to a modified Hill equation

(Newton and Koshland, 1989) using Systat 5.0 where y is the value (PDBu binding or kinase activity), a is the maximum value, x is the mole %, k is the mole % resulting in half-maximal binding and n is the Hill coefficient. $y=a[x^n/(k^n+x^n))$.

IV. RESULTS

Lipid specificity for enzyme activation of PKC Apl I and PKC Apl II

PKC Apl II requires more PS for activation than does PKC Apl I (Table 3.1) (Sossin et al., 1996a). To examine the ability of other lipids to activate the two enzymes, we determined the lipid specificity of enzyme activation of PKC Apl I and PKC Apl II in the presence of 1 mole % dioctylglycerol (DOG) using the mixed micelle kinase assay (Hannun et al., 1986). PS was the most effective lipid for both kinases (Fig. 1A). PI activated PKC Apl I better than PKC Apl II, although the concentration of PI required was significantly higher than that of PS (Fig. 1B). PA only stimulated kinase activity of PKC Apl I and PKC Apl II at high concentrations (Fig. 1B and C). Neither PE nor PC could stimulate kinase activity.

One possible explanation for the difference between PKC Apl I and PKC Apl II in the amount of PS required for activty could be that PKC Apl I has a higher affinity for DOG than PKC Apl II, since the concentration of DOG affects the amount of PS required for maximal activity (Table 3.1). However, in the presence of saturating amounts of DOG, or using PDBu (150 nM) instead of DOG, the difference in lipid required by the two enzymes is not diminished (Fig. 1D and data not shown).

Although the results above suggested that for both PKC Apl I and PKC Apl II only PS could effectively synergize with DOG to stimulate enzyme activity, it is possible that in the presence of some PS, other lipids would become more effective. We used levels of PS (5 mole % for PKC Apl I and 10 mole % for PKC Apl II) that gave 10-20% of the maximal

activity with 1 mole % DOG and compared addition of more PS to addition of other lipids (Fig. 1E and F). For PKC Apl I, addition of PA was more effective than addition of PS, despite the poor ability of PA to activate PKC Apl I on its own. PI could replace PS at an equimolar level, while PC and PE were ineffective (Fig. 1E). For PKC Apl II, PA was equivalent to PS, PI was poorer than PS, and PC and PE had no ability to replace PS (Fig. 1F).

These experiments demonstrate several similarities between the isoforms. For both enzymes, PS was the most effective lipid at increasing PKC activity, while PC and PE had no effects on either enzyme. These experiments also revealed several differences between the enzymes: 1) PI was more effective at stimulating PKC Apl I kinase activity than PKC Apl II; 2) PA was more effective at stimulating kinase activity in the presence of small amounts of PS in PKC Apl I than PKC Apl II; and 3) PKC Apl I required less lipid for activation than PKC Apl II.

PKC Apl I and PKC Apl II require similar levels of lipid to induce PDBu binding

To determine the lipid specificity for PKC binding, we compared the ability of lipids to induce PDBu binding to PKC Apl I and PKC Apl II using the mixed micelle PDBu binding assay (Hannun and Bell, 1987), since this assay allows us to use the same lipid compositions used to measure kinase activity. In this assay, bound ^{3}H-PDBu is separated from free ^{3}H-PDBu by gel filtration since micelles bound to protein and PDBu are larger than micelles bound to PDBu alone. This assay will only detect protein bound to PDBu that is also bound to micelles and thus PDBu binding in this assay is directly related to the ability of lipids to

support enzyme binding to the mixed micelles (Quest et al., 1995; Newton and Koshland, 1989; Lee and Bell, 1989).

We found that the lipid specificity for PDBu binding differed from that for kinase activation. In contrast to the superiority of PS in stimulating kinase activity, PS and PA were equally effective at inducing PDBu binding (Fig. 2A). PI supported PDBu binding to a greater extent for PKC Apl II than for PKC Apl I, while the reverse was true for enzyme activity. Neither PC nor PE could support PDBu binding.

There was no difference in the $K_{1/2}$ for PS to stimulate PDBu binding between the two isoforms, as opposed to the large difference in the $K_{1/2}$ for PS measured for enzyme activity (Fig. 2B and Tables 3.1 and 3.2). Thus, the difference in the amount of PS needed to stimulate enzyme activity of PKCs Apl I and Apl II was not reflected in the ability of PS to stimulate PDBu binding to the two enzymes.

Lipid specificity for PDBu binding can be attributed to lipid specificity of the C1 domain

In order to determine whether the lipid specificity of the enzymes could be explained by the lipid specificity of the isolated regulatory domains, we measured PDBu binding to fusion proteins containing the C1 or C2 domains of PKC Apl I and PKC Apl II. Proteins of the correct molecular weight were observed for all constructs (Fig. 3), along with several degradation products the size of GST. Since GST does not bind to PDBu (data not shown), these proteins should not interfere with the PDBu binding assays.

The lipid specificity for PDBu binding to the C1 domains was very similar to that of the purified enzymes (Fig. 4A). The only exception was that PDBu binding to the C1 domains of PKC Apl II was induced more strongly by PA than PS compared to the full length enzyme. The GST-C2 domain fusion proteins did not bind to PDBu (data not shown). Similar amounts of PS were required for PDBu binding to the C1 domains of PKC Apl I and PKC Apl II (Fig. 4B) as to the full length enzymes (Fig. 2B and Table 3.2). These results suggest that the lipid specificity for PDBu binding to the kinases can be largely explained by the lipid specificity of the C1 domains, and that PS interacts with the C1 domains of PKC Apl I and PKC Apl II in a similar manner.

The C2 domain of PKC Apl I, but not PKC Apl II binds directly to lipid

Since the C2 domains do not bind to PDBu, we determined their lipid specificity by measuring the ability of liposomes to bind to fusion proteins that were still attached to glutathione beads. This is a well characterized assay that has been used previously to measure Ca^{2+}-dependent lipid binding to the synaptotagmin C2 domain and the C2 domain of PKCβ (Davletov and Sudhof, 1993; Shao et al., 1996). The liposomes are made up of 80% PC and 20% of each of the other lipids. The C2 domain of PKC Apl I bound liposomes containing PS or PA, but not other lipids, while the C2 domain of PKC Apl II bound liposomes no better than the GST control (Fig. 5A). The ability of the PKC Apl I C2 domain to bind to liposomes did not require Ca^{2+}, but could be increased by Ca^{2+} ions (Fig. 5A). However, the concentration of Ca^{2+} ions required to stimulate additional liposome binding to the C2 domain was very high (1-2 mM Ca^{2+}) (Fig. 5B). Nevertheless, liposome binding to the GST control was not affected by this concentration of Ca^{2+} (Fig. 5B).

We also compared the ability of the C1 domains to bind liposomes using this assay. Both GST-C1 domain fusion proteins had higher background binding to PC liposomes than did the GST-C2 domain fusion proteins (Fig. 5C). However, the ability of the C1 domains to bind the different liposomes broadly agreed with the specificity of lipids to stimulate PDBu binding to the C1 domains. Liposomes containing PI and PA bound better to the PKC Apl II C1 domains than to the PKC Apl I C1 domains, similar to results with PDBu binding. Liposomes containing PS bound equally well to PKC Apl I and PKC Apl II and PS was also equipotent at inducing PDBu binding to the two C1 domain fusion proteins.

The studies of fusion protein lipid interactions can be summarized as follows: First, the C1 domains of PKC Apl I and PKC Apl II had similar affinities for PS suggesting that differences in the C1 domains could not explain the differential activation of the two enzymes by PS. Second, PS bound to the C2 domain of PKC Apl I, but not of PKC Apl II, possibly explaining why less PS is required to stimulate PKC Apl I activity than PKC Apl II.

The role of C2 domains in PKC Apl I and PKC Apl II

Binding of lipids to the C2 domain could remove an inhibitory effect of the C2 domain, facilitate the effects of PS acting through the C1 domain, or do both. To differentiate between these models we compared the amount of PS required to activate PKC Apl I, PKC Apl II and a PKC Apl II construct with the C2 domain removed (Apl IIΔC2) (Sossin et al., 1996a). If the C2 domain is simply an inhibitory domain, activation of PKC Apl IIΔC2 should require the same amount of PS as that of PKC Apl I, since the C1 domains of PKC Apl I and PKC Apl II have similar interactions with PS. In fact, PKC Apl IIΔC2 required an intermediate

amount of PS, less than that of PKC Apl II, but more than that of PKC Apl I, both at low and high concentrations of DOG (Table 3.1). This finding suggests that the C2 domain has both effects, inhibiting PKC Apl II but also facilitating activation of PKC Apl I. This analysis was complicated, however, by the fact that PS is acting both at the C1 domain and the C2 domain of PKC Apl I.

PA specifically reduces the inhibitory effect of the C2 domain in PDBu binding experiments with a fusion protein containing both the C1 and C2 domains of PKC Apl II (Pepio and Sossin, 1998), but our data suggest that PA does not interact with the C2 domain directly (Fig. 5A). To investigate whether PA binding to the C1 domain of PKC Apl II could specifically remove the inhibition of enzyme activity mediated by the C2 domain, we examined the ability of PA to decrease the concentration of DOG required to activate PKC Apl II and PKC Apl IIΔC2. Indeed, deletion of the C2 domain or addition of PA did decrease the requirement of PKC Apl II for DOG (Fig. 6A and B and Table 3.3). However, PA also decreased the requirement for DOG in PKC Apl IIΔC2 (Fig. 6C and Table 3.3). Thus, although adding PA had the same effect as removing the C2 domain, removing the C2 domain did not occlude the effect of PA.

A small amount of PA (0.5 mole %) has a large affect on PDBu binding to both the PKC Apl II GST-C1-C2 domain fusion protein and the purified enzyme in the presence of PS (Pepio and Sossin, 1998). The same amount of PA also had a large effect on the amount of PS required for enzyme activity at saturating concentrations of DOG (Fig. 6C and Table 3.1). However, this was true in the enzyme with or without the C2 domain (Fig. 6C and Table

3.1). Thus, although small amounts of PA did have significant effects on PKC Apl II activity, this is not through removal of C2 domain mediated inhibition.

Another way of testing the hypothesis that lipid binding to the C2 domain in PKC Apl I facilitates the effects of PS acting through the C1 domain is to examine the role of PA. Recall that PA interacts with the C2 domain of PKC Apl I, but not PKC Apl II, and does not synergize with DOG to stimulate enzyme activity through the C1 domain of either enzyme (Fig. 1). In the presence of saturating levels of DOG and PA, PKC Apl I required less PS for activation than did PKC Apl II or PKC Apl IIΔC2 (Fig. 7). This experiment illustrates both the inhibitory role of the C2 domain in the absence of an effector at the C2 domain (PKC Apl II vs PKC Apl IIΔC2) and the facilitatory role of the C2 domain in the presence of an effector (PA) at the C2 domain (PKC Apl I vs PKC Apl IIΔC2). In the presence of PA, the $K_{1/2}$ for PS in PKC Apl I was near 1 mol % (or approximately two molecules in a Triton X-100 micelle) (Table 3.1), suggesting that only a few PS molecules are specifically required to act at the C1 domain in synergy with DOG in the presence of lipids interacting with the C2 domain.

Effect of Ca^{2+} on PKC Apl I

The C2 domain of PKC Apl I contains the aspartic acid residues predicted to confer Ca^{2+}-specificity, while the C2 domain of PKC Apl II does not. Indeed, Ca^{2+} activated PKC Apl I, but not PKC Apl II which was actually inhibited at high concentrations of Ca^{2+} ions (Fig. 8A and B) (Sossin et al., 1996a). Under conditions of limiting PS and DOG, the $K_{1/2}$ for Ca^{2+} was 390 μM and the Hill coefficient was 2 (Fig. 8A). Although this concentration is high

compared to vertebrate PKCs, it is lower than the amount of Ca^{2+} required to increase lipid binding to the isolated C2 domain (Fig. 5B). As another approach to examine the effect of the C2 domain in PKC Apl I, we examined the effects of Ca^{2+} on the levels of PS and DOG required for activity. The effect of Ca^{2+} is to lower the lipid requirement of PKC Apl I at constant DOG, or to decrease the requirement for DOG at constant lipid (Fig. 8C and D). However, PKC Apl I did not require Ca^{2+} for activity and the presence of Ca^{2+} did not increase the maximal amount of PKC activity at saturating concentrations of PS and DOG (Fig. 8C).

V. DISCUSSION

Lipid specificity of PKC Apl I. The lipid specificity for stimulation of cPKCs *in vitro* has been well studied (Newton and Keranen, 1994; Lee and Bell, 1989,1992; Mosior et al., 1996; Newton, 1993,1995a; Orr and Newton, 1992a,b). Both Ca^{2+} and DOG act independently to increase the affinity of the enzyme for PS, presumably through C1 domains (DOG) and C2 domains (Ca^{2+}) (Newton, 1995a). These results led to a model where the C1 domain and C2 domain act independently to translocate the enzyme to the membrane, where specific binding to PS activates the enzyme by inducing a conformational shift of the pseudosubstrate (Newton, 1995a). These studies also distinguished binding of the enzyme to membranes, which was specific for acidic lipids and not specific for PS from the synergy with DAG, which was specific for PS (Newton and Keranen, 1994). Our results with PKC Apl I are in general agreement with these results. PS stimulates enzyme activity in the presence of DOG much better than PA despite the ability of PA to bind to both C1 and C2 domains as well as, if not better than, PS. One major difference between PKC Apl I and vertebrate cPKCs is that Ca^{2+} is not necessary for activation of PKC Apl I in the mixed micelle kinase assay. This may be explained by the ability of the C2 domain of PKC Apl I, but not of vertebrate cPKCs (Nalefski et al., 1994; Shao et al., 1996) to bind to lipids in the absence of Ca^{2+} ions.

Lipid specificity of PKC Apl II. There have been few studies examining the lipid specificity for activation of nPKCs *in vitro* using the mixed micelle assay or comparing cPKCs and nPKCs in a systematic manner. Our results suggest that the lipid specificity conferred by the C1 domain is similar but not identical to that of cPKCs. Similar to cPKCs, PS is the most

effective lipid for stimulating enzyme activation of PKC Apl II and PA is more effective at binding the enzyme than it is at stimulating enzyme activity. In contrast, PI had different effects on PKC Apl II and PKC Apl I. PI was better at stimulating enzyme activity in PKC Apl I than PKC Apl II, but better at inducing PDBu binding to PKC Apl II than PKC Apl I.

Role of PA in activation of PKC Apl II. Previously, we had suggested that PA may specifically activate PKC Apl II by removing inhibition mediated by the C2 domain (Pepio and Sossin, 1998). Our results demonstrate that this is not the case, since effects of PA on enzyme activity are similar in constructs with or without the C2 domain. Previous studies also suggested a role for PA in binding of PDBu to PKC Apl II (Pepio and Sossin, 1998). Our results are consistent with a role for PA in activation of PKC Apl II. First, PA bound to the C1 domain of PKC Apl II better than to the C1 domain of PKC Apl I (Fig. 4). Second, at saturating levels of DOG, 0.5 mole % of PA (approximately 1 molecule in a Triton X-100 micelle) significantly reduced the amount of PS required for PKC activity from a $K_{1/2}$ of 14 to a $K_{1/2}$ of 9 mole % (Table 3.1). There was also not much of an additional effect after raising PA from 0.5 to 5 mole % (Table 3.1). Additionally, PA was very effective at replacing PS in PKC Apl I, but since PA interacts with the C2 domain of PKC Apl I the results are difficult to compare.

There is a specific requirement for PS in enzyme activation for both PKC Apl I and PKC Apl II. This agrees with the ability of DOG to increase affinity for PS, but not other lipids in both vertebrate cPKCs and nPKCs (Newton and Keranen, 1994). This requirement for PS is not conferred solely by the C2 domain since DOG increases affinity for PS even when the C2

domain has been mutated to ablate lipid binding (Edwards and Newton, 1997a) and there is still specificity for activation by PS in PKC Apl II without the C2 domain (data not shown). However, our results suggest that other lipid binding sites that are not specific for PS are also important for enzyme activation, and that PA can replace PS at these sites. Furthermore, since the PS levels in *Aplysia* are somewhere between 12 and 18 mole % (Piomelli et al., 1987), the effect of PA on the C1 domain of PKC Apl II may be important for physiological enzyme activation. At these levels of PS, addition of 0.5 mole % PA can double enzyme activity (Fig. 6C).

Role of C2 domains. The difference in the amount of PS required for stimulation of PKC Apl I compared with PKC Apl II can be explained by lipid binding to the C2 domain of PKC Apl I, but not that of PKC Apl II. Our data are consistent with the idea that lipid binding to the C2 domain increases the ability of lipid binding to the C1 domain to induce the conformational shift required for enzyme activity. Whether binding to the C2 domain is necessary to remove an inhibitory effect of the C2 domain in cPKCs as in nPKCs was difficult to determine from our experiments, since the PS required for binding to the C1 domain of PKC Apl I also bound to the C2 domain. However, the requirement of Ca^{2+} for PKC activity in cPKCs from vertebrates is consistent with an inhibitory effect of the C2 domain in these isoforms. Mutating the Ca^{2+}-binding site in a vertebrate cPKC greatly increases the amount of lipid required for enzyme activity (Orr and Newton, 1992b). It will be interesting to see if a similar amount of lipid would be required after removal of the C2 domain. Our results with PKC Apl II suggest that less lipid would be required to activate cPKCs when the C2 domain is absent compared to when it is present and cannot bind lipid.

The lipids examined did not bind to the C2 domain of PKC Apl II. While we cannot rule out the possibility that this was due to incorrect folding of the fusion protein, it is unlikely given the stability of this fusion protein (Fig. 3). Furthermore, the pattern of partial trypsin cleavage of the C2 domain is similar regardless of whether the C2 domain is derived from purified PKC Apl II or the GST-C2 domain fusion protein (Fig. 9) suggesting that the domain is folded similarly in both proteins. Our results examining kinase activation with and without the C2 domain are also consistent with the lack of lipid binding to this domain. Removing the C2 domain allows PKC Apl II to be activated at lower concentrations of PS, but this difference is not alleviated by increasing DOG concentrations or adding PA since these compounds decrease the amount of PS required in PKC Apl II without the C2 domain to a similar extent. These experiments argue against a model where lipids are acting through the C2 domain of PKC Apl II to facilitate enzyme activity. Moreover, there have been no reports of lipid binding to the related C2 domain of PKCε or PKCη, although it has been speculated that they bind lipids constitutively (Newton, 1995a), or that they bind to hydrophobic lipids due to the prevalence of hydrophobic residues in the loops corresponding to the calcium binding regions (CBRs) (Perisic et al., 1998). Other lipid activators of PKC like PIP_2, PIP_3, or oleic acid stimulate nPKC activity similarly in the presence or absence of the C2 domain suggesting that they also do not interact with the C2 domain (Palmer et al., 1995; Sossin et al., 1996a). Identifying other lipids or proteins that do interact with its C2 domain will be important in understanding how PKC Apl II is activated physiologically. One candidate for this interaction is the protein β-COP which binds to the C2 domain of PKCε (Csukai et al., 1997), although it has not yet been shown that the presence of this protein can increase PKC activity.

The physiological role of Ca^{2+} in stimulating PKC Apl I. Although the amount of Ca^{2+} required for stimulation of PKC Apl I does not appear to be physiological, it is still possible that under some conditions increases in Ca^{2+} concentrations are important for activation of PKC Apl I since the amount of Ca^{2+} required is also dependent on levels of PS and DOG. For example, during pathological events such as axonal damage, Ca^{2+} concentrations may reach the required level. Short treatments of serotonin activate PKC Apl I (Sossin and Schwartz, 1992), but do not appear to raise Ca^{2+} levels significantly in the absence of action potentials (Eliot et al., 1993). It is interesting that unlike several vertebrate isoforms (Hannun et al., 1986), PKC Apl I does not require Ca^{2+} for activity in the mixed micelle assay. This parallels the ability of the C2 domain of PKC Apl I to bind to lipid in the absence of Ca^{2+}, while isolated C2 domains from enzymes that require Ca^{2+} do not bind lipid in the absence of Ca^{2+} (Davletov and Sudhof, 1993). The difference in the C2 domain that confers this change is not clear. There are three regions of the C2 domain that are defined as CBRs from crystallographic studies (Nalefski and Falke, 1996; Perisic et al., 1998; Grobler et al., 1996). CBR1 and CBR3 are extremely well conserved between PKC Apl I and vertebrate cPKCs, while CBR2 is similar in PKC Apl I compared to vertebrate kinases (Fig. 10). The crystal structure of the cPKC C2 domain has not yet been solved, but CBR2 is important for Ca^{2+} coordination in $cPLA_2$ and PLCδ1, but not synaptotagmin. Recently, the autophosphorylation of a serine near the carboxyl-terminal of a vertebrate cPKC has been shown to alter the concentration of Ca^{2+} required for PKC activation suggesting that interactions between the C2 domain and the carboxyl-terminus of the enzyme occur (Edwards and Newton, 1997b). This serine is conserved in PKC Apl I. A role for the carboxyl-terminus may explain the

large difference in the amount of Ca^{2+} required for C2 domain binding alone, compared to the amount of Ca^{2+} required to stimulate enzyme activity.

The physiological role of lipids in stimulating PKC Apl II. In *Aplysia* activation of PKC by serotonin leads to the reversal of synaptic depression, but does not play a role in the augmentation of synaptic responses also mediated by serotonin (Ghirardi et al., 1992). In contrast, PDBu causes both augmentation and the reversal of synaptic depression (Braha et al., 1990). Short applications of serotonin activate PKC Apl I, but not PKC Apl II leading to a model whereby PKC Apl II may lead to augmentation stimulated by other transmitters (Sossin and Schwartz, 1992). Indeed, prolonged incubation with serotonin can activate both isoforms of PKC (Sossin et al., 1994). Furthermore, addition of insulin to the bag cell neurons of *Aplysia* transiently activates PKC Apl I, but then later activates PKC Apl II (Sossin et al., 1996b). These results suggest that a transient increase in DAG, either through G-protein activated phospholipase C-β or tyrosine kinase activated phospholipase C-γ is sufficient to activate PKC Apl I, but not PKC Apl II which appears to require an additional stimulus. The activation of PKC Apl II by insulin is blocked by wortmannin which implies a role for either lipids downstream of PI 3-kinase or PA downstream of phospholipase D (Sossin et al., 1996b) in activation of PKC Apl II. Either lipid may help activate PKC Apl II through binding to the C1 domain, but perhaps more likely, these stimuli may lead to the production of a signaling molecule that can interact with the C2 domain of PKC Apl II and activate the enzyme. Identifying this molecule will be important in the study of PKC regulation.

VI. ACKNOWLEDGEMENTS

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Table 3.1 Lipid dependence of PKC activation

Enzyme	DOG (mol%)	Lipid	Lipid for half-maximal activation (mol%) ± SD	Hill number (n) ± SD	N
Apl I	1	PS	11 ± 2	5 ± 1	6
	10	PS	5 ± 0.3	3 ± 0.4	4
	10	PS (5 mole % PA)	1 ± 0.2	1 ± 0.5	2
Apl II	1	PS	19 ± 5	7 ± 3	6
	10	PS	14 ± 2	6 ± 2	4
	10	PS (0.5 mole % PA)	9.2 ± 1	5 ± 1	3
	10	PS (5 mole % PA)	8.5 ± 1	3 ± 1	2
Apl IIΔC2	1	PS	15 ± 2	8 ± 2	5
	10	PS	8.5 ± 0.8	5 ± 1	4
	10	PS (0.5 mole % PA)	6.5 ± 0.6	4 ± 1	3
	10	PS (5 mole % PA)	4.1 ± 0.3	3 ± 1	2

Values for the Hill coefficient and for half-maximal binding were calculated by non-linear regression (see Materials and Methods) for each individual experiment and the means ± S.D. are presented. N refers to the number of independent experiments.

Table 3.2 Lipid dependence of PDBu Binding

Protein	PDBu (nM)	Lipid	Lipid for half-maximal PDBu binding (mol%) ± SD	Hill number (n) ± SD	Maximal PDBu binding (mol/mol) ± SD	N
Apl I	150	PS	16 ± 1	7.4 ± 1.1	0.38 ± .09	3
Apl II	150	PS	16 ± 0.3	4.1 ± 0.2	0.19 ± .06	3
Apl I GST-C1	150	PS	15 ± 2	4.9 ± 0.9	0.26 ± .14	3
Apl II GST-C1	150	PS	16 ± 1	3.0 ± 0.8	0.10 ± .06	3

Values for the Hill coefficient and for half-maximal binding were calculated by non-linear regression (see Materials and Methods) for each individual experiment and the means ± S.D. are presented. N refers to the number of independent experiments.

Table 3.3 DOG dependence of PKC activity

Enzyme	Lipid	Mole %	DOG for half-maximal activation (mole %) ± SD	Hill number (n) ± SD	N
Apl II	PS	16	5.2 ± 1	1.1 ± 0.3	2
	PS	16 (2.5 mole % PA)	2.0 ± 1	1.0 ± 0.2	2
Apl IIΔC2	PS	16	3.4 ± 2	1.1 ± 0.3	2
	PS	16 (2.5 mole % PA)	1 ± .3	1.0 ± 0.1	2

Values for the Hill coefficient and for half-maximal binding were calculated by non-linear regression (see Materials and Methods) for each individual experiment and the means ± S.D. are presented. N refers to the number of independent experiments.

VIII. FIGURE LEGENDS

Figure 1. *Lipid specificity of enzyme activation.* A) Activation of PKC Apl I (white bars) and PKC Apl II (dark bars) in the presence of 1 mole % DOG and 30 mole % of PS, PC, PI, PE, or PA. Activity in the absence of DOG was subtracted. Values are standardized to activity in the presence of PS. Values are means ± S.E., (n=3). Activation curves of PKC Apl I (B) or PKC Apl II (C) in the presence of 1 mole % DOG and various concentrations of PS (open circles), PI (open squares) or PA (closed circles). Activity in the absence of DOG was subtracted. Values are means ± S.E., (n=2-4). D) Activation of PKC Apl I (open circles) and PKC Apl II (closed circles) in the presence of 10 mole % DOG and increasing amounts of PS. Activity in the absence of PS was subtracted. Values are means ± S.E., (n=3). Activation curve of PKC Apl I (E) in the presence of 1 mole % DOG, 5 mole % PS or PKC Apl II (F) in the presence of 1 mole % DOG, 10 mole % PS and increasing amounts of either PS (open circles) PI (open squares), PA (closed circles), PE (closed squares) and PC (open diamonds). The amount of additional lipid added is indicated on the *x* axis and is different for PKC Apl I and PKC Apl II. The levels of PA added were different than the other lipids, but were the same for PKC Apl I and PKC Apl II. Values are means ± S.E., (n=3-5).

Figure 2. *Phospholipid dependence of PDBu binding to PKC Apl I and PKC Apl II.* A) Binding of ^{3}H-PDBu (150 nM) to PKC Apl I (white bars) or PKC Apl II (dark bars) in the presence of 20 mole % of PS, PC, PI, PE or PA. Values are standardized to binding in the presence of PS. Values are means ± S.D., (n=3). B) Binding of ^{3}H-PDBu (150 nM) to PKC

Apl I (open circles) or PKC Apl II (closed circles) at different mole % PS. Values are means ± S.E., (n=3).

Figure 3. *Expression of C1 and C2 domain fusion proteins.* A) Coomassie stained gel of fusion proteins after elution from glutathione beads. 6 μg of purified protein preparations were run in corresponding lanes. Lane 1 GST-Apl I C1, Lane 2 GST-Apl II C1, Lane 3 GST-Apl I C2; Lane 4 GST-Apl II C2. B) Coomassie stained gel of fusion proteins before elution from glutathione beads. 10 μg of purified protein preparations were run in corresponding lanes. Lane 1; GST; Lane 2 GST-Apl I C1, Lane 3 GST-Apl II C1, Lane 4 GST-Apl I C2; Lane 5 GST-Apl II C2.

Figure 4. *Phospholipid dependence of PDBu binding to GST-C1 fusion proteins.* A) Binding of ^{3}H-PDBu (150 nM) to GST-Apl I-C1 (white bars) or GST-Apl II-C1 (dark bars) in the presence of 20 mole % of PS, PC, PI, PE or PA. Values for GST-Apl II-C1 are from Pepio and Sossin, 1998. Values are means ± S.D., (n=3). Binding of ^{3}H-PDBu to GST-Apl I-C1 (open circles) and GST-Apl II-C1 (closed circles) at various concentrations of PS (B) Values are means ± S.E., (n= 3).

Figure 5. *Phospholipid dependence of liposome binding by the C1 and C2 domains of PKC Apl I and PKC Apl II.* A) GST (open bars), GST-Apl I C2 (gray bars), or GST-Apl II C2 (dark bars) were incubated with ^{3}H-labeled liposomes composed of PC only or PC mixed with 20 % PS, PA, PI, or PE in the presence of 1 mM $MgCl_2$ (-) or 1 mM $CaCl_2$ (+). Values are means ± S.E., (n=2-4). B) Binding of the GST-Apl I C2 domain at different

concentrations of Ca^{2+} to the liposomes was measured. Note that in the presence of EGTA (0 Ca^{2+}) there is higher background binding to GST and higher binding to GST-Apl I C2 compared to binding in the presence of 1 mM $MgCl_2$ as seen in A. Values are means ± S.E., (n=3). C) Binding of GST (open bars), GST-Apl I C1 (gray bars) and GST-Apl II C1 (dark bars) to ^3H-labeled liposomes composed of PC only or PC mixed with 20 % PS, PA, PI, or PE. There were no differences in binding using 1mM $MgCl_2$ or 1 mM $CaCl_2$ and results were pooled. Values are means ± S.E., (n=4).

Figure 6. *Effect of PA on PKC Apl II.* Activation of PKC Apl II (A) or PKC Apl IIΔC2 (B) in the presence of 16 mole % PS and increasing amounts of DOG in the presence (closed circles/squares) or absence (open circles/squares) of 2.5 mole % PA. Values are means ± S.E., (n=2). C) Activation of PKC Apl II (circles) or PKC Apl IIΔC2 (squares) in the presence of 10 mole % DOG in the presence (closed) or absence (open) of 0.5 mole % PA. Activity in the absence of PS was subtracted. Values for PKC Apl II in the absence of PA are the same as in Fig. 1D. Values are means ± S.E., (n=3).

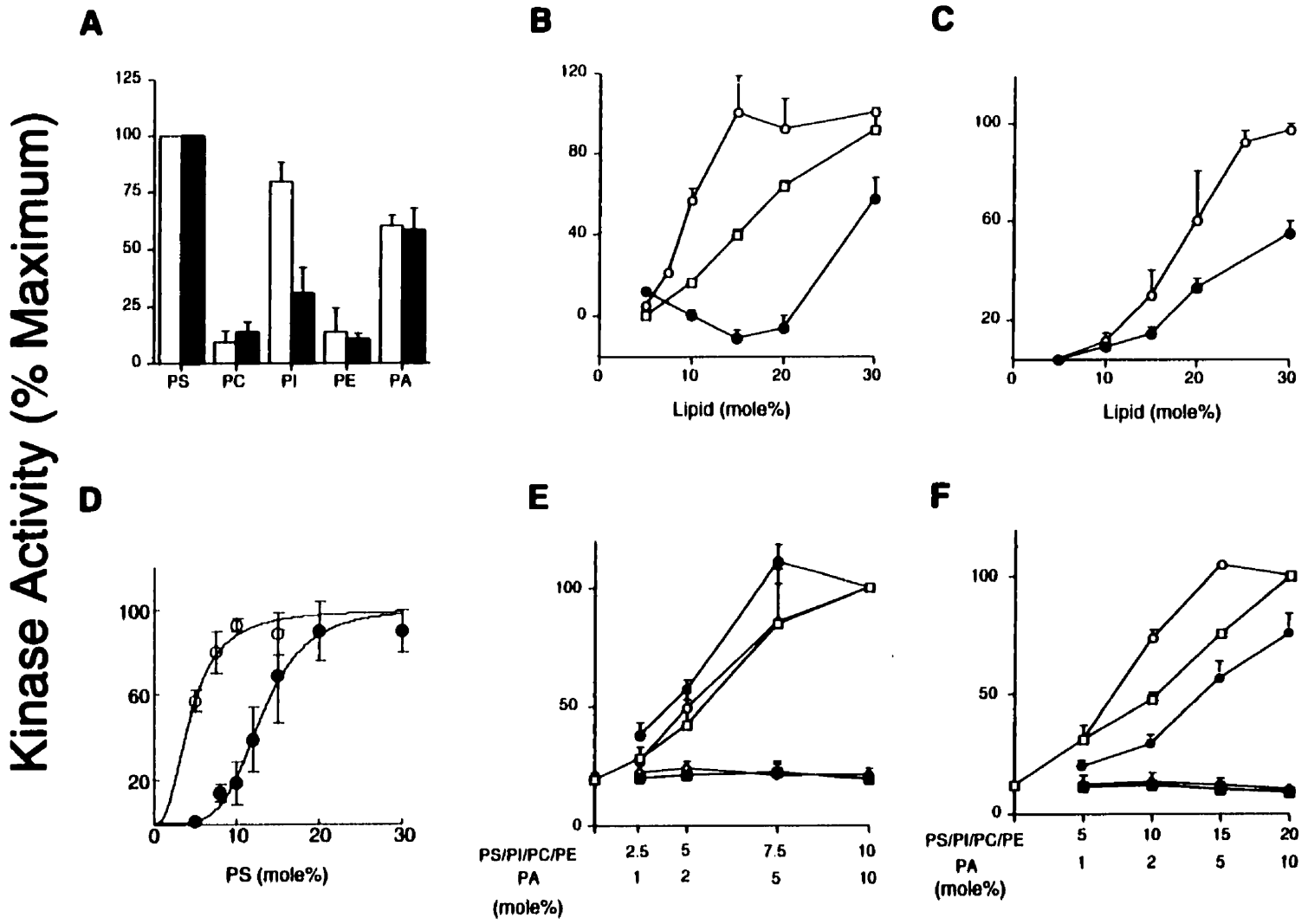
Figure 7. *Role of the C2 domain in kinase activation.* Activation of PKC Apl I (open circles), PKC Apl II (closed circles) and PKC Apl IIΔC2 (open squares) in the presence of 10 mole % DOG, 5 mole % PA, and increasing amounts of PS. Activity in the absence of PS was subtracted. Values are means ± S.E., (n=2).

Figure 8. *Effects of Ca^{2+} on PKC Apl I and PKC Apl II.* A) Activation of PKC Apl I in the presence of 5 mole % PS and 1 mole % DOG and increasing concentrations of Ca^{2+} ions.

Value in the absence of Ca^{2+} was subtracted. Values are means ± S.E., (n=3). B) Activation of PKC Apl II in the presence of 20 mole % PS and 1 mole % DOG and increasing amounts of Ca^{2+} ions. Values are means ± S.E., (n=2). C) Activation of PKC Apl I with 1 mole % DOG in the presence (closed circles) or absence (open circles) of 500 μM Ca^{2+} and increasing amounts of PS. Activity in the absence of PS was subtracted. Values are means ± S.E., (n=2). D) Activation of PKC Apl I with 5 mole % PS in the presence (closed circles) or absence (open circles) of 500 μM Ca^{2+} and increasing amounts of DOG. Activity in the absence of DOG was subtracted. Values are means ± S.E., (n=2).

Figure 9. *Trypsin digests of PKC Apl II and GST-C2.* GST (50 ng), PKC Apl II (200 ng), or GST-Apl II-C2 (50 ng) were cleaved with increasing concentrations of trypsin (0, 0.4, 1, 2, 5, and 10 μg/ml), separated on a 17% SDS-PAGE gel and immunoblotted with an antibody specific for the C2 domain of PKC Apl II. 0.4 μg/ml trypsin was sufficient to cleave the C2 domain from both PKC Apl II and the GST-C2 domain fusion protein (arrowhead). Increasing concentrations of trypsin show a similar pattern of fragmentation whether the C2 domain is derived from PKC or the GST fusion protein (arrows). The predicted size of the C2 domain core is 17.5 kDa. The antibody exhibited a cross-reaction with trypsin seen at the highest concentrations of trypsin, but did not cross-react with GST.

Figure 10. *Comparison of the C2 domain of PKC Apl I and vertebrate C2 domains.* Strand positions and CBRs are as described (Nalefski and Falke, 1996; Perisic et al., 1998). Shaded residues are conserved in all isoforms. D represents conserved aspartic acids involved in Ca^{2+} binding.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100
75
50
25
0
PS
PC
PI
PE
PA
B
120
80
40
0
0
10
20
30
Lipid (mole%)
C
100
60
20
0
10
20
30
Lipid (mole%)
D
100
60
20
0
10
20
30
PS (mole%)
E
100
50
0
PS/PI/PC/PE
PA
(mole%)
2.5
5
7.5
10
1
2
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(mole%)
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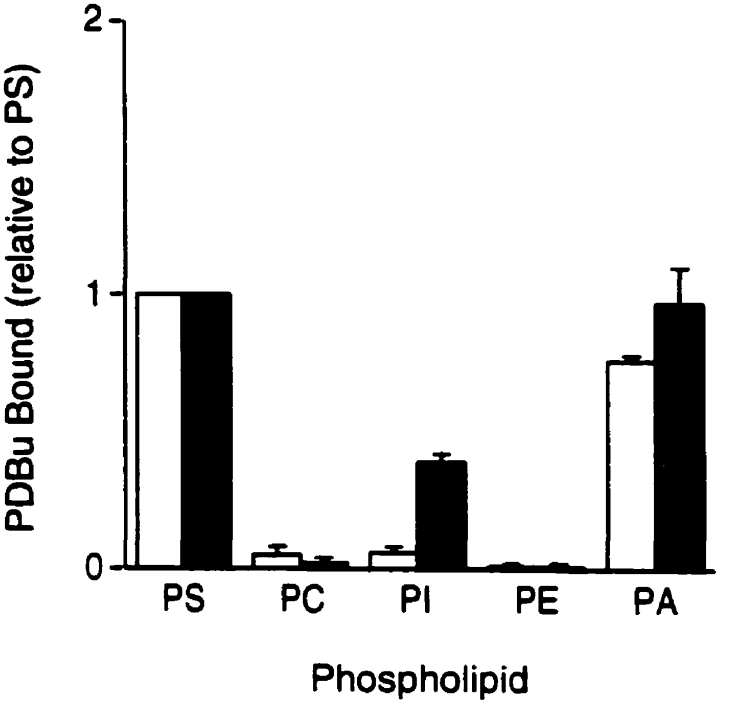
1
2
5
10

A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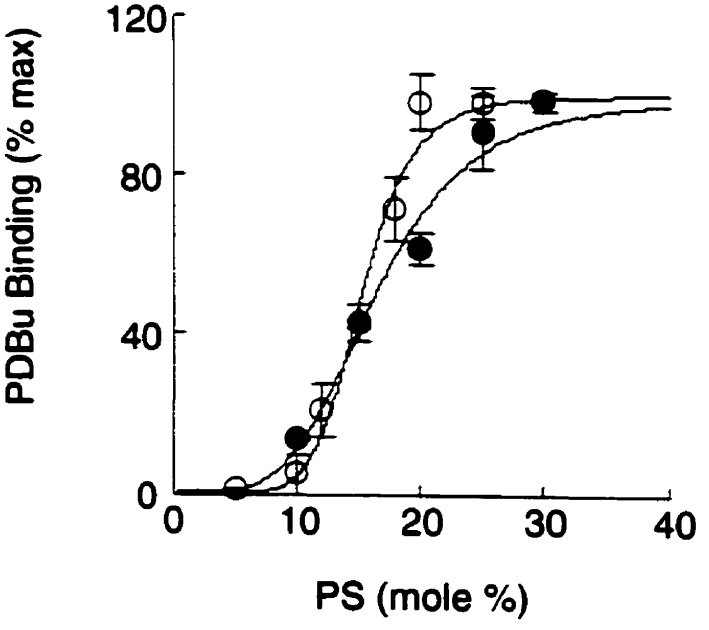
PI
PE
PA
Phospholipid

B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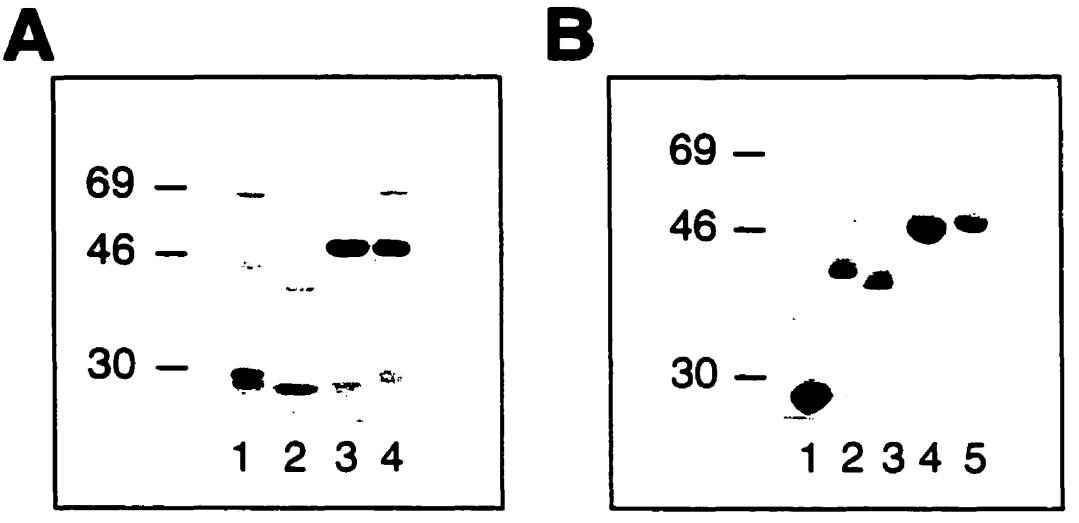
40
0
0
10
20
30
40
PS (mole %)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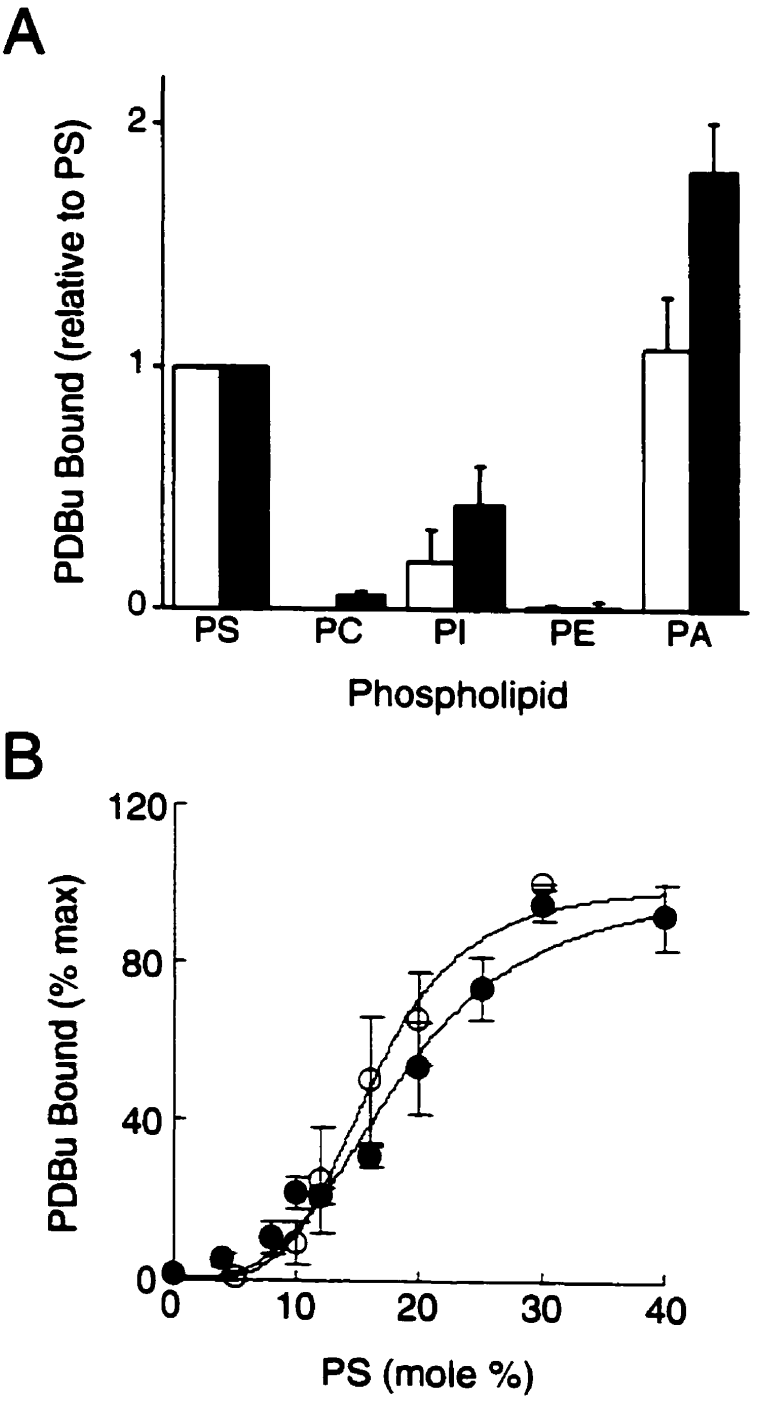
1 2 3 4 5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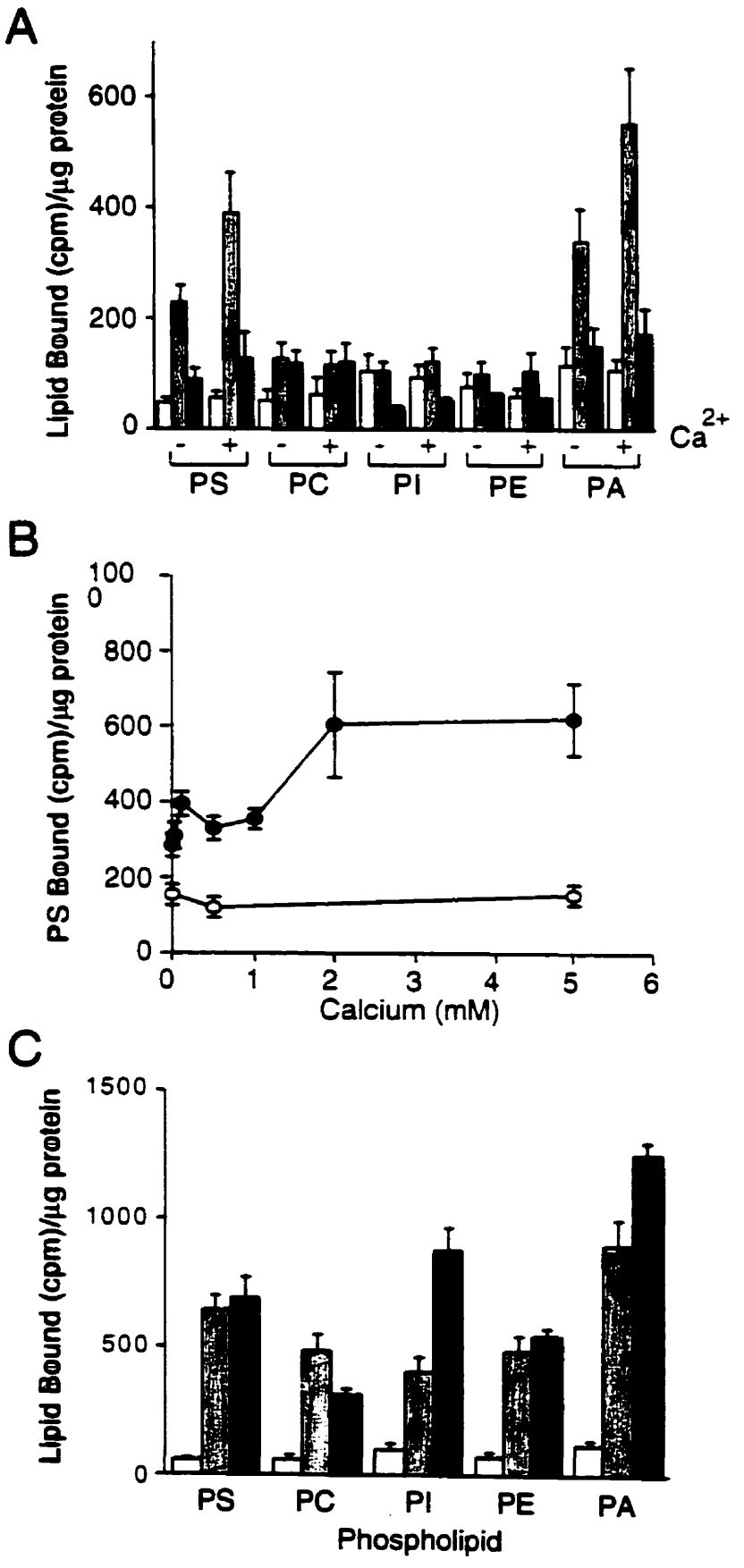
0
0
10
20
30
40
PS (mole %)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Ca2+
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B
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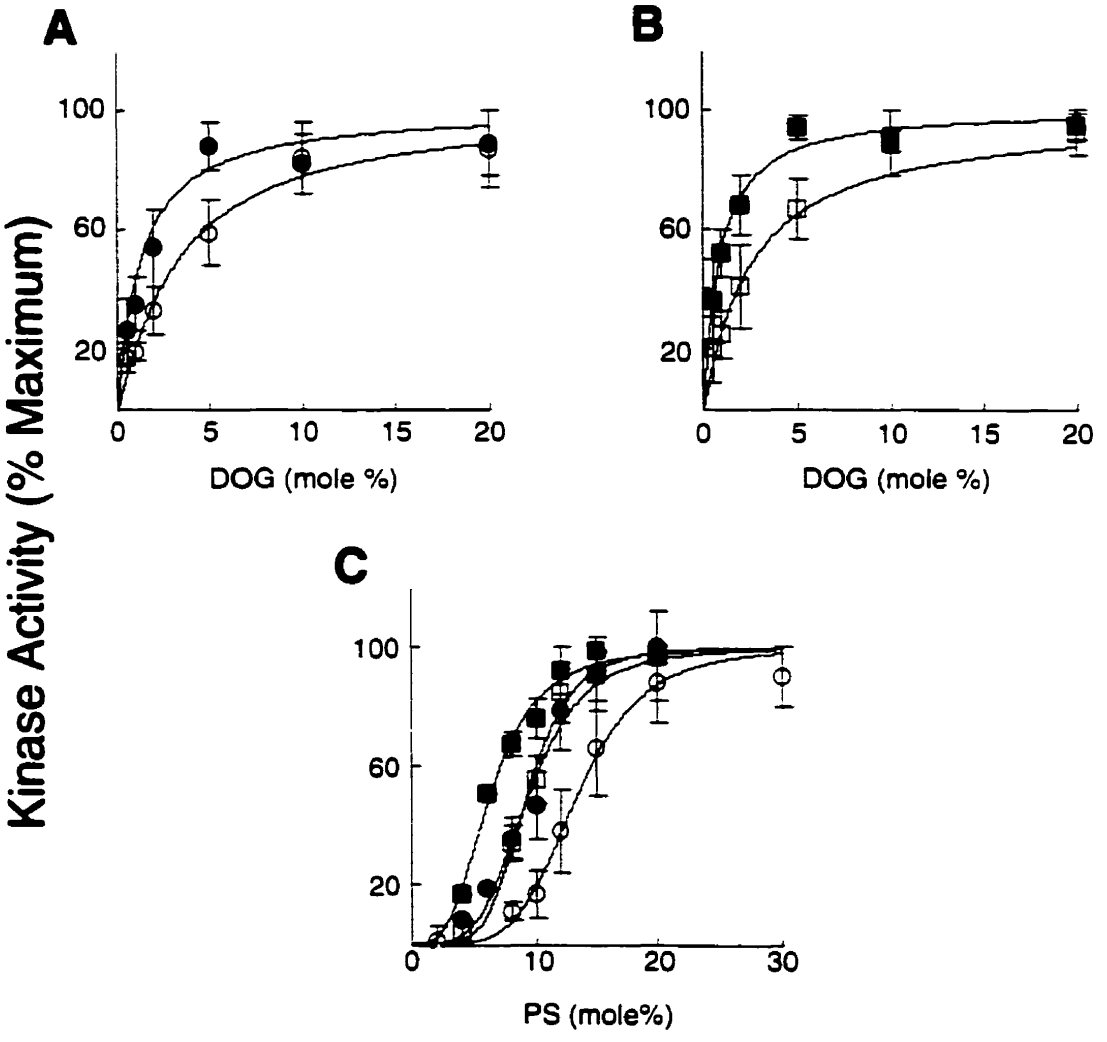
0
800
600
400
200
0
0 1 2 3 4 5 6
Calcium (mM)
C
Lipid Bound (cpm)/µg protein
1500
1000
500
0
PS PC PI PE PA
Phospholipid


A
B
C
Kinase Activity (% Maximum)
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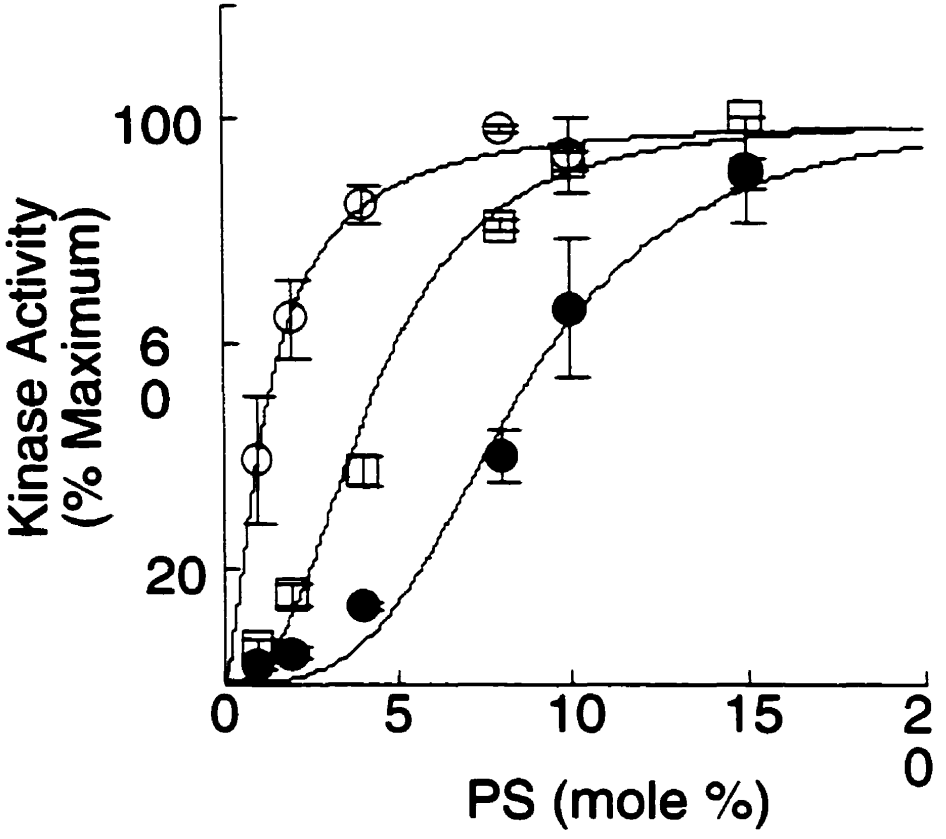
60
20
0
5
10
15
20
DOG (mole %)
30
PS (mole%)


Kinase Activity
(% Maximum)
100
60
20
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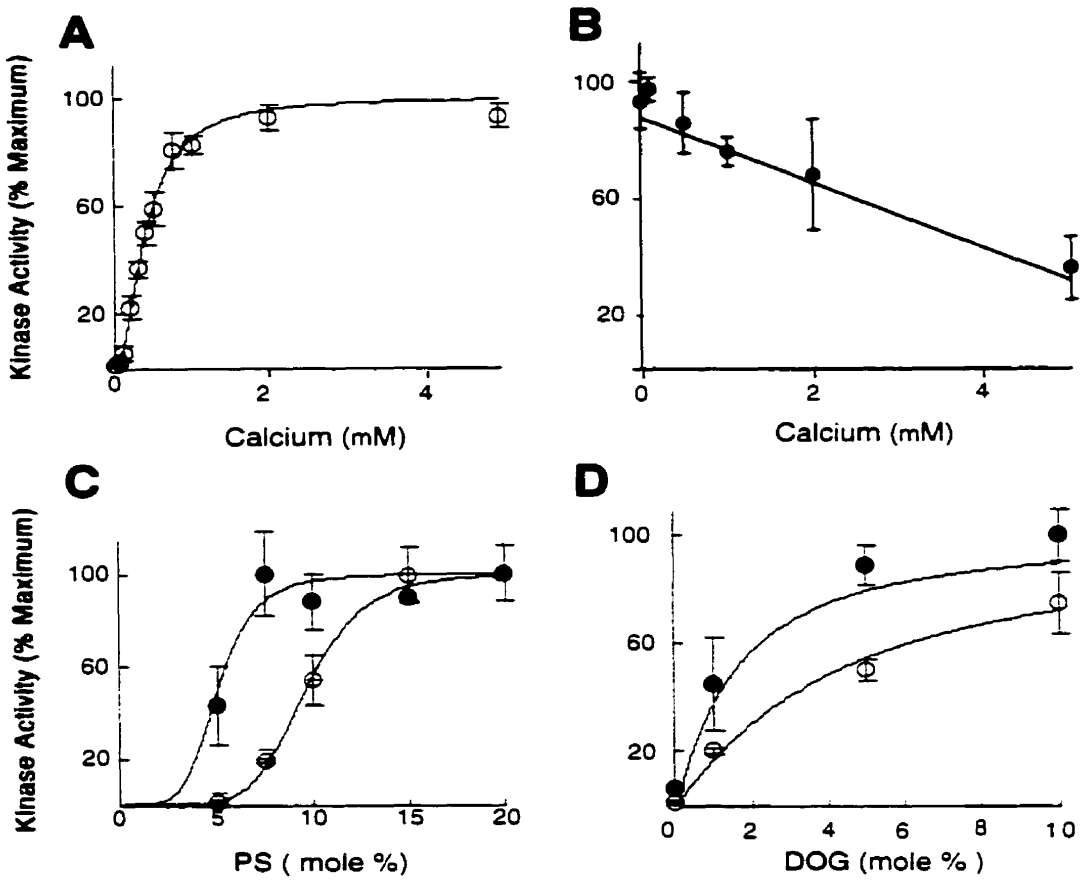
5
10
15
20
PS (mole %)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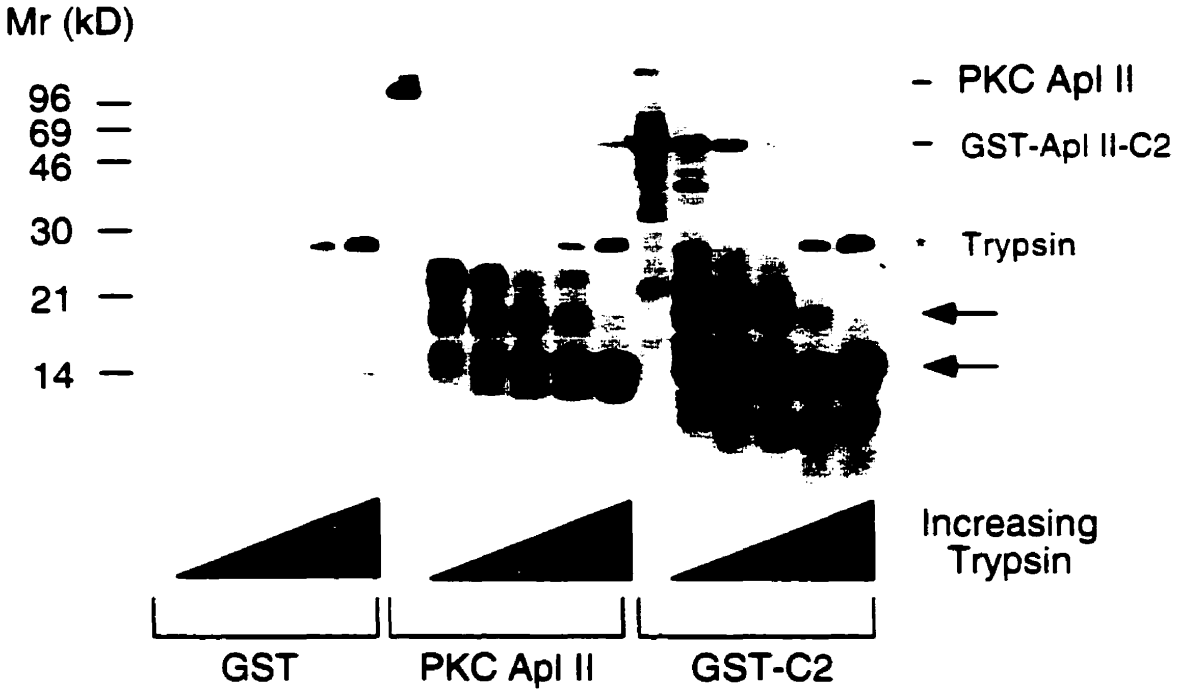
B
C
D
Kinase Activity (% Maximum)
Calcium (mM)
Calcium (mM)
Kinase Activity (% Maximum)
PS (mole %)
DOG (mole %)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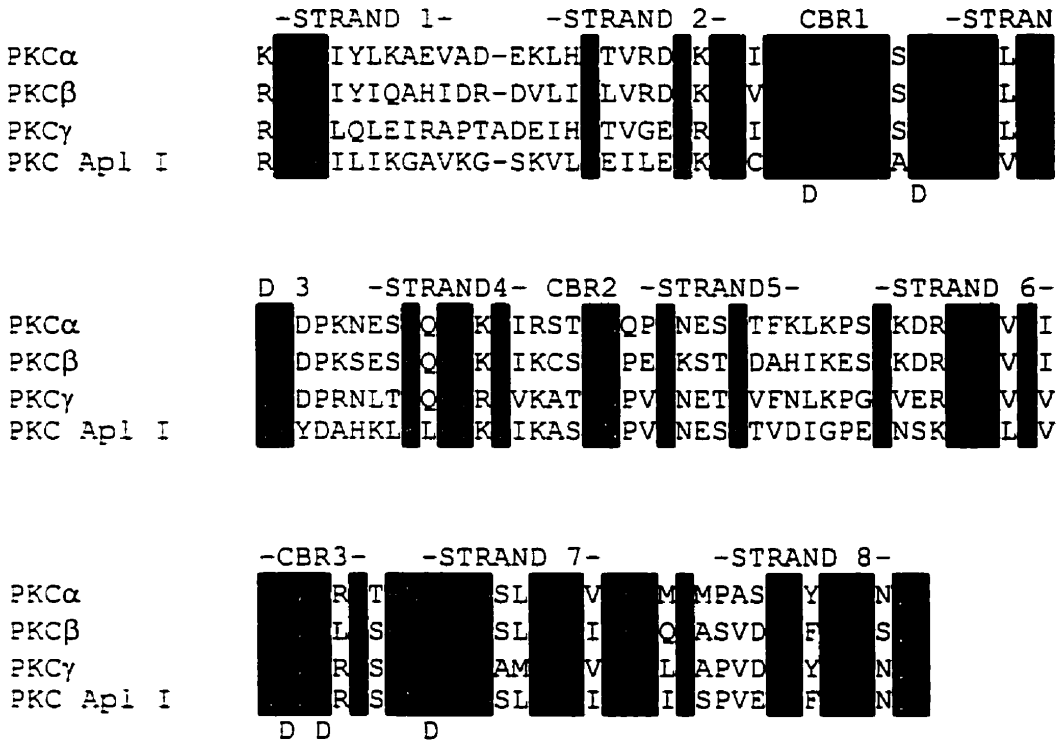
69
46
30
21
14
PKC Apl II
GST-Apl II-C2
Trypsin
Increasing Trypsin
GST
PKC Apl II
GST-C2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PKCγ
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D
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-STRAND 6-
-CBR3-
-STRAND 7-
-STRAND 8-
D D
D

PREFACE TO CHAPTER 4

Together, the previous two chapters reveal an inhibitory role for the C2 domain of the Ca^{2+}-independent PKC Apl II in *Aplysia*. Evidence suggests that this inhibitory role of the C2 domain is a consequence of the domain's inability to bind lipid. In cells, the lack of C2 domain-lipid interactions may prevent kinase translocation to membranes or localization to cellular microdomains. Although Ca^{2+}-independent PKCs do not bind Ca^{2+}, they do possess a phosphorylation site in loop 1, supporting an alternate mechanism of lipid interaction. Indeed, differences between C2 domain types have led to the suggestion that there are diverse sub-classes of C2 domains that have evolved equally diverse lipid binding mechanisms (Pappa et al., 1998).

In this final chapter, we identify two autophosphorylation sites, serine 2 and serine 36, in the C2 domain of the Ca^{2+}-independent PKC Apl II. Importantly, phosphorylation of serine 36 increased binding of the C2 domain to phosphatidylserine membranes *in vitro*. In cells, nPKC Apl II phosphorylation at serine 36 was increased by PKC activators and nPKC Apl II phosphorylated at this position translocated more efficiently to membranes. Moreover, mutation of serine 36 to alanine significantly reduced membrane translocation of nPKC Apl II. We suggest that translocation of Ca^{2+}-independent PKCs is regulated by phosphorylation of the C2 domain.

CHAPTER 4

Membrane translocation of novel PKCs is regulated by phosphorylation of the C2 domain

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I. ABSTRACT

Ca^{2+}-independent or novel PKCs (nPKCs) contain an N-terminal C2 domain of unknown function. Removal of the C2 domain of the *Aplysia* nPKC Apl II allows activation of the enzyme at lower concentrations of phosphatidylserine, suggesting an inhibitory role for the C2 domain in enzyme activation. However the mechanism for C2 domain-mediated inhibition is not known. Mapping of the autophosphorylation sites for PKC Apl II reveals four phosphopeptides in the regulatory domain of PKC Apl II, two of which are in the C2 domain at serine 2 and serine 36. Unlike most PKC autophosphorylation sites, these serines could be phosphorylated in trans. Interestingly, phosphorylation of serine 36 increased binding of the C2 domain to phosphatidylserine membranes *in vitro*. In cells, PKC Apl II phosphorylation at serine 36 was increased by PKC activators and PKC phosphorylated at this position translocated more efficiently to membranes. Moreover, mutation of serine 36 to alanine significantly reduced membrane translocation of PKC Apl II. We suggest that translocation of nPKCs is regulated by phosphorylation of the C2 domain.

II. INTRODUCTION

Protein kinase Cs (PKCs) are a family of lipid-activated enzymes that play important roles in many cellular processes including regulation of synaptic strength in the nervous system (Tanaka and Nishizuka, 1994). We have studied the regulation of PKC in *Aplysia*, an important model system for synaptic plasticity, where PKC plays several important roles in regulating synaptic strength (Ghirardi et al., 1992; Manseau et al., 1998; Sossin et al., 1994; Sugita et al., 1992, 1997). The structure of PKCs is conserved in *Aplysia*. Ca^{2+}-activated PKCs (cPKCs) in vertebrates (α, β, γ) and invertebrates (cPKC Apl I in *Aplysia*) have two C1 domains and a C2 domain. Ca^{2+}-independent PKCs (nPKCs) in vertebrates (δ, ε, η, and θ) and invertebrates (nPKC Apl II in *Aplysia*) also have two C1 domains and a C2 domain. However, the C2 domain in these kinases is N-terminal to the C1 domains and lacks several of the critical aspartic acids necessary for Ca^{2+} binding (Nalefski and Falke, 1996).

In cPKCs, the C2 domain mediates Ca^{2+}-dependent binding to the membrane lipid phosphatidylserine (PS) (Newton, 1995a,b; Pepio et al., 1998). This binding is believed to be the primary step in kinase activation: First, it transiently recruits the enzyme to the membrane where its physiological activator, diacylglycerol (DAG), resides; second, in conjunction with the C1 domain interacting with DAG, binding of the C2 domain to PS induces a conformational change that activates the enzyme (Medkova and Cho, 1999; Oancea and Meyer, 1998). Finally, the C2 domain binds to the receptor for activated C kinase (RACK) and this binding is important for the location of PKC translocation and for cellular functions (Mochly-Rosen and Gordon, 1998).

In contrast to cPKCs, a detailed model for the role of the N-terminal C2 domain in nPKC activation is not available. C2 domains of nPKCs do not bind constitutively to lipids and are not regulated by calcium (Pepio et al., 1998). However, similar to cPKCs, C2 domain derived peptides from nPKCs act as isoform specific inhibitors and activators in cells, implicating the C2 domain in the regulation of enzymatic activity (Dorn et al., 1999; Gray et al., 1997; Yedovitzky et al., 1997). Removal of the C2 domain of PKCη does not perturb the enzyme's substrate specificity or response to PI 3-kinase activity (Dekker et al., 1993; Palmer et al., 1995). However, removal of the C2 domain of nPKC Apl II, decreases the amount of PS necessary for kinase activity, implicating the C2 domain in kinase regulation (Sossin et al., 1996a).

In vitro, nPKC Apl II is activated more poorly than cPKC Apl I, even when calcium is removed from cPKC Apl I, suggesting a requirement of additional activators for stimulation of nPKC Apl II (Sossin et al., 1996a; Pepio et al., 1998). Comparisons between nPKCε and cPKCα also indicate that nPKCs require additional factors to bind efficiently to lipid membranes (Medkova and Cho, 1998a). nPKC Apl II can be activated under a number of physiological conditions in cells, usually after prolonged activation of signal transduction pathways (Sossin et al., 1996b; Sossin et al., 1994). Prolonged activation also induces autonomous activity of nPKC Apl II (Sossin, 1997). This activation is specific, as cPKC Apl I does not become autonomous under these conditions. The autonomous activity does not result from proteolysis to form a PKM, but probably results from post-translational modifications in the regulatory domain of nPKC Apl II (Sossin, 1997).

PKCs are regulated by phosphorylation. Phosphorylation in the activation loop by phosphoinositide-dependent protein kinase I (PDK-1) is required for subsequent PKC "maturation" by autophosphorylation at two residues critical for enzyme stability and activity (Dutil et al., 1998; Le Good et al., 1998; Newton, 1995b). Phosphorylation of nPKCδ on tyrosine residues is thought to be important for activation of the kinase (Blake et al., 1999; Brodie et al., 1998; Song et al., 1998). Upon activation, PKCs additionally phosphorylate themselves on several residues. The roles for these phosphorylations are still unknown. Autophosphorylation at a conserved site in the carboxyl-terminal domain has been associated with persistent activation of PKC and may be involved in removal of the enzyme from the membrane (Nakhost et al., 1999; Sweatt et al., 1998). A phosphorylation site has also been identified in the C2 domain of vertebrate cPKCα that is correlated with PKC activation (Ng et al., 1999). Autophosphorylation of the epsilon-eta family of nPKCs, that includes the invertebrate kinases nPKC Apl II in *Aplysia*, *Drosophila* PKC98F, and *C. elegans* PKCI has not been investigated.

In this paper, we identify two autophosphorylation sites, serine 2 and serine 36, in the C2 domain of nPKC Apl II. Phosphorylation at serine 36 increases lipid binding to the C2 domain and increases translocation of PKCs in cells. We suggest that phosphorylation of C2 domains in nPKCs is important for regulating their translocation.

III. MATERIALS AND METHODS

Reagents. 4-β-Phorbol 12,13-dibutyrate (LC Services); dioleoyl phosphatidylserine (PS), dioleoyl phosphatidylcholine (PC), (Avanti Polar Lipids Inc., Alabaster, AL); Triton X-100 (Avanti); prestained molecular weight markers (Amersham/Pharmacia, Oakville, ON); N-tosyl-L-phenylalanine chloromethyl ketone (TPCK) -treated trypsin (Worthington Biochemicals, Freehold, NJ), thin layer cellulose plates (Merck, darmstadt, Germany). All other reagents were of the highest grade available.

Plasmid construction of PKCs, fusion proteins, and mutants. Full-length clones of nPKC Apl II were present in bluescript SK and the baculovirus vector BB3 (Sossin et al, 1996a). Initially, nPKC Apl II was excised from baculovirus BB3 using Sac I/Sal I and inserted into the new vector BB4 (Invitrogen) at Sac I/Sal I. The C2 domain S36A and S36E mutations were generated with a two-step mutagenic procedure using the polymerase chain reaction (PCR). First round PCR used the C2 domain of nPKC Apl II in the pMALC-C2 plasmid (New England Biolabs) as a template and either the outside 5' primer (O5) and the inside 3' primer (I3) or the inside 5' primer (I5) and the outside 3' primer (O3) (see Table 4.1 for primers). The products from the first round synthesis were combined and used as the template for second round synthesis using O5 and O3. The resultant product was cut with appropriate enzymes (Table 4.1) and inserted into nPKC Apl II in the BB4 vector. A new site was formed by the mutagenesis (Table 4.1) and was used to confirm the cloning. The mutations were also made in the pMAL-C2 vector encoding the MBP-C2 fusion protein using a similar procedure. The final product was cut with appropriate enzymes (Table 4.1)

and inserted into the MBP-C2 domain at the indicated cloning sites (Table 4.1). Serine 68 → alanine and serine 2 → alanine were both made with a similar strategy using different inside primers and the same outside primers (Table 4.1). A clone of human nPKCε was a kind gift of A. Toker (Harvard Medical School). The construct was amplified with PCR using VENT DNA polymerase (New England Biolabs, Beverly, MA) and inserted into BB4 at Kpn I and BamH I. The construct lacking the C2 domain used an alternative 5' primer containing the initiating methionine of nPKCε followed by amino acid 145. GST and MBP-C2 fusion proteins and MBP-C2 deletion constructs were made previously (Pepio et al, 1998; Sossin et al, 1996a).

Baculovirus construction. *Spodoptera frugiperda* (Sf9, Sf21; Invitrogen) cells were grown in suspension cultures with supplemented Grace's media (Life Technologies, Inc., Gaithersburg, MD) and 10% fetal bovine serum (Life Technologies). The baculovirus transfer vectors were cotransfected with linearized baculovirus (Invitrogen), and the resultant blue colonies were plaque-purified. Positive colonies were confirmed by PCR and by immunoblotting with anti-PKC Apl II or anti-PKCε antibodies (Kruger et al., 1991; CalBiochem). High titer viral stocks (10^8-10^{10} plaque-forming units [PFU]/ml) were generated as described (Sossin et al., 1996a).

PKC purification from baculovirus. Biochemical purification of all *Aplysia* PKCs, human nPKCε and mutants was performed as previously described (Sossin et al., 1996a) with the following modifications. Briefly, 250 ml of Sf21 cells (2×10^6 cells/ml) were infected with 1 $\times$ 10^9 PFUs of baculovirus and incubated for 64 h for optimal PKC expression. Cells were

pelleted, resuspended and sonicated, and centrifuged as described. The supernatant solution was passed through a 0.22 μm filter, diluted to 50 ml with buffer A (20 mM Tris-HCL, pH 8.0, 0.5 mM EGTA, 0.5 mM EDTA, 10 mM 2-mercaptoethanol, 10% glycerol) and loaded onto a Mono-Q column (Bio-Rad, Richmond, CA) previously equilibrated in buffer A. The remaining steps were performed following previous protocols (Sossin et al., 1996a). All baculovirus expressed enzymes were aliquoted and stored frozen at -70 °C until needed.

Fusion protein synthesis. Both a maltose binding protein (MBP) and a glutathione S-transferase (GST) fusion protein were expressed in bacterial DH5α cells from either a pMAL-C2 plasmid (New England Biolabs) or a pGEX 5x.1 plasmid (Pharmacia). These plasmids contained an insert of nPKC Apl II amino acids 1-155 corresponding to the C2 domain (Pepio et al, 1998; Sossin et al, 1996a). The MBP-C2 domain and GST-C2 domain fusion proteins were purified by affinity chromatography on amylose (New England Biolabs) or GST resin columns (Pharmacia), respectively.

Antibody production and immunoblotting. We synthesized a peptide (CRLQKG[pS]TKEK) corresponding to amino acids 30-40 of nPKC Apl II's C2 domain with Ser-36 converted to phosphoserine (Quality Controled Biochemicals, MA) to generate a phosphopeptide antibody. The peptide was conjugated to bovine serum albumin maleimide (Pierce) via the cysteine, and injected intramuscularly into rabbits along with TitreMax Gold (Cytrx, Norcross, GA) three times at 4 week intervals. Serum was affinity purified and used in western blots as described (Dyer et al., 1998) with the phosphorylated peptide antibody at 1μg/ml dilution, and goat anti-rabbit, horseradish peroxidase-conjugated secondary antibody

at 1 μg/ml. The phosphopeptide antibody was preabsorbed using the nonphosphorylated form of the peptide at 1 μg/μl for 30 min prior to its addition to the immunoblot. Results were visualized by enhanced chemiluminescence (NEN Life Science Products).

Lipid preparation. Sucrose-loaded large unilamellar vesicles containing trace [^{3}H]PC were prepared. Briefly, lipid mixtures in chloroform were dried under a stream of nitrogen and resuspended in buffer (20 mM HEPES, pH 7.5, 170 mM sucrose). They were then subjected to 5 freeze-thaw cycles in liquid nitrogen, and extruded through 2 stacked 0.1 μm polycarbonate filters using a Liposofast microextruder (Avestin, Inc., Ontario, Canada) as described (Mosior and Newton, 1995; Rebecchi et al., 1992). Phosphatidylserine liposomes were prepared by resuspending dried lipids in buffer and vortexing (Sossin et al., 1996a).

PKC assays. Protein kinase C (5 nM) activity was assayed using phorbol esters (Sossin and Schwartz, 1992) or the mixed micelle assay (Sossin et al., 1996a).

PKC and fusion protein in vitro phosphorylation. PKCs (200-500nM) or PKC derived C2 domain fusion proteins (2 μM) were incubated in reaction buffer (50 mM Tris-HCL, pH. 7.5, 10 mM $MgCl_2$, 5 mM EGTA) with 50 μg/ml dioleoyl phosphatidylserine, 20 nM 12-O-tetradecanoyl phorbol-13-acetate (TPA), and [γ-^{32}P]ATP (4 μCi in 50 μM ATP) for 30 min at RT. Phosphorylation was quenched by the addition of 5 × Laemmli sample buffer and boiled.

C2 domain membrane-binding assay. Membrane binding of C2 domain constructs was determined by measuring the binding to sucrose-loaded vesicles or liposomes, as described

(Johnson et al., 1997; Mosior and Newton, 1995; Rebecchi et al., 1992, Sossin et al., 1996a). MBP-fusion proteins on amylose beads were phosphorylated by PKC as described above. PKC, ATP, and lipids were removed by sedimenting the beads at low speeds (3000 x g for 1 min) followed by three washes with sedimentation buffer (0.3 mg ml^{-1} Ovalbumin, 100 mM KCl, 1 mM DTT, 5 mM $MgCl_2$, 20 mM HEPES pH 7.5). Fusion proteins were eluted from the beads with sedimentation buffer containing 10 μM maltose. The purified fusion proteins were prespun to pellet aggregates for 30 min at 100,000 × g and 25 °C in a TLA-100 ultracentrafuge (Beckman, Palo Alto, CA). Soluble fusion proteins (2 μM) were incubated with sucrose-loaded vesicles (20 μM lipid) for 10 min at 15 °C. The proteins associated with the sucrose loaded vesicles were then separated from free protein by ultracentrifugation at 100,000 × g for 30 min at 15 °C. Pelleting of lipids was confirmed using scintillation counting of ^{3}H-PC which was included as a tracer in the sucrose loaded vesicles or liposomes. Membrane-bound (pellet fraction) and free (supernatant fraction) C2 domain fusion protein was separated by SDS-PAGE and transferred to nitrocellulose membranes. Total protein was visualized by Ponceau-S staining and phosphorylated protein by autoradiography using Kodak BioMax MS film on the membrane. In several cases, quantitation of the Ponceau-S staining was confirmed by immunoblotting using an antibody to the maltose binding protein. In some experiments, the fusion protein was excised from the gels and the amount of radioactivity incorporated calculated by scintillation counting with correction for quenching. This experiment demonstrated that less than 5% of the total fusion protein was phosphorylated under these conditions.

Cell fractionation. Recombinant baculovirus encoding wild type or S36A nPKC Apl II was incubated with Sf-21 cells in media at 27 °C. After 72 h of infection, cells were incubated with vehicle (control) or 4-β-PDBu (experimental) for 1 hr at 27 °C. Cells were then harvested and lysed in homogenization buffer (50 mM Tris, pH 7.5, 1 mM EGTA, 10 mM $MgCl_2$, 2.6 mM 2-mercaptoethanol, 20 mg/ml aprotinin, 5 mM benzamadine, 0.1 mM leupeptin, 50 mM NaF, 5 mM sodium pyrophosphate (pH 8.5), and 1 μM microcystin). The lysate was centrifuged at 100,000 × g for 30 min at 4 °C to separate the cytoplasmic fraction (supernatant) from the membrane/cytoskeleton fraction (pellet). Equal protein fractions of the supernatant and pellet fractions were analyzed by SDS-PAGE (9% acrylamide gels), transferred to nitrocellulose membranes, and immunoblotted with the nPKC Apl II antibody and the phospho-S36 antibody.

Phosphorylation and partial tryptic digestion. Purified nPKC Apl II or nPKC Apl IIΔC2 was autophosphorylated as described (Sossin et al., 1996a). The phosphorylation reaction contained 10 pmoles of nPKC Apl II or nPKC Apl IIΔC2, 50 μM ATP, reaction buffer (80 mM Tris, pH 7.5, 10 mM EGTA, 20 mM $MgCl_2$, 40 nM TPA, and 150 μg/ml dioleoyl phosphatidylserine) and proceeded for 30 min at RT in the presence of [γ-^{32}P]ATP. Phosphorylation was quenched by the addition of 5 μM inhibitory peptide. The 75 μl proteolysis reaction contained 10 pmoles of either phosphorylated nPKC Apl II or nPKC Apl IIΔC2 and 0.1% Triton X-100. The phosphorylated kinase component was incubated for 3 min at 30 °C and proteolysis was initiated by addition of 2 μg/ml of TPCK-treated trypsin and incubated for an additional 5 min at 30 °C. Reactions were quenched by addition of 20 μl Laemmli sample buffer and boiled. Proteins were separated by SDS-PAGE on 7% gels

and electrophoretically transferred to nitrocellulose. Membranes were subjected to autoradiography to visualize phosphate incorporation into proteins and immunoblot with the anti-C2 domain antibody for identification of C2 domain containing protein fragments (Pepio et al., 1998).

Analysis of tryptic peptides by two-dimensional thin layer chromatography. Analysis was performed as described with several modifications (Boyle et al., 1991; Dyer et al., 1998). Membrane fragments containing protein bands were preblocked in 0.5% PVP-360 in 100 mM acetic acid, washed, and subsequently digested overnight with 10 μg of TPCK-treated trypsin in 10 mM Tris (pH 7.5) and 5% acetonitrile. An additional 10 μg of trypsin was added to each band for 3 h the following day and the supernatant solution containing desired peptides was lyophilized to completion. Peptides were resuspended in pH 1.9 buffer (0.6 M formic and 1.4 M acetic acid) and analyzed by two-dimensional TLC. Analysis was performed using a Hunter-3000 HTLC for primary dimension electrophoresis and phosphochromatography (n-butanol:pyridine:acetic acid:water; 37.5:25:7.5:30) for secondary dimension peptide separation. Labeled peptides were visualized by autoradiography using Kodak BioMax MS film on the plate. Predicted migrations of phosphopeptides were calculated as described (Boyle et al., 1991).

Phosphoamino acid analysis. After tryptic digestion and lyophilization, nPKC Apl II or C2 fusion protein peptides derived from *in vitro* phosphorylations were hydrolyzed to constituent amino acids using constant boiling 6 N HCl for 2.5 h at 110 °C. Sample amino acids were mixed with phosphoserine, phosphothreonine, and phosphotyrosine standards

prior to running the mixture in the primary TLC dimension using standard buffers (Boyle et al., 1991). Phosphoamino acid standards were stained with 0.25% ninhydrin in acetone, and autoradiography was performed on the completed plate.

Data analysis. Immunoblots and autoradiograms were scanned, and analysis was performed using the public domain NIH Image program (developed at the National Institutes of Health and available on the Internet). We calibrated the program with the uncalibrated OD feature of NIH Image, which transforms the data using the formula $y = \log_{10} (255/(255-x))$, where x is the pixel value (0-254). Control experiments demonstrated that after this calibration, values were linear with respect to the amount of protein over a wide range of values (Nakhost et al., 1998).

IV. RESULTS

The C2 domain of nPKC Apl II contains 2 autophosphorylation sites

In order to determine whether the C2 domain of nPKC Apl II is regulated by autophosphorylation, we compared tryptic phosphopeptide maps generated after autophosphorylation of purified nPKC Apl II or a purified nPKC Apl II mutant lacking the C2 domain (nPKC Apl IIΔC2). nPKC Apl II had 5 reproducible phosphopeptides (Fig. 1A). Two of these phosphopeptides (#2 and #3) were not found in the enzyme lacking the C2 domain (Fig. 1B and C). The absence of phosphopeptides #2 and #3 could be due to their location in the C2 domain, or conformational changes due to the loss of the C2 domain. To obtain further evidence these sites were actually located in the C2 domain, we obtained tryptic phosphopeptide maps of C2 domain-containing regions of nPKC Apl II after partial tryptic digests of autophosphorylated nPKC Apl II and nPKC Apl IIΔC2 (Fig. 2A). The C2 domain containing fragments were identified based on immunoreactivity to a C2 domain-specific antibody (Fig. 2B) and by their absence in partial tryptic digests of nPKC Apl IIΔC2. (Fig. 2B, lane 2). Three bands were isolated from the gel and completely digested with trypsin for phosphopeptide mapping analysis (Fig. 2C). The largest C2 domain fragment, corresponding to the size of the intact regulatory domain contained phosphopeptides #2, #3, #4 and #5 suggesting that all four of these sites are present in the regulatory domain (Fig. 2C, upper panel). Phosphopeptide #1 was not further investigated but based on predicted mobility it may correspond to the carboxyl-terminal fragment that contains two predicted phosphorylation sites (Nakhost et al., 1999). The C2 domain core itself is quite resistant to tryptic digestion (Pepio et al., 1998). Phosphopeptide mapping of the smallest C2 domain

fragment corresponding to the size of the core exhibited only phosphopeptide #3 (Fig. 2C, lower panel). Thus, phosphopeptide #3 is contained in the C2 domain, while phosphopeptide #2 is either near the end of the C2 domain and is sensitive to tryptic digestion, or is outside the C2 domain and its phosphorylation is decreased by C2 domain removal.

The autonomous nPKC Apl II is not autophosphorylated at site #4

Purified nPKC Apl II contains both regulated and autonomous activities (Sossin, 1997). The autonomous activity generated by purification is similar to the autonomous form of nPKC Apl II that is found in the nervous system. To determine differences between autonomous and regulated kinase activities, we compared tryptic phosphopeptide maps of nPKC Apl II that was autophosphorylated in the absence of activators (autonomous activity), or after activation by phorbol esters (Fig. 3A). Autophosphorylation of peptide #4 was greatly decreased in the autonomous kinase (Fig. 3B). Quantitation of the relative phosphorylation of peptide #4 in three separate experiments from two different purified kinase preparations confirmed this result (Fig. 3C). This could be due to existing phosphorylation of the site corresponding to peptide #4 in this kinase, or to a conformational change in the regulatory domain that reduces phosphorylation of this site.

Fatty acids do not preferentially autophosphorylate the C2 domain

nPKC Apl II also can be stimulated by fatty acids. Autophosphorylation induced by fatty acids can be distinguished from that induced by phorbol esters since removal of the C2 domain greatly decreases fatty acid induced autophosphorylation without affecting phosphorylation induced by phorbol esters (Sossin et al, 1996a). To determine if this

difference is due to selective fatty-acid induced phosphorylation of the C2 domain, we compared PKC that was autophosphorylated in the presence of oleic acid, or in the presence of phorbol esters (Fig. 3A). Phosphorylation of phosphopeptides #2 and #3 by oleic acid and PS-TPA was comparable (Fig. 3B). Quantitation of the relative phosphorylation in three separate experiments showed no significant difference between oleic acid and phorbol ester stimulation (Fig. 3C). Thus, the selective requirement of the C2 domain for fatty-acid induced phosphorylation is not due to preferential autophosphorylation of this domain.

Sites in the C2 domain can be trans-phosphorylated

Incubation of nPKC Apl II, or cPKC Apl I (data not shown) with fusion proteins containing the C2 domain led to trans-phosphorylation of the C2 domain (Fig. 4A). Phosphopeptide mapping results of both trans-phosphorylated GST- and MBP-C2 domain fusion proteins indicated two major C2 domain phosphorylation sites (Fig. 4B and C) that are not present in GST or MBP alone (Fig. 4B and C and data not shown). Co-application of phosphopeptides derived from autophosphorylated nPKC Apl II and the trans-phosphorylated GST-C2 domain fusion protein demonstrated that phosphopeptide #3 was identical in the autophosphorylated kinase and in the trans-phosphorylated C2 domain (Fig. 4D). An additional phosphopeptide (#2') was also observed in both fusion proteins, whose migration was slightly altered from phosphopeptide #2 in autophosphorylated nPKC Apl II.

Site #2 is serine 2

We used a series of C2 domain deletion constructs (Sossin et al., 1996a) to attempt to delineate the sites of trans-phosphorylation that corresponded to the various

phosphopeptides. However, deleting any area of the C2 domain prevented phosphorylation of peptide #3 suggesting that phosphorylation of this site is sensitive to conformation of the C2 domain (data not shown). In contrast, an N-terminal deletion but not a C-terminal deletion (data not shown) removed the site that produced phosphopeptide #2'. Site #2 is predicted to be at the N-terminal of the C2 domain. This tryptic peptide, in the C2 domain fusion proteins, would be altered by the addition of two amino acids to the sequence between the fusion partner and the C2 domain (MSR → ASMSR). This is consistent with the change from site #2 to the new site #2'. An N-terminal location would also be consistent with the loss of phosphopeptide #2' from the C2 domain by the partial tryptic digestion (Fig. 2C, lower panel). Indeed, conversion of serine 2 to alanine prevented site #2 phosphorylation (data not shown).

The major autophosphorylation site of nPKC Apl II is serine 36

Phosphoamino acid analysis of phosphopeptides #2, #3 and #5 revealed them all to be serines (data not shown). Using a hypothetical phosphopeptide map of regulatory domain tryptic peptides, and assuming phosphopeptide #2 is M[pS]R, there were only two possible serines (serine 36 and serine 68) that were consistent with the migration of phosphopeptide #3 (Fig. 5A). Conversion of serine 68 to alanine did not affect peptide #3 phosphorylation (data not shown). In contrast conversion of serine 36 to alanine prevented peptide #3 phosphorylation in both the trans-phosphorylated fusion protein (Fig. 5B-D) and the intact kinase (Fig. 5E-G).

C2 domain phosphorylation at serine 36 promotes lipid binding

The C2 domain of nPKC Apl II does not bind to phosphatidylserine membrane preparations using vesicles (Sossin et al., 1996a), lipid micelles (Pepio et al., 1998) or extruded sucrose loaded vesicles (Fig. 6A; quantitated in Fig. 6C). In contrast, the C2 domain showed significant binding to extruded sucrose loaded vesicles (60%PS:40%PC) after trans-phosphorylation by nPKC Apl II (Fig. 6B; quantitated in Fig. 6C). This result was also observed using PS vesicles (data not shown). In these experiments less than 5% of the fusion protein was phosphorylated (see Materials and Methods), and thus phosphorylated protein did not contribute significantly to the measured translocation of total fusion protein. The binding was reduced if the vesicles contained only PC (Fig. 6B; quantitated in Fig. 6C). Conversion of serine 36 to alanine significantly decreased binding of the phosphorylated fusion protein to the lipid vesicles (Fig. 6B; quantitated in Fig. 6C, $p<0.05$ unpaired two-tailed students t-test between the percentage of phosphorylated protein pelleted of wild type and S36A fusion proteins)., while conversion of serine 68 to alanine did not decrease binding (data not shown). In contrast, conversion of serine 36 to glutamic acid significantly increased binding of the fusion protein to lipids, even in the absence of phosphorylation (Fig. 6A-C). These results suggest that phosphorylation of serine 36 increased lipid binding. Phosphorylation of serine 2 may also be involved in binding since the phosphorylated form of both the S36-E and S36-A fusion proteins bound lipids better than did their non-phosphorylated form (Fig. 6A, quantitated in Fig. 6B).

The conversion of serine 36 to glutamic acid caused a small shift in the position of the protein on SDS-PAGE gels (Fig. 6A). This band co-migrated with a phosphorylated form of

the wild-type fusion protein (Fig. 6A). The form of the fusion protein migrating at this position bound better to the lipid vesicles than did the lower band (Fig. 6A). These results suggest that phosphorylation caused a conformational change involved in lipid binding. However, phosphorylation was neither necessary nor sufficient to cause the shift, since i) phosphorylated wild type protein incubated with PC vesicles alone rarely exhibited the shift in molecular weight (Fig. 6A) and ii) in experiments using higher concentrations of fusion proteins and sucrose loaded vesicles, the shifted band was seen in the non-phosphorylated wild type protein, and even in the non-phosphorylated S36A fusion protein (data not shown). These results are consistent with a model whereby a conformational shift is required for lipid binding and phosphorylation of the fusion protein enhances the stability of this conformation.

Conversion of serine 36 to alanine or glutamic acid does not affect enzyme activation

Removal of the C2 domain allows kinase activation at lower concentrations of PS using the mixed micelle assay. To determine if mutations at serine 36 could mimic this effect we assayed nPKC Apl II S36A and S36E and wild type nPKC Apl II in the mixed micelle assay. No difference was seen in the concentration of PS required for activation of nPKC Apl II S36A or S36E compared to the wild type control (Fig. 7). The lack of an effect of the S36E mutation suggests that either phosphorylation of this site is not important for removing C2 domain inhibition or that the glutamic acid does not mimic phosphorylation of serine 36. While the conversion to glutamic acid does allow for lipid binding of the isolated C2 domain, there may be differences between the phosphorylated residue and the glutamic acid in the context of the whole protein.

Characterization of a phospho-specific antibody to serine 36

In order to examine phosphorylation of serine 36 in cells we generated a phosphopeptide antibody to serine 36 (phospho-S36). We tested the specificity of the antibody using autophosphorylated, purified nPKC Apl II and nPKC Apl II S36A (Fig. 8A). Purified nPKC Apl II showed some reactivity with the phospho-peptide antibody and stimulation of nPKC Apl II by either PS-TPA or oleic acid resulted in a large increase in immunoreactivity with the antibody (Fig. 8A, upper panel). In contrast, no immunoreactivity was seen with Apl II S36A either before or after stimulation despite the ability of this enzyme to autophosphorylate itself as measured using incorporation of [γ-^{32}P]ATP (Fig. 8A, middle panel). Thus, this antibody was specific for phosphorylation of serine 36.

Phosphorylation of serine 36 in cells

The phosphopeptide antibody to serine 36 recognized nPKC Apl II over-expressed in Sf21 cells (Fig. 8B). Immunoreactivity was specific for phosphorylated proteins as no immunoreactivity is seen when nPKC Apl II S36A was expressed to the same levels (Fig. 8B and C). Immunoreactivity increased after addition of PDBu to the cells (150% ± 30% n=3, S.E.M.) consistent with serine 36 being autophosphorylated in cells (Fig. 8C). PDBu also translocated serine 36 phosphorylated PKC (Fig. 8C). The relative percent translocation of serine 36 phosphorylated PKC was increased dramatically compared to total nPKC Apl II (690% ± 300% compared to 90% ± 30% n=3, S.E.M.) consistent with a role for phosphorylation in translocation. However, this large increase in percentage translocation is probably also related to a pool of misfolded PKC after overexpression in Sf21 cells. Misfolded PKC would neither translocate nor be autophosphorylated at serine 36, thus

decreasing the relative translocation of the non-phosphorylated pool of nPKC Apl II. Phosphorylation was not sufficient for translocation, since in the absence of PDBu, phosphorylated protein was found mainly in the supernatant. Similarly, the distribution of nPKC Apl II S36E was not different than that of the wild-type enzyme (data not shown) and translocation of Apl II S36E was similar to wild type PKC Apl II (60% ± 18% n=3, S.E.M). Most importantly, the relative percent translocation of nPKC Apl II S36A was impaired compared to wild type nPKC Apl II (20% ± 20% compared to 90% ± 30% n=3, S.E.M.), suggesting that phosphorylation is required for proper translocation of PKC. Thus, even though only a small percentage of wild-type enzyme is phosphorylated at any given point in time, wild-type protein translocation can be assisted by transient phosphorylation, leading to increased translocation of wild-type protein compared to the S36A mutation.

Conservation of C2 domain phosphorylation in vertebrate nPKCε

To determine if other nPKCs contain C2 domain phosphorylation sites we examined the autophosphorylation of purified wild type vertebrate nPKCε and of a mutant nPKCε lacking the C2 domain (nPKCεΔC2) after expression in Sf21 cells as we did for nPKC Apl II. Autoradiograms indicate that nPKCεΔC2 is considerably less autophosphorylated than wild type nPKCε (Fig. 9A and B) and it appears that the major autophosphorylation site of nPKCε is in the C2 domain (Fig. 9C). While these results are consistent with a conserved role for C2 domain phosphorylation, future studies are needed to determine whether this phosphorylation regulates C2 domain lipid binding and whether phosphorylation of the C2 domain is present in all nPKCs.

V. DISCUSSION

Autophosphorylation of nPKC Apl II. A major site for autophosphorylation in nPKC Apl II is serine 36 in the C2 domain, and nPKC Apl II is probably also autophosphorylated at serine 2 in the C2 domain. While these autophosphorylations are likely due to cis-autophosphorylation (initial studies suggest single order kinetics), unlike many PKC phosphorylations, serines 2 and 36 can be phosphorylated in trans. Furthermore, serine 36 is contained in a consensus PKC phosphorylation site (Fig. 10A). At this time, we do not know whether serine 2 and serine 36 are cis or trans-phosphorylated in cells. Serine 36 was trans-phosphorylated by cPKC Apl I *in vitro*, however we did not see phosphorylation of a kinase-dead mutant of nPKC Apl II when co-expressed in Sf21 cells with cPKC Apl I (data not shown).

The autonomous kinase was not autophosphorylated at peptide #4. We have not directly determined the sequence of peptide #4, but based on partial tryptic digests it is likely to be in the regulatory domain. Furthermore, based on predicted tryptic phosphopeptide mobilities, both peptides #4 and #5 are in the hinge domain (Fig. 5A). It seems unlikely, that phosphorylation in the hinge domain would be sufficient to produce an autonomous kinase, and thus we favor a model for autonomous kinase formation where a conformational change in the regulatory region no longer presents the site in peptide #4 for autophosphorylation. This is consistent with experiments using proteolysis as an assay for hinge domain conformation that found discrete constraints on sensitivity of proteolytic sites in the hinge domain. These constraints changed with conformational changes of the whole enzyme

(Keranen and Newton, 1997). We could consider this as additional evidence that autonomous enzyme formation is a result of conformational changes in nPKC Apl II.

Mechanistic model for C2 domain phosphorylation. The C2 domains of nPKCs contain an extended region in what would be the calcium binding region (CBR) loop 1 as defined in cPKCs. This region forms an alpha helix in PKCδ (Pappa et al., 1998), but there is little sequence conservation in this region in the epsilon/eta family (Nalefski and Falke, 1996), and without a crystal structure of an epsilon-like C2 domain the organization of the loops is still speculative. Serine 36 is contained in the extended carboxyl-terminal region of loop 1 (Fig. 10A). Phosphorylation of serine 36 increases *in vitro* lipid binding and cellular translocation of nPKC Apl II. However, since phosphorylation increases negative charge, it would not be expected to increase binding to acidic lipids by electrostatic interactions directly. We propose a model (Fig. 10B) where phosphorylation of this residue leads to a conformational shift in the loops of the C2 domain that expose a cryptic lipid-binding site. The amino-terminal portion of loop 1 is highly conserved in PKCε-like PKCs and contains a peptide used as a specific inhibitor of PKCε binding to RACK (Fig. 10A) (Gray et al., 1997; Yedovitzky et al., 1997; Dorn et al., 1999). This region might act as the cryptic lipid binding/RACK-binding domain for these nPKCs. In contrast, the extended region of the loop that contains serine 36 is not as well conserved. However, all isoforms contain a phosphorylation site in this region (Fig. 10A) and we have shown that nPKCε contains at least one autophosphorylation site in the C2 domain (Fig. 9).

This hypothesis is consistent with earlier results measuring lipid binding to C2 domain deletion constructs of nPKC Apl II. In these studies, removal of the carboxyl-terminal of the C2 domain permits lipid binding, while removal of the amino-terminal of the C2 domain (containing the phosphorylation site and proposed lipid binding site) does not permit binding (Sossin et al., 1996b). This suggests that the amino-terminal region of the C2 domain contains a site for lipid binding that is normally inhibited by the carboxyl-terminal of the C2 domain. A model for the actions of the PKCε activating peptide also predicts intra-C2 domain interactions (Dorn et al., 1999). This peptide leads to increased isoform-specific membrane translocation of PKCε (Dorn et al., 1999). The peptide is made from a conserved sequence around loop 3 (Fig. 10A) and is believed to interact with loop 1 to increase access of PKCε to RACKS (Dorn et al., 1999). We suggest that phosphorylation of the C2 domain serves the same purpose as the peptide, inhibiting intra-loop interactions to expose a cryptic lipid or RACK binding site. Indeed loop 3 is negatively charged suggesting that introducing a negative charge in loop 1 would inhibit loop 1-loop 3 interactions. Intra-C2 domain interactions have also been shown to be important in regulating synaptotagmin's C2-B domain (Ibata et al., 1998). Different synaptotagmin isoforms show distinct abilities to bind to inositol polyphosphates through a region in the C2 domain. However, differences in binding are not due to the actual inositol polyphosphate binding sequence, which is well conserved in all isoforms. Instead isoform-specific differences map to the carboxyl-terminal of the C2 domain where some isoforms have residues that interact with the inositol polyphosphate binding domain and inhibit binding, while other isoforms lack these residues and exhibit binding (Ibata et al., 1998). Deletion of the carboxyl-terminal of the C2 domain allows inositol polyphosphate binding to all the isoforms (Ibata et al., 1998).

Role of C2 domain phosphorylation in translocation by PDBu. Phorbol esters translocate nPKC Apl II to the membrane in cells, and this translocation appears to be partially dependent on C2 domain phosphorylation at serine 36. While one might expect PDBu mediated translocation to depend only on the C1 domain, mutations in the C2 domain of cPKCs can decrease PDBu mediated translocation, suggesting that both domains are required for translocation (Medkova and Cho, 1998b; Oancea and Meyer, 1998). Moreover, the C2 domain is required for translocation of cPKCs by natural stimuli (Feng et al., 2000). Since there are few differences in lipid and/or phorbol ester-binding between the C1 domains of cPKCs and nPKCs (Pepio et al., 1998), it is likely that the C2 domain is also required for nPKC translocation *in vivo*. Deletions in the C2 domain can block PDBu binding and the presence of the C2 domain decreases the affinity of the C1 domain for PDBu in a PS-dependent fashion (Pepio and Sossin, 1998; Quest and Bell, 1994). The phosphorylation at serine 36 may not only increase lipid binding to the C2 domain, but may decrease C2 domain-mediated inhibition of the C1 domain. However, while conversion of serine 36 to glutamic acid was sufficient to increase binding of the C2 domain to lipids, it did not appear to decrease C2 domain-mediated inhibition of the C1 domain in kinase assays and did not have a dramatic effect in the translocation of PKC. The conformational change in C2 domain that allows binding to lipid may not effect the C2-C1 domain interaction. Alternatively, the conformations may not be identical in the phosphorylated protein and the S36-E fusion protein. Thus, while S36-E is sufficient to induce a lipid binding conformation, it is not sufficient to remove the C2-C1 domain interaction. Future work will be needed to address this issue.

It has also been demonstrated that the C1 and C2 domains work in a concerted fashion to facilitate prolonged activation of PKC (Medkova and Cho, 1998a,b; 1999; Oancea and Meyer, 1998). The transient translocation of PKC is mediated by the C2 domain of the soluble kinase binding to the membrane in the presence of calcium ions (Oancea and Meyer, 1998). However, a more stable and prolonged translocation was associated with DAG binding to the C1 domain in addition to membrane binding by the C2 domain. Thus, there exist two distinct states of PKC membrane association: a low affinity state mediated by the C2 domain alone and a high affinity state mediated by both the C1 and C2 domains acting together. We propose that where calcium recruits the aid of conventional C2 domains to translocate cPKCs, phosphorylation may recruit the aid of novel C2 domains to bind lipids and perpetuate PKC membrane association (Fig. 11). This requirement of the C2 domain to perpetuate nPKC membrane association would act to help rather than hinder kinase activity and may indeed be a mechanism for persistent kinase activation.

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Table 4.1 Wild type and mutant PKC primers and cloning sites

Construct Name	Primers	Cloning sites	Introduced sites
MBP-C2 S36A	I5 5'-CAGAAGGGCGCCACGAAAGAAAAAGCC I3 5'-TTCTTTCGTGGCGCCCTTCTGCAATCG	EcoR I Kpn I	Nar I
MBP-C2 S36E	I5 5'-CAGAAGGGCGCCACGAAAGAAAAAGCC I3 5'-TTCTTTCGTGGCGCCCTTCTGCAATCG	EcoR I BamH I	Esp 31
MBP-C2 S68-A	I5 5'-CCCAAGGCCGTTAAACCACAGTGG I3 5'-GGTTTAACGGCCTTGGGCTTCGTAG	EcoR I EcoR I	Hae III
MBP-C2 S2-A	I5 5'-GCTTCAATGGCGCGCAGGGCCAAAATG I3 5'-GGCCCTGCGCGCCATTGAAGCCCGCTC	Xmn I Xmn I	BssH II
Apl II S36A	I5 5'- same as MBP-C2 S36A I3 5'- same as MBP-C2 S36A	EcoR I Kpn I	Nar I
Apl II S36E	I5 5`- same as MBP-C2 S36E I3 5`- same as MBP-C2 S36E	EcoR I Kpn I	Esp 31
All MBP + PKC Apl II Mutants	O5 5'-GGGAATTCCATGGTCTTCAACGGTTCGGT O3 5'-CGCCAGGGTTTTCCCAGTCACGAC	EcoR I Kpn I	-NA-
PKCε	O5 5'-GTAATACGACTCACTATAGGGC O3 5'-GGGGTACCCTCTCAGGGCATCAGGT	Kpn I BamH I	-NA-
PKCε Δ C2	O5 5'-GGGGATCCGACCATCGAGCGTGTGTTCAGGGAA O3 5'- same as PKCε	Kpn I BamH I	-NA-

VIII. FIGURE LEGENDS

Figure 1. PKC Apl II autophosphorylates on 5 primary peptides (#1-5) two of which are in the C2 domain. Proteins were phosphorylated, digested and analyzed as described under "Materials and Methods". A) Phosphopeptide map of wild type PKC Apl II. B) Phosphopeptide map of the mutant PKC Apl II lacking its C2 domain (PKC Apl IIΔC2) illustrating that it does not phosphorylate peptides #2 and #3. C) Phosphopeptide map of co-applied PKC Apl II wild type and PKC Apl IIΔC2 illustrating that the spots from wild type PKC Apl II are the same as those from PKC Apl IIΔC2 with the exception of peptides #2 and #3. + and – refer to the polarity of the primary electrophoresis dimension in buffer pH 1.9; vertical arrows indicate direction of liquid chromatography; *S* signifies origin of sample application or coapplication.

Figure 2. Phosphopeptide #3 originates in the core of the C2 domain. Proteins were phosphorylated, digested and analyzed as described under "Materials and Methods". A) PKC Apl II wild type (lane 1) and PKC Apl IIΔC2 (lane 2), were phosphorylated *in vitro* for 30 min in the presence of [γ-^{32}P]ATP. The samples were partially digested with 2 μg/ml trypsin for 5 min at 30 °C, separated by SDS-PAGE, transferred to nitrocellulose and exposed to x-ray film (Autoradiogram). B) Western-blot of the membrane in panel A using an antibody directed to the C2 domain of PKC Apl II (Pepio et al., 1998) confirming that the bands positive for ^{32}P incorporation in panel A are C2 domain containing fragments. C) Phosphopeptide maps of C2 domain containing bands from panel B lane 1 (upper to lower) illustrating that peptide #3 is isolated as the C2 domain core is isolated. + and – refer to the

polarity of the primary electrophoresis dimension in buffer pH 1.9; vertical arrows indicate direction of liquid chromatography; *S* signifies origin of sample application.

Figure 3. Autophosphorylation of peptide #4 is absent in the autonomous kinase. Proteins were phosphorylated, digested and analyzed as described under "Materials and Methods". A) Wild type PKC Apl II was *in vitro* autophosphorylated in the presence of [γ-^{32}P]ATP and either no activators, PS/TPA, or oleic acid for stimulation. The samples were separated by SDS-PAGE, transferred to nitrocellulose and exposed to x-ray film (Autoradiogram). B) Phosphopeptide mapping analysis of bands from panel A. + and – refer to the polarity of the primary electrophoresis dimension in buffer pH 1.9; vertical arrows indicate direction of liquid chromatography; *S* signifies origin of sample application. C) Quantitation of NIH image analyzed data from panel B. Values are means ± S.E.M., (n=3).

Figure 4. C2 domain fusion proteins are transphosphorylated on phosphopeptides #2 and #3. Proteins were phosphorylated, digested and analyzed as described under "Materials and Methods". A) GST-C2 and MBP-C2 domain fusion proteins were transphosphorylated by PKC Apl II in the presence of [γ-^{32}P]ATP. The samples were separated by SDS-PAGE, transferred to nitrocellulose and exposed to x-ray film (Autoradiogram). B) Phosphopeptide mapping analysis of the GST-C2 domain fusion protein in panel A illustrating that the GST-C2 domain fusion protein yields phosphopeptides #2' and #3 as well another phosphopeptide that was seen in GST alone (Letter A). C) Phosphopeptide mapping analysis of the MBP-C2 domain fusion protein in panel A indicating that the MBP-C2 domain fusion protein also yields phosphopeptides #2' and #3. D) Phosphopeptide analysis of co-applied wild type PKC

Apl II and the GST-C2 domain fusion protein illustrating that phosphopeptide #3 is identical in the autophosphorylated kinase and the fusion protein. + and – refer to the polarity of the primary electrophoresis dimension in buffer pH 1.9; vertical arrows indicate direction of liquid chromatography; *S* signifies origin of sample application.

Figure 5. Identification of serine 36 as the primary phosphorylated residue in the C2 domain of PKC Apl II. A) Hypothetical phosphopeptide mapping of the regulatory domain of PKC Apl II identifies probable peptide #3. The regulatory region of PKC Apl II was divided into constituent tryptic peptides and their mass/charge ratio calculated based on singular phosphorylations. The relative hydrophobicity of each peptide was then calculated in the phospho-chromatography buffer as described in the methods. The resulting values for regulatory domain peptides (black circles), C2 domain specific peptides (gray circles), and C2 domain peptide TTTK (white circle) were plotted against each other to generate a phosphopeptide "map" where the location of each peptide is relative. B-D) The residue within peptide #3 responsible for its heavy phosphorylation is serine 36. Proteins were phosphorylated, digested and analyzed as described under "Materials and Methods". B) Phosphopeptide analysis of the wild type MBP-C2 domain. C) Phosphopeptide analysis of the MBP-C2 domain fusion protein with a serine 36 → alanine mutation (MBP-C2 S36A). D) Phosphopeptide analysis of co-applied wild type MBP-C2 domain and MBP-C2 S36A. E-G) PKC Apl II autophosphorylates at serine 36. E) Phosphopeptide analysis of wild type PKC Apl II. F) Phosphopeptide map of the mutant PKC Apl II containing the serine 36 → alanine mutation (PKC Apl II S36A). G) Phosphopeptide map of co-applied PKC Apl II wild type and PKC Apl II S36A. + and – refer to the polarity of the primary electrophoresis

dimension in buffer pH 1.9; vertical arrows indicate direction of liquid chromatography; *S* signifies origin of sample application or coapplication.

Figure 6. Sucrose loaded lipid vesicle assay for C2 domain membrane binding. Proteins were phosphorylated on beads, washed, eluted, pre-centrifuged to remove aggregates and then incubated with sucrose loaded vesicles as described under "Materials and Methods". A-B) The S36A mutation in the C2 domain reduces phosphorylation dependent lipid binding. Purified MBP-Apl II C2 (wt), MBP-Apl II C2 with serine 36 converted to alanine (S36A), or MBP-Apl II C2 with serine 36 converted to glutamic acid (S36E) were incubated with sucrose loaded vesicles (20 μM lipid) consisting of either 60% PS:40% PC (PS/PC) or 100%PC (PC) for 10 min at 15 °C. The sucrose vesicles were subsequently sedimented in a Beckman TLA-100 ultracentrifuge at 100,000 x g for 30 min and the supernatant and pellet fractions separated and analyzed by PAGE and membrane transfer. Total protein was visualized by Ponceau-S staining (Panel A) while phosphorylated protein was measured by autoradiography (Panel B). While very little of the nonphosphorylated wild type fusion protein domain is sedimented, a considerable amount of the phosphorylated protein does sediment. The S36A mutation reduced sedimentation of the phosphorylated protein significantly, while converting serine 36 to glutamic acid increased sedimentation of the non-phosphorylated protein. C) Graphic illustration of quantitated results from panels A and B. Data is displayed as the percentage of total protein found in the bound (pellet) fraction for both total protein (white bars) and phosphorylated protein (black bars). Values are means ± S.E.M., (n=6).

Figure 7. Substrate phosphorylation is unaffected by the S36A or S36E mutations. Protein kinase C activity assays were performed as described under "Materials and Methods". PKC assays were performed for wild type PKC Apl II (white circles), PKC Apl II S36A (black circles), and PKC Apl II S36E (white squares) illustrating no significant change in phosphatidylserine dependent substrate phosphorylation in the mutant kinases.

Figure 8. Serine 36 is phosphorylated *in vivo* and is necessary for PKC membrane translocation. Proteins were phosphorylated and kinase assays performed as described under "Materials and Methods". A) The phospho-S36 antibody is specific for PKC Apl II phosphorylated at serine 36. Purified wild type PKC Apl II and PKC Apl IIΔC2 were autophosphorylated *in vitro*. Enzymes were stimulated by no activators (BL), 50 μg/ml dioleoyl phosphatidylserine and 20 nM tetra decanoic acid (PS/TPA), or oleic acid (Oleic) in the absence (-) or presence (+) of 50 μM ATP and [γ-^{32}P]ATP. A, upper panel) Immunoblot using the phospho-S36 antibody illustrating the increase in immunoreactivity with PS/TPA and Oleic acid stimulation of the wild type PKC that is absent in the mutant, PKC Apl II S36A. A, middle panel) Autoradiogram of the upper panel illustrating an increase in ^{32}P incorporation for the stimulated wild type PKC that parallels that seen in the anti phospho-S36 western blot. Also illustrated is the reduction in oleic acid stimulation of PKC Apl II S36A but not wild type PKC Apl II. A, lower panel) Immunoblot of the upper panel using the PKC Apl II antibody indicating that approximately equal protein is loaded in each lane and between PKC constructs. B) Cells were fractionated as described under "Materials and Methods". Anti-PKC Apl II immunoblot of fractionated Sf21 cells infected with baculovirus coding for wild type PKC Apl II (lanes 1-4) and PKC Apl II S36A (lanes 5-8) after treatment

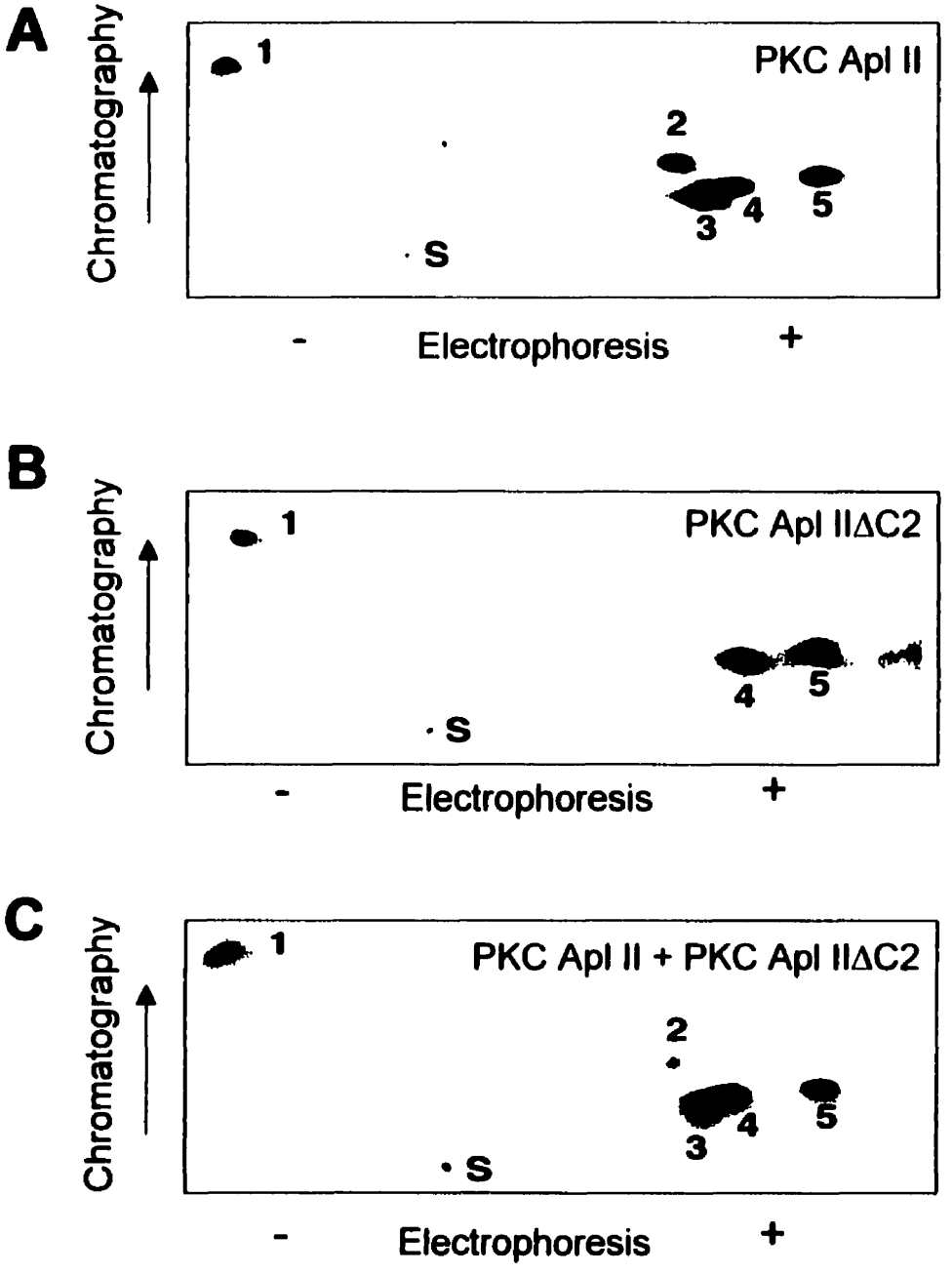
in the absence (-) or presence (+) of 4-β-PDBu. C) Anti- phospho-S36 immunoblot of the experiment in panel 8B illustrating serine 36 phosphorylation *in vivo* of only wild type PKC Apl II and the preferential PDBu induced membrane translocation of serine 36 phosphorylated PKC.

Figure 9. Conservation of C2 domain phosphorylation in vertebrate nPKCε. Proteins were expressed, purified from baculovirus, and phosphorylated as described under "Materials and Methods". A) Immunoblot of purified PKCε and the mutant lacking its C2 domain, PKCεΔC2 after autophosphorylation and SDS-PAGE analysis illustrating the difference in size between wild type PKCε and PKCεΔC2. B) Autoradiogram of the blot in panel 10A illustrating heavy ^{32}P incorporation into wild type PKCε and the dramatic reduction in labeling when the C2 domain is removed. C, upper panel) Phosphopeptide map of wild type PKCε depicting two phosphorylated peptides (#1 and #2). C, lower panel) Phosphopeptide map of PKCεΔC2 illustrating the apparent loss of heavily phosphorylated peptide #1 when the C2 domain is removed.

Figure 10. Sequence alignment of novel C2 domains and model of phosphorylation induced lipid binding. A) Comparison of the C2 domains of PKC Apl II and novel vertebrate PKCs. Strand positions are based on those previously described (Nalefski and Falke, 1996; Perisic et al., 1998; Pepio et al., 1998). Black shaded residues are conserved in all isoforms. D represents hypothetical locations of aspartic acid residues used by conventional C2 domains for calcium binding. Gray shaded residues indicate serine 36 and other putative phosphorylation sites in loop 1 of the C2 domain of PKC Apl II and other Ca^{2+}-independent

C2 domains. B) Model of phosphorylation induced lipid binding. B, left panel) A diagrammatic representation of PKC Apl II's C2 domain with a positively charged lipid binding surface on loop 1 whose access is restricted by the position of loop 3. B, right panel) Phosphorylation of serine 36 in the putative alpha helix of loop 1 shifts the position of loop 3 to expose the lipid binding surface on loop 1. C2 domain structure adapted from (Perisic et al., 1998).

Figure 11. Model of the role for C2 domain phosphorylation in Ca^{2+}-independent PKC membrane translocation and activation. A) Fully mature PKC Apl II that is phosphorylated on its activation loop (T500 in vertebrate $PKC\beta_{II}$) by PDK1, autophosphorylated on its turn motif (T641 in vertebrate $PKC\beta_{II}$) and its hydrophobic motif (S660 in vertebrate $PKC\beta_{II}$) resides in the cytoplasm awaiting activation. The pseudosubstrate sequence (PS) is occupying the catalytic pocket and the C2 domain restricts activator binding to the C1 domain. B) PKC Apl II can become activated through production of DAG by PLC_{γ} or PLC_{β} and high levels of phosphatidylserine binding to its C1 domain. Solely PKC Apl II's C1 domain mediates this membrane translocation and as a result its membrane affinity would be low and lead to transient kinase activation. C) Alternatively, PKC Apl II can become autophosphorylated (in cis or perhaps trans) on serine 36 in loop 1 of its C2 domain. D) This phosphorylation may expose a cryptic C2 domain lipid binding site allowing phosphatidylserine binding and now both the C1 and C2 domains are responsible for recruiting and anchoring the kinase to the membrane. This C1 and C2 mediated activation produces a high membrane affinity and may be responsible for persistent activation of Ca^{2+}-independent PKCs like PKC Apl II.


A
Chromatography
PKC Apl II
1
2
3
4
5
S
-
Electrophoresis
+
B
Chromatography
PKC Apl IIΔC2
1
4
5
S
-
Electrophoresis
+
C
Chromatography
PKC Apl II + PKC Apl IIΔC2
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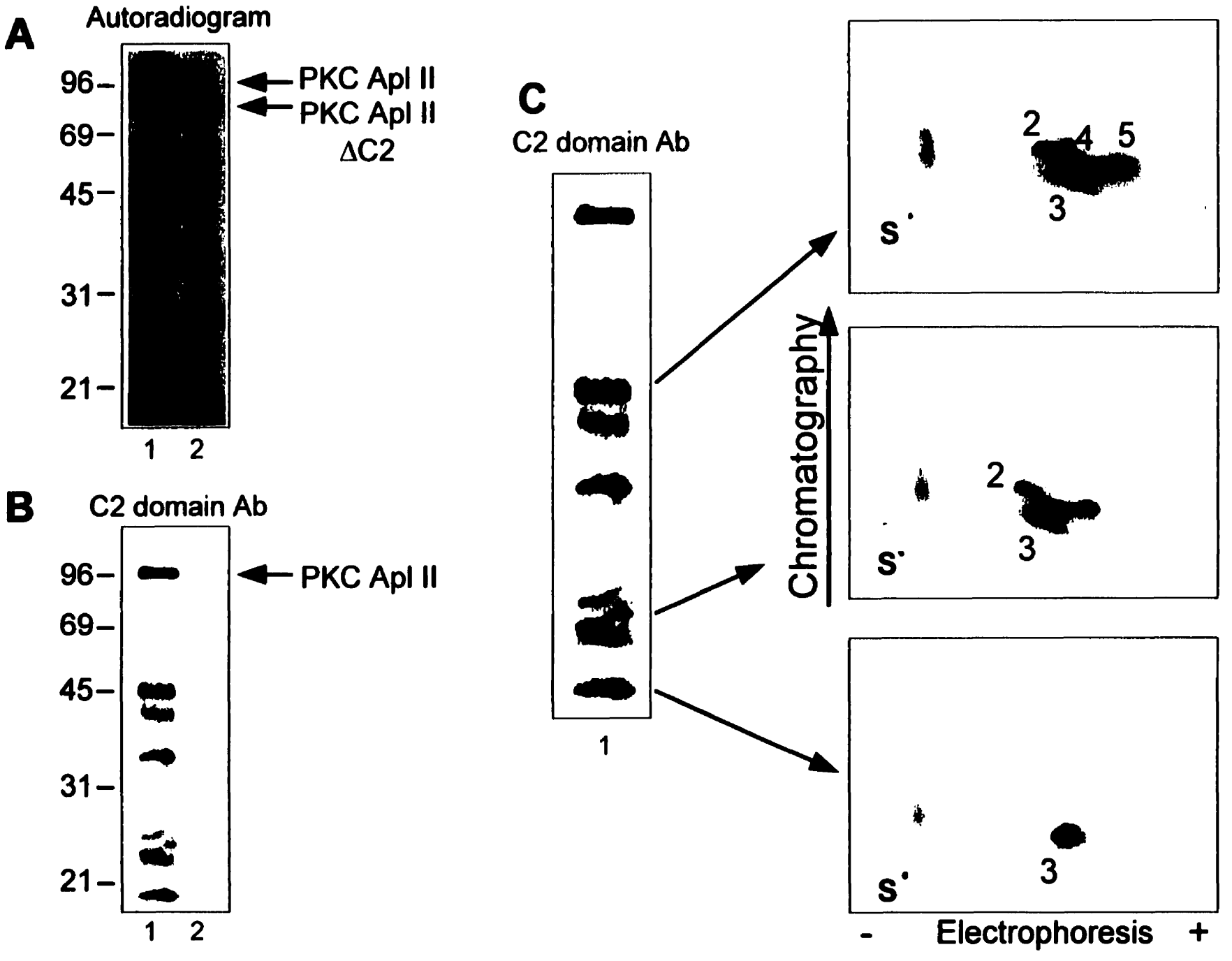
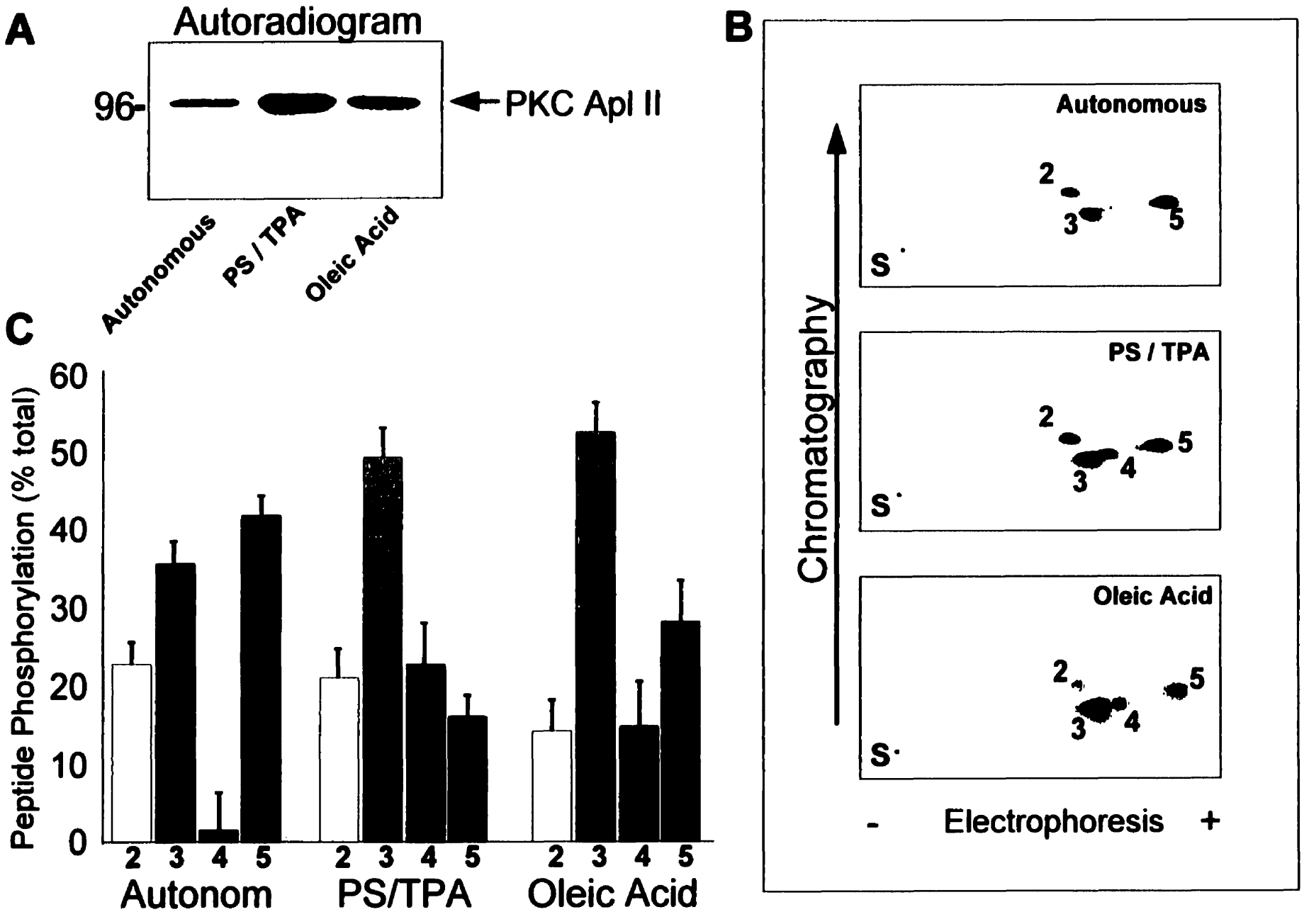
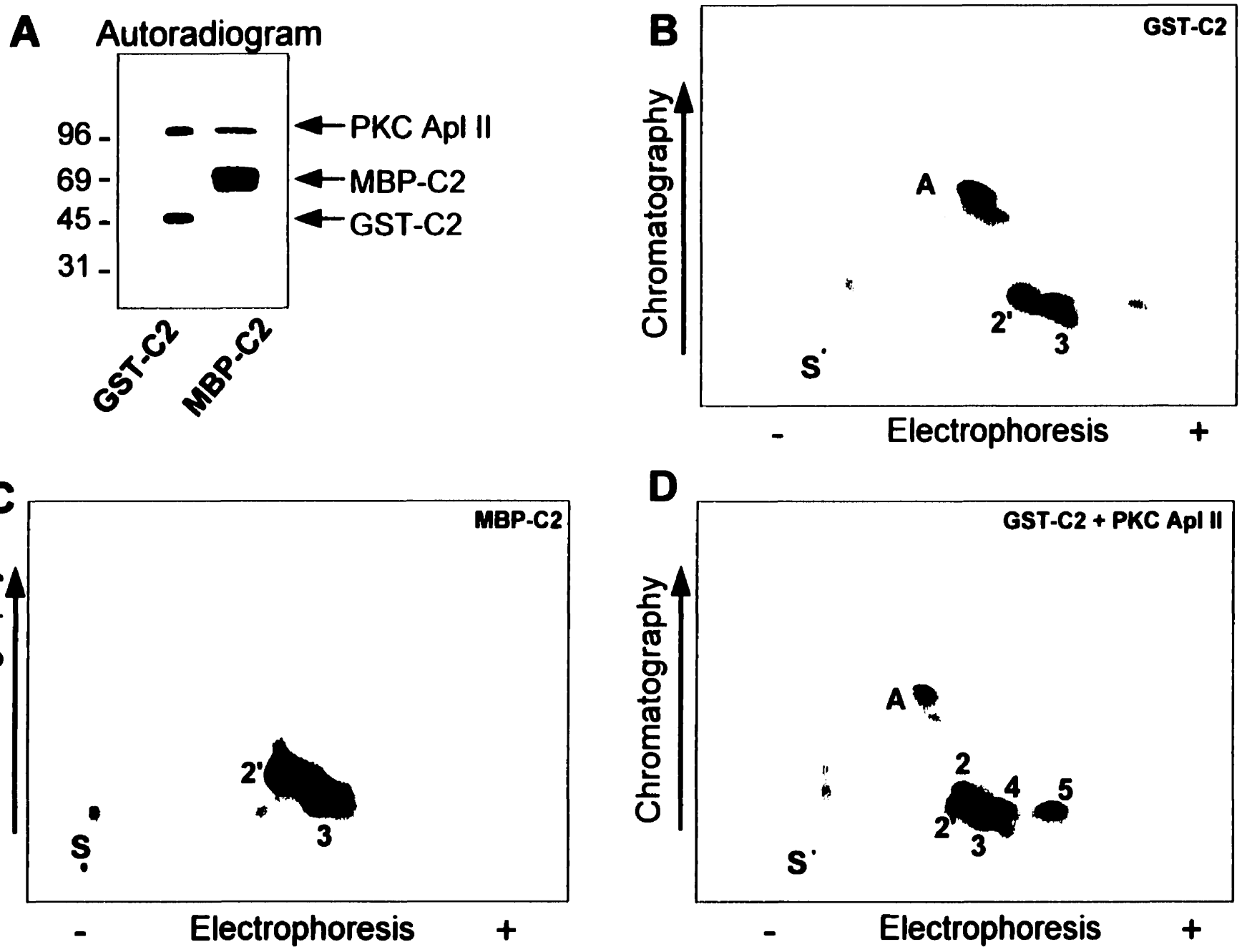
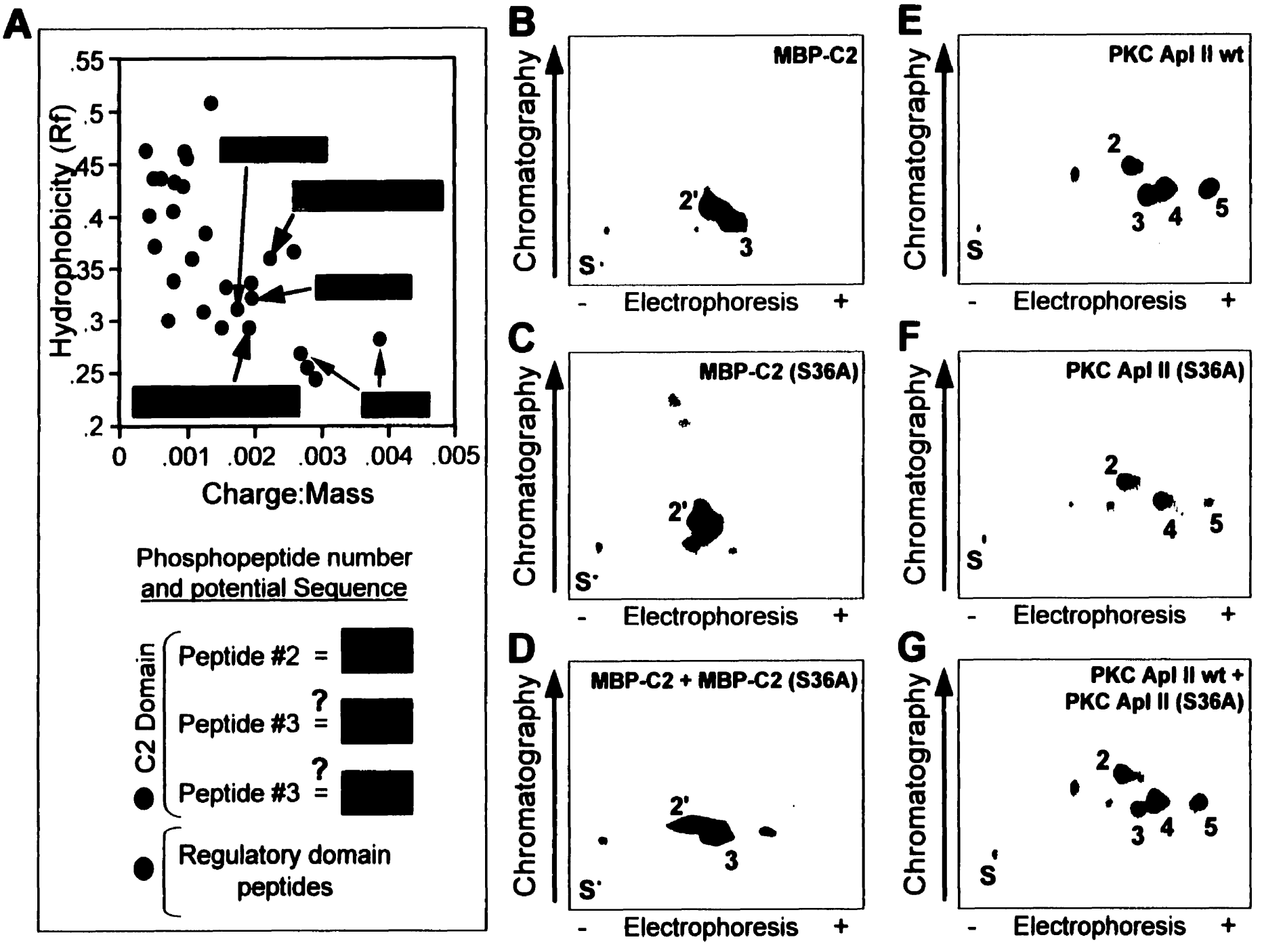
2
3
4
5
S
-
Electrophoresis
+


A
Autoradiogram
96–
69–
45–
31–
21–
PKC Apl II
PKC Apl II
ΔC2
1 2
B
C2 domain Ab
96–
69–
45–
31–
21–
PKC Apl II
1 2
C
C2 domain Ab
1
Chromatography
2 4 5
3
S
2
3
S
3
S
- Electrophoresis +


A
Autoradiogram
96-
PKC Apl II
Autonomous
PS / TPA
Oleic Acid
B
Autonomous
PS / TPA
Oleic Acid
Chromatography
Electrophoresis
C
Peptide Phosphorylation (% total)
Autonom
PS/TPA
Oleic Acid


A
Autoradiogram
96 -
69 -
45 -
31 -
PKC Apl II
MBP-C2
GST-C2
GST-C2
MBP-C2
B
GST-C2
Chromatography
A
2'
3
S
-
Electrophoresis
+
C
MBP-C2
Chromatography
2'
3
S
-
Electrophoresis
+
D
GST-C2 + PKC Apl II
Chromatography
A
2
4
5
2'
3
S
-
Electrophoresis
+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Hydrophobicity (Rf)
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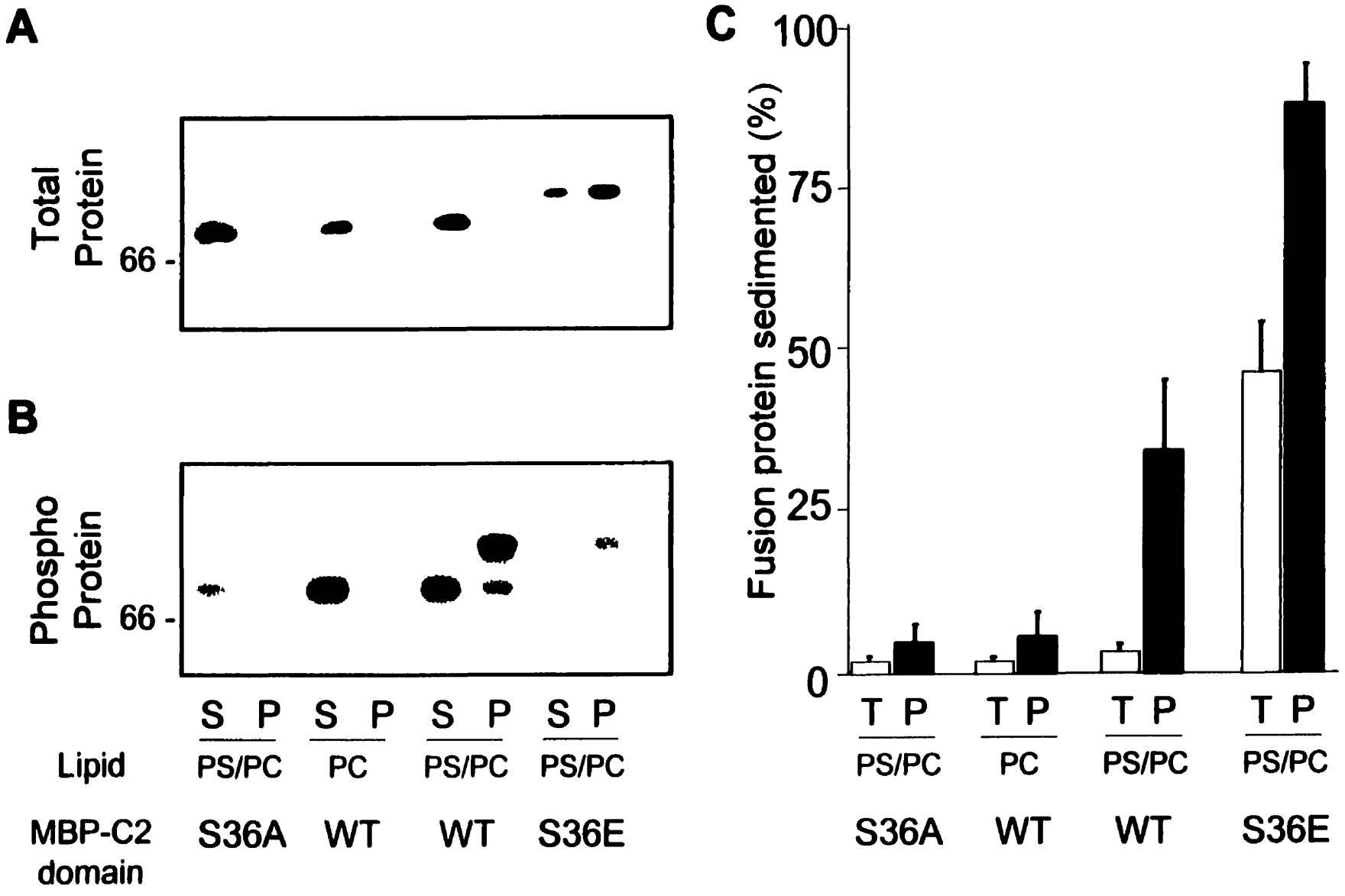
.5
.45
.4
.35
.3
.25
.2
0
.001
.002
.003
.004
.005
Charge:Mass
Phosphopeptide number
and potential Sequence
C2 Domain
Peptide #2 =
Peptide #3 ?=
Peptide #3 ?=
Regulatory domain
peptides
B
MBP-C2
2'
3
S
Chromatography
- Electrophoresis +
C
MBP-C2 (S36A)
2'
S
Chromatography
- Electrophoresis +
D
MBP-C2 + MBP-C2 (S36A)
2'
3
S
Chromatography
- Electrophoresis +
E
PKC Apl II wt
2
3
4
5
S
Chromatography
- Electrophoresis +
F
PKC Apl II (S36A)
2
4
5
S
Chromatography
- Electrophoresis +
G
PKC Apl II wt +
PKC Apl II (S36A)
2
3
4
5
S
Chromatography
- Electrophoresis +


A
Total Protein
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B
Phospho Protein
66 -
S P S P S P S P
Lipid
PS/PC PC PS/PC PS/PC
MBP-C2 domain
S36A WT WT S36E
C
Fusion protein sedimented (%)
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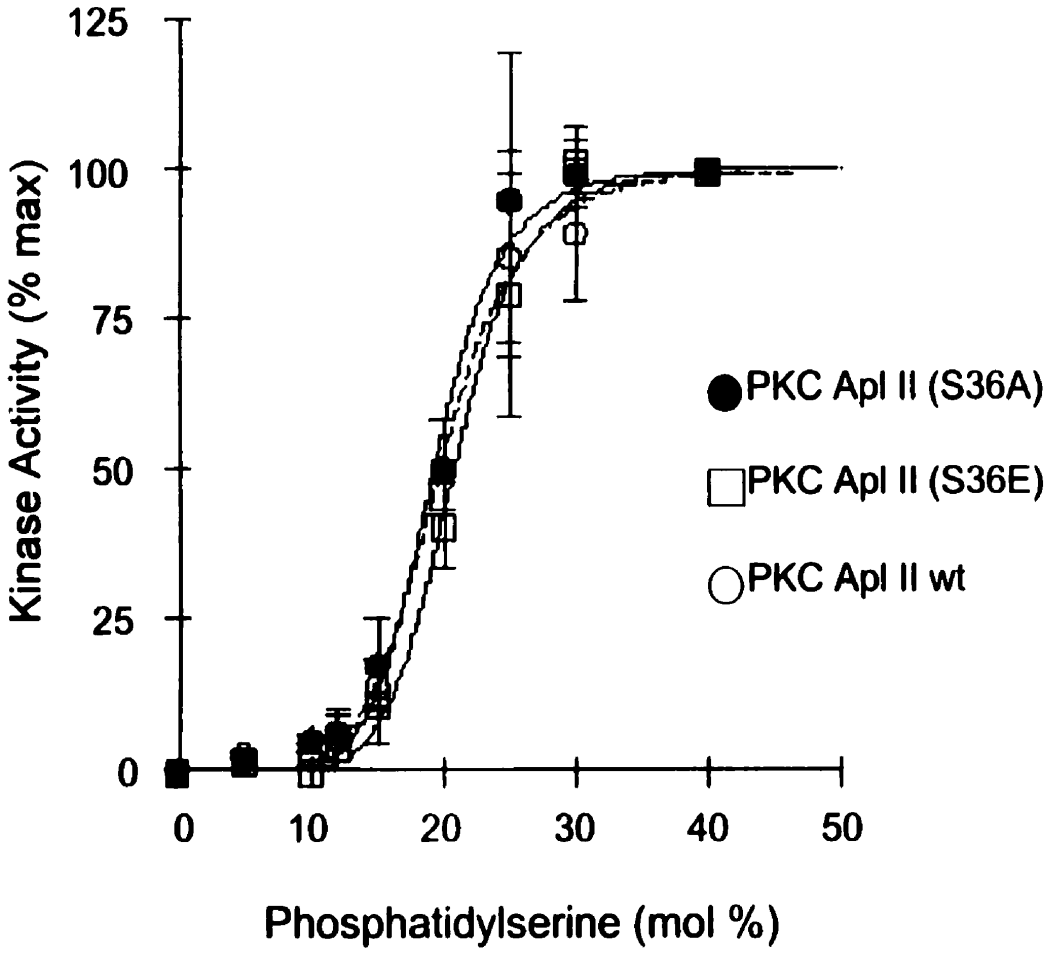
75
50
25
0
T P T P T P T P
PS/PC PC PS/PC PS/PC
S36A WT WT S36E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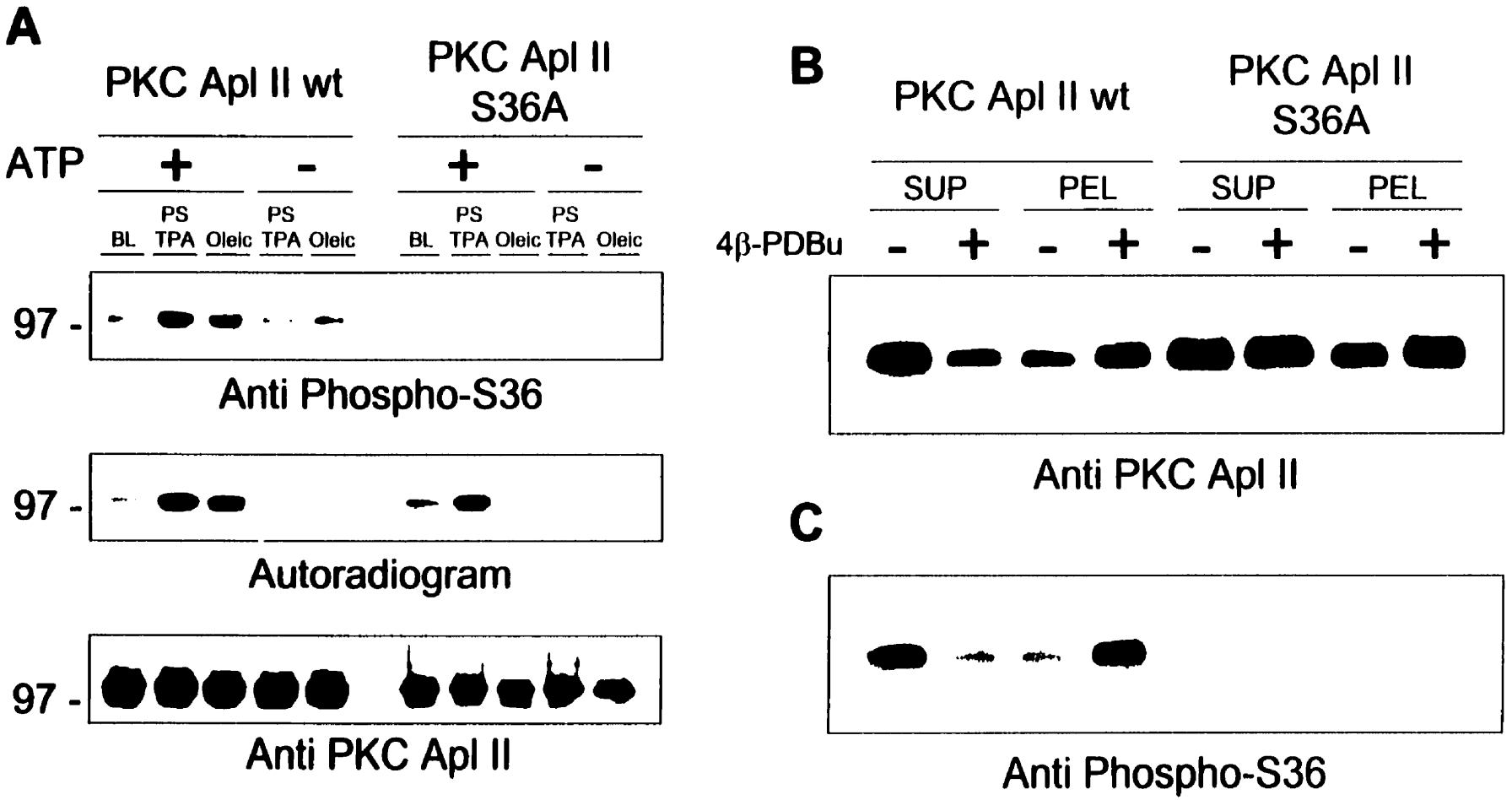
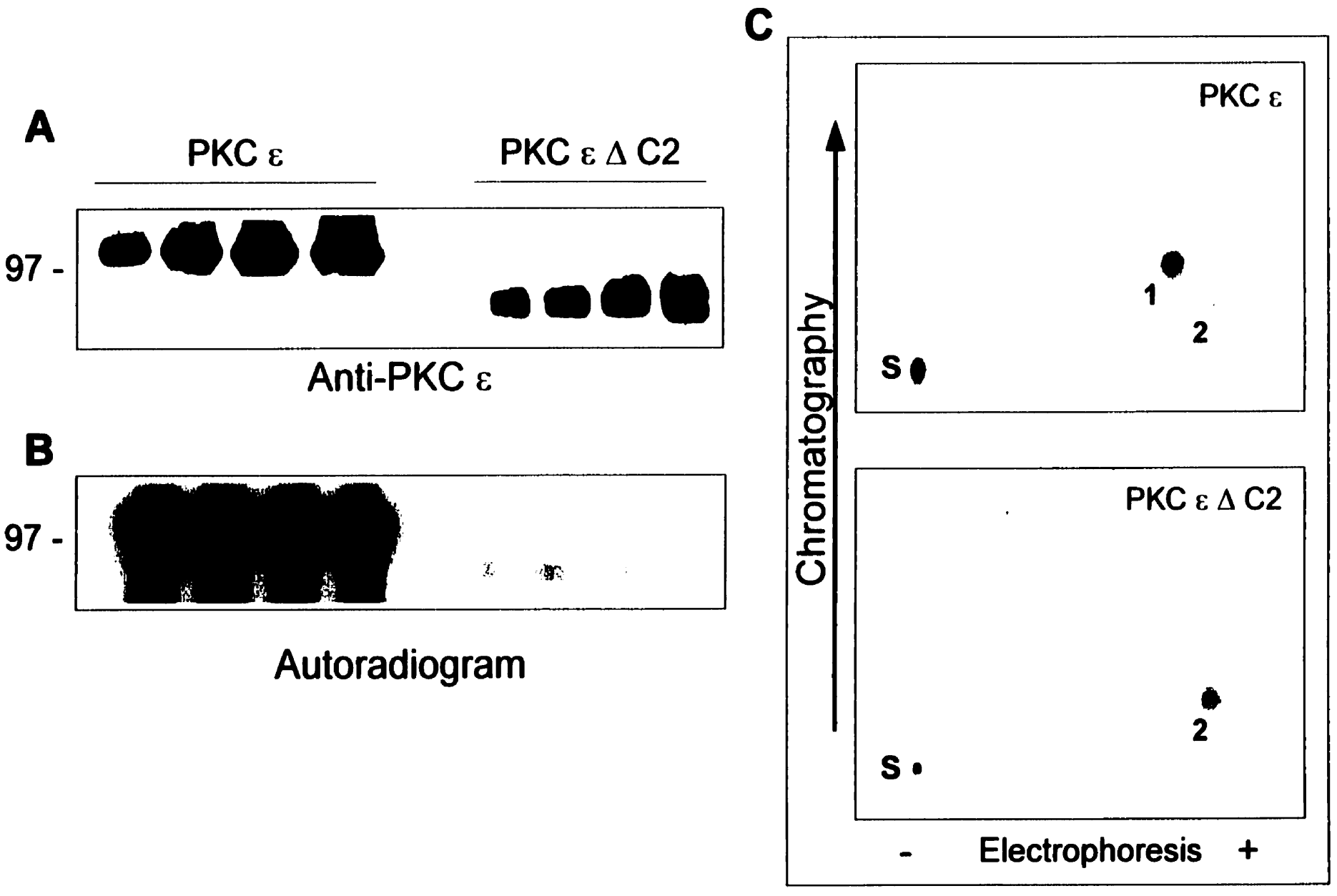
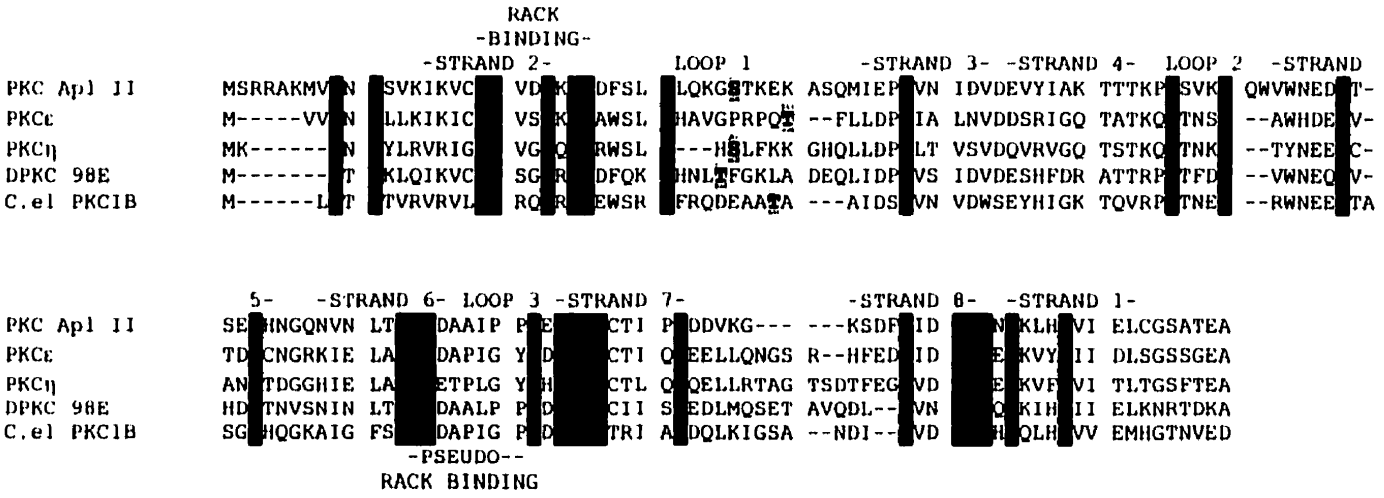
100
75
50
25
0
Kinase Activity (% max)
0
10
20
30
40
50
Phosphatidylserine (mol %)
PKC Apl II (S36A)
PKC Apl II (S36E)
PKC Apl II wt


A
PKC Apl II wt
PKC Apl II S36A
ATP
+
-
+
-
PS
BL
TPA
Oleic
PS
TPA
Oleic
PS
BL
TPA
Oleic
PS
TPA
Oleic
97 -
Anti Phospho-S36
97 -
Autoradiogram
97 -
Anti PKC Apl II
B
PKC Apl II wt
PKC Apl II S36A
SUP
PEL
SUP
PEL
4β-PDBu
-
+
-
+
-
+
-
+
Anti PKC Apl II
C
Anti Phospho-S36


A
PKC ε
PKC ε Δ C2
97 -
Anti-PKC ε
B
97 -
Autoradiogram
C
PKC ε
1
2
S
PKC ε Δ C2
2
S
Chromatography
- Electrophoresis +

A



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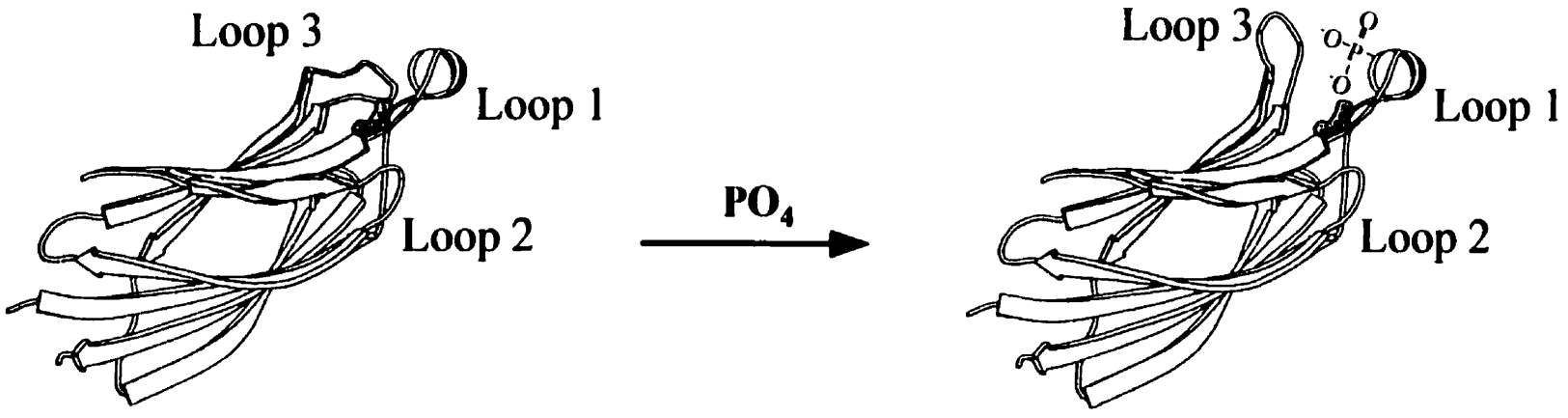
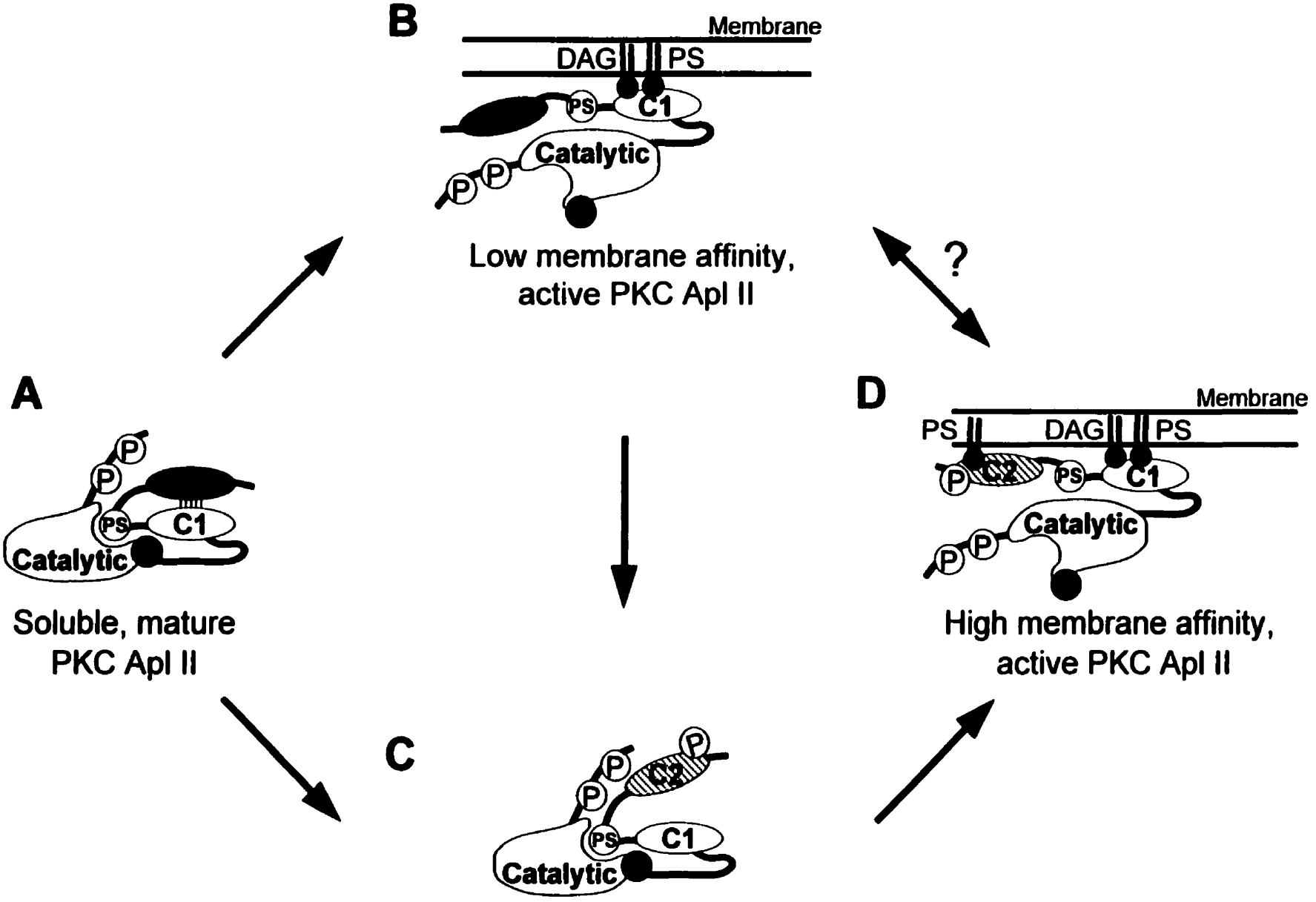


nPKC C2 Domain

loop 3 covers lipid binding site

Phosphorylated C2 Domain

PO_4 of S36 in loop 1 exposes lipid binding site


B
Membrane
DAG
PS
PS
C1
Catalytic
P
P
Low membrane affinity,
active PKC Apl II
?
A
P
P
PS
C1
Catalytic
Soluble, mature
PKC Apl II
D
Membrane
PS
DAG
PS
P
C2
PS
C1
Catalytic
P
P
High membrane affinity,
active PKC Apl II
C
P
P
P
C2
PS
C1
Catalytic
Phosphorylation disrupted
"C2 domain mediated inhibition"

CHAPTER 5

CHAPTER 5: General Discussion

I. DISCUSSION

This thesis has explored i) whether the C2 domain of PKC Apl II could modulate activator binding to the C1 domain and ii) potential modulatory mechanisms of the C2 domain of PKC Apl II. In the first study, fusion proteins containing both the C1 and C2 domains were found to have a variable affinity for phorbol ester that was highly dependent on phosphatidylserine levels. The affinity of these C1-C2 domain proteins for phorbol ester was significantly reduced at lower phosphatidylserine concentrations and when PS concentrations were elevated this could increase the affinity dramatically. Although we found the affinity for phorbol ester to be highly dependent on phosphatidylserine levels, we also found that phosphatidic acid (PA) is much more effective at increasing this phorbol ester affinity. Together, our results confirm the inhibitory model of C2 domain mediated inhibition of the C1 domain.

The second study addressed whether the difference in the lipid requirements for kinase activation of PKC Apl I and PKC Apl II stems from differences in their structural domains. Using both a direct liposome binding assay and an assay measuring the ability of lipids to induce phorbol ester binding, we examined lipid interactions of fusion proteins containing C1 or C2 domains of the cPKC Apl I and the nPKC Apl II. Our results indicate that the major difference between cPKCs and nPKCs is that lipids act through the C2 domain to enhance activity of cPKCs but not nPKCs.

The final study identified two autophosphorylation sites, serine 2 and serine 36, in the C2 domain of the Ca^{2+}-independent PKC Apl II. Phosphorylation of serine 36 increased binding of the C2 domain to phosphatidylserine membranes *in vitro*. In cells, nPKC Apl II phosphorylation at serine 36 was increased by PKC activators and nPKC Apl II phosphorylated at this position translocated more efficiently to membranes. Moreover, mutation of serine 36 to alanine significantly reduced membrane translocation of nPKC Apl II. We propose that translocation of Ca^{2+}-independent PKCs is regulated by phosphorylation of the C2 domain.

A) Roles of Phospholipase D

Phospholipase D activation has been shown to preceed the activation of Ca^{2+}-independent PKCs and follow the activation of Ca^{2+}-dependent PKC isoforms. The signal-dependent activation of PLD has been observed in a wide variety of brain and neural-derived cells. The activation of PLD by cell surface receptor ligands and the rapid nature of its effects indicates a potentially important role for the enzyme in signal transduction. In some cell types, PLD is activated by tyrosine kinase receptor agonists such as epidermal growth factor (EGF), platelet-derived growth factor (PDGF) and fibroblast growth factor (FGF) (Klein et al., 1995). It has been demonstrated that both G-protein coupled and intrinsic tyrosine kinase receptors are capable of stimulating PLD activity (Natarajan et al., 1996). PLD stimulation has been observed downstream of hormones, neurotransmitters, growth factors, and cytokines (Liscovitch and Chalifa-Caspi, 1996). As well, there is an equally diverse list of cell responses which PLD activity has been noted to effect including secretion, contraction, and proliferation (Olson and Lambeth, 1996). The wide range of PLD agonists and effects

dictate that PLD activity is probably not specific to a cellular function but is instead involved in some common aspect of signal transduction. As well, PLD is known to be stimulated, probably through direct interactions, by Ca^{2+}-dependent PKCs, phosphatidylinositol 4, 5 bisphosphate (PIP_2), and the small guanine nucleotide-binding protein ADP ribosylation factor-1 (ARF-1) (Liscovitch et al., 1994; Cockcroft, 1996; Kiss, 1996; Hammond et al., 1997). Studies have found that phorbol ester stimulation of PLD was unaffected by tyrosine kinase inhibitors, suggesting that RTK phosphorylation is upstream of PKC in the signaling cascade (Olson and Lambeth, 1996).

PI 3-kinase activation and accumulation of the second messenger phosphatidylinositol 3,4,5-triphosphate (PIP_3) has been reported in many cell neutrophils and other cells to be correlated with PLD activation (Olson and Lambeth, 1996). A potent inhibitor of PI 3-kinase, wortmannin, blocks PIP_3 production and PLD activation (Liscovitch and Chalifa-Caspi, 1996). A recent study on PIP_3 signaling by Czech's group reports the cloning of a general receptor for phosphoinositides (GRP-1), a pleckstrin homology (PH) domain containing protein (Klarlund et al., 1997). GRP-1 binds PIP_3 and catalyzes nucleotide exchange of ARF-1. This protein indirectly links PI 3-K activation to PLD stimulation as follows: RTK activity recruits the p85/p110 type PI 3-K to tyrosine phosphate sites, promoting the generation of PIP_3, which then binds the PH domain of GRP-1 (Klarlund et al., 1997). Membrane bound GRP-1 may interact through Sec7 homology regions with ARF1, a known PLD activator, to cause guanine nucleotide exchange (Klarlund et al., 1997). From this point in the pathway, PLD activity would produce PA and eventually DAG to activate PKC.

The enzymatic function of choline specific PLD is to catalyze the hydrolysis of phosphatidylcholine at its terminal phosphodiester bond, thus producing phosphatidic acid and releasing the free polar headgroup, choline (Ella et al., 1994). PA has been implicated as a second messenger activating PKCs and we have found the Ca^{2+}-independent PKC Apl II to be sensitive to this phospholipid. As well, increases in DAG levels necessary for persistent kinase activity have been reported following phosphatidic acid phosphohydrolase (PAP) activity via conversion of PA to DAG (Liscovitch and Chalifa-Caspi, 1996). Billah and others reported the potential for this pool of DAG to activate Ca^{2+}-dependent PKCs and Ca^{2+}-independent PKCs that in turn feedback and enhance PLD activity. The persistent nature of this DAG may produce the prolonged activation PKC by sustaining its association with the lipid membrane. As well, the slowly hydrolysed form of DAG is thought to be produced from phosphatidylcholine hydrolysis, since the fatty acid composition of this DAG matches that of PC (Holbrook et al., 1992; Qian and Drewes, 1989). The persistent form of DAG may be generated by growth factor stimulation of cell receptors (Exton, 1990; Cockcroft, 1992) which correlates with PKC activation (Nishizuka, 1995; Olson and Lambeth, 1996). As well, this long-lasting form of DAG can be generated in the absence of the transient form of DAG (Exton, 1990; Cockcroft, 1992; Liscovitch, 1992), suggesting that independent mechanisms may underlie rapid and prolonged PKC stimulation (Billah, 1993).

B) Functions of Phosphatidic Acid

A variety of functional roles have been proposed for PA and its phospholipase A_2 metabolite, lysophosphatidic acid (LPA) (Stasek et al., 1993; Lang et al., 1995; English et al., 1996). One of phosphatidic acid's mitogenic effects can be explained by its ability to be metabolized

by PA phosphohydrolase (PAP) to generate DAG, the known activator of PKC (Billah et al., 1989; Brindley et al., 1996). It has been shown recently in vertebrate systems that PAP coimmunoprecipitates with PKCε, providing not only functional linkage between PA and Ca^{2+}-independent PKCs but also subcellular localization (Jiang et al., 1996). Another role of PA as a signaling molecule is to activate phosphoinositide 4,5-kinase (PI 4-K) which produces PIP_2 (Jenkins et al., 1994). Since PIP_2 is a direct activator of PLD, an increase in PA levels would stimulate a positive feedback loop by increasing the activity of both PI 4-K and PLD. Finally, it has been reported that PA binds Raf-1 kinase, a serine/threonine kinase activated by growth factor stimulation (Ghosh et al., 1996). Via PA activation, Raf-1 may initiate cell proliferation by activating the MAP kinase cascade. There is some controversy however, since PA has been shown to inhibit the activity of the GTPase activating protein (GAP), which functions to turn off the Ras monomeric GTPase, regulating upstream events of the MAP kinase cascade (Ghosh et al., 1996; Kuroda et al., 1996).

C) Insulin Dependent Calcium Modulation

In the bag cell neurons, it has been demonstrated that insulin persistently activates and then downregulates PKC Apl II (Sossin et al., 1996a). The changes in Ca^{2+} channel currents coincide with the time-course of PKC Apl II activation (Jonas et al., 1996). As well, the activation of PKC Apl II is blocked by the phosphoinositide 3-kinase (PI 3-K) inhibitor wortmannin, suggesting that phosphatidylinositol signaling is important in the activation of PKC Apl II (Sossin et al., 1996a). Similar results have been seen in the vertebrate nervous system where RTK activation enhances activation of Ca^{2+}-independent PKCs through PI 3-kinase (Ha and Exton, 1993; Toker et al., 1994; Moriya et al., 1996).

In summary, evidence suggests that insulin increases a Ca^{2+} current in the bag cell neurons of *Aplysia* through activation of PKC Apl II and membrane insertion of the Ca^{2+} channels. As well, short term treatment of *Aplysia* sensory neurons with phorbol esters cause a long term increase in excitability that could also be mediated by membrane insertion events (Manseau et al., 1998). This is likely to be mediated by PKC Apl II because short applications of 5-HT, which activate Apl I and not Apl II do not cause a long-term increase in excitability. These observations implicate PKC Apl II in a novel mechanism of synaptic plasticity that may serve as an 'intermediate' memory. Thus, it is important to understand how physiological activation of PKC Apl II is regulated.

D) Roles of C1 and C2 Domain Interactions

Our hypothesis of C2 domain function generalizes to distinguish a different signaling cascade for each of the two classes of PKC isoforms. For Ca^{2+}-dependent PKCs, Ca^{2+} serves to remove C2 domain-mediated inhibition. As a result, Ca^{2+}-dependent PKCs are activated by phospholipase C, which produces both DAG and IP_3, releasing Ca^{2+} from intracellular stores (Nishizuka, 1995). For the Ca^{2+}-independent PKCs, PA produced through the activation of PLD is one mechanism to remove inhibition by the C2 domain, although not through direct C2 domain binding. Another mechanism is through the specific phosphorylation of the C2 domain to enhance membrane affinity. This model would explain some of the results from *Aplysia*: Serotonin, acting through phospholipase C produces DAG and eventually Ca^{2+}, thus stimulating PKC Apl I but not PKC Apl II (Sossin et al., 1996b); prolonged insulin treatment activates PKC Apl II (Sossin et al., 1996a) maybe through a combination of DAG and PA produced by phospholipase D acting downstream of a receptor tyrosine kinase. Perhaps the

initial stimulation of PKC Apl I is required to phosphorylate the C2 domain of PKC Apl II, or conversely, the DAG formed from PA may act to preferentially prolong nPKC activity.

The membrane translocation and activation of conventional PKC requires Ca^{2+}, PS, and DAG under physiological conditions. Conventional PKCs have two membrane-targeting modules, the C1 and C2 domains, which are responsible for its membrane binding and activation. A consensus mechanism of *in vitro* PKC activation is that the C1 and C2 domains work in concert to bring the PKC molecule to the membrane surface, where the protein undergoes conformational changes to remove the pseudosubstrate region from the active site, resulting in PKC activation (Newton, 1995, 1997). Extensive structural and mutational studies have helped understand the roles of individual domains in the membrane targeting and activation of PKCs and identified those amino acids that are critically involved in these processes. For instance, a structure-function study of the C2 domain of PKCα defined the role of the C2 domain as a membrane docking unit as well as a module that triggers conformational changes of the protein for its activation (Medkova and Cho, 1998a). Also, extensive mutagenesis studies on the C1b domain of PKCδ identified the essential amino acids for phorbol ester binding (Kazanietz et al., 1995; Wang et al., 1996). Less is known, however, about the temporal and spatial sequences of membrane targeting and activation of PKCs. A recent elegant cell study indicated that the activation of PKCγ follows well defined sequential steps in which the Ca^{2+}-dependent membrane binding of the C2 domain is followed by the DAG/phorbol ester binding of the C1 domain (Oancea and Meyer, 1998). Similarly, Medkova and Cho described systematic structure-function studies of the two

domains of PKCα that provide the first detailed analysis of the temporal and spatial sequences of *in vitro* membrane targeting and activation of PKCα (Medkova and Cho, 1999).

For most peripheral membrane binding proteins, both electrostatic and hydrophobic interactions play roles in their membrane binding, although their relative contributions vary with the type of proteins (Lichtenbergova et al., 1998; Lee et al., 1996; Snitko et al., 1997). It has been shown that the membrane binding of PKCα is also driven by these interactions; hydrophobic and electrostatic interactions of the calcium ions bound to PKCα with anionic phospholipids mediate Ca^{2+}-dependent penetration of PKC into the hydrophobic core of the membrane (Medkova and Cho, 1998a,b). C2 domain phosphorylation may play a similar role exposing a lipid binding core in the C2 domain (Fig. 1) (Pepio and Sossin, 2001). Thus, the Ca^{2+} and PS are involved not only in direct electrostatic interaction but also in eliciting hydrophobic interactions. Extensive *in vitro* studies have shown that the activation of conventional PKC requires the binding of multiple PS molecules (Mosior and Newton, 1998) and a single DAG (phorbol ester) molecule to PKC (Hannun et al., 1985). Also, the PS specificity of conventional PKC is much more pronounced in the presence of DAG in the membrane, suggesting the synergism between PS-binding site(s) and a DAG-binding site (Newton and Keranen, 1994). Structural and mutational analyses have clearly identified the DAG binding site in each of two C1 domains, although it is unclear which of the two binding sites is actually involved in binding to a single DAG molecule (Zhang et al., 1995; Kazanietz et al., 1995; Wang et al., 1996). The presence and location of PS-specific binding site(s) remains controversial, however, because some synthetic phospholipids, such as dansyl-PE, are also able to simulate the effects of PS (Mosior et al., 1996).

The one-to-one stoichiometry of conventional PKC-DAG (or phorbol ester) binding indicates that only one of two DAG-binding sites is actually involved in the DAG binding and PKC activation. A recent binding study using a fluorescent phorbol ester analog suggested that PKCα has two discrete phorbol ester-binding sites with different affinities (Slater et al., 1994). It was also found that DAG and phorbol esters bind to the two discrete sites with opposite affinity, so that a high affinity DAG-binding site is a low affinity phorbol ester binding site and vice versa (Slater et al., 1996). Structure-function analyses of the two zinc finger domains show that the C1a domain contains the high affinity DAG-binding site (Medkova and Cho, 1999). The lipid monolayer penetration of PKCα indicated that the upper part of the C1a domain penetrates into the membrane, whereas its counterpart in the C1b domain does not (Medkova and Cho, 1999). Suggesting that only the C1a domain would be allowed to interact with DAG. The C1a domain is immediately linked to the pseudosubstrate region, and thus conformational changes accompanying the penetration of C1a domain into the membrane might provide a mechanical force to remove the pseudosubstrate region from the active site (Medkova and Cho, 1999).

There is evidence for the alternative splicing of the gene for the novel vertebrate PKCε. Ono and collegues isolated a cDNA clone of PKCε that was truncated by 240 amino acids from its N-terminal (Ono et al., 1988). This truncation would correspond to a natural deletion of its entire C2 domain and approximately 90 additional amino acids into the regulatory region (Ono et al., 1988).

It has been demonstrated that the C1 and C2 domains work in a concerted fashion to facilitate prolonged activation of PKC (Medkova and Cho, 1998a,b; 1999) *in vivo* (Oancea and Meyer, 1998). The transient translocation of PKC is mediated by the C2 domain of the soluble kinase binding to the membrane (Oancea and Meyer, 1998). However, a more stable and prolonged translocation was associated with membrane binding by the C1 domain in addition to that mediated by the C2 domain alone. Thus, there exist two distinct states of PKC membrane association: a low affinity state mediated by the C2 domain alone and a high affinity state mediated by both the C1 and C2 domains acting together. We propose that where calcium recruits the aid of conventional C2 domains to translocate cPKCs, phosphorylation may recruit the aid of novel C2 domains to bind lipids and perpetuate PKC membrane association (see Fig. 1). This requirement of the C2 domain to perpetuate nPKC membrane association would act to help rather than hinder kinase activity and may indeed be a mechanism for persistent kinase activation.

We have demonstrated that removal of the C2 domain of PKC Apl II does not occlude the effect of PA to reduce PS necessary for kinase activity. PA could still act to remove C2 domain-mediated inhibition by binding to the C1 domain. We proposed that on the C1 domain of the Ca^{2+}-independent PKC Apl II there may be a specific site that has a much higher affinity for PA (Pepio and Sossin, 1998).

E) Regulation of PKCs by Phosphorylation

i) Core phosphorylation sites

Recently, attention has been drawn to the phosphorylation of PKC itself. Intriguingly, what was once considered a purely effector-driven signal transducer is revealed to possess a complex mechanism for regulation. This idea holds true for both cPKC and nPKC phosphorylation mediated kinase modulation. Evidence that cPKCα activity is under control by phosphorylation has been available for some time, with the findings that a phorbol ester-induced fast migrating (dephosphorylated) form of PKC was inactive (Borner et al., 1988) and that the purified protein could be inactivated following phosphatase treatment (Pears et al., 1992). Subsequent mutagenesis of PKCα defined a threonine residue (T497) within the activation loop of the kinase domain that was essential for activity (Cazaubon et al., 1994). This phosphorylation site is also conserved within PKA (T197), a member of the ACG kinase superfamily. Based upon detailed structural analysis of PKA, the phosphorylated T197 has been shown to play a role in aligning the catalytic site of the enzyme for catalysis (Knighton et al., 1991). Structural models proposed for PKCα and PKCβ suggest an equivalent role for these activation loop phosphorylation sites (Orr and Newton, 1994; Srinivasan et al., 1996). As for PKCα, an absolute requirement for phosphorylation in the activation loop (T500 site in PKCβ) has been established (Orr and Newton, 1994). Interestingly, a similar phenomeon has been demonstrated for nPKCs as well. Stempka and coworkers demonstrated that a similar residue in the activation loop of PKCδ (G500) that mimics phosphorylation may take over the role of the phosphate groups on T497/T500 in PKCα and PKCβ (Stempka et al., 1999). Thus, for these PKCs, there is evidence that activation loop phosphorylation is required for activity. Furthermore, analysis of

recombinant proteins expressed in insect cells demonstrates that these sites are occupied to some degree (Keranen et al., 1995; Tsutakawa et al., 1995). Expression in mammalian cells also reveals phosphorylation of these sites in other PKC isotypes including cPKCγ (Hansra et al., 1999).

One of the original autophosphorylation sites defined for baculovirus-expressed PKCβ_I, threonine 642, is also occupied in purified recombinant protein and in intact cells (Flint et al., 1990). The phosphorylation of this site has been reported to have various effects on activity. The original mutational analysis for PKCβ_I indicated that T642 phosphorylation was essential for activity. However, the solubility of the expressed non-phosphorylated protein can be problematic; removal of phosphates from recombinant PKCα induces kinase aggregation (Bornancin and Parker, 1996). It was subsequently shown that for PKCβ_{II}, if the homologous site (T641) is mutated to alanine, other local sites become phosphorylated to compensate, yielding a fully functional protein (Newton, 1997). For PKCα, the equivalent T638A mutated kinase is not fully functional, displays a high specific activity, thermal unstability, sensitivity to oxidation and phosphatases (Bornancin and Parker, 1996). This phenotype suggests that even if compensating phosphoryaltions occur in PKCα the phosphorylation of T638 itself plays a unique role. These observations of PKCα suggest that phosphorylation of T638 induces a conformational change in the catalytic domain that is also induced by activation loop phosphorylation. Thus, there appears to be an interaction between this C-terminal region and the kinase core.

A third phosphoryaltion site that is critical in PKCα and PKCβ was identified by direct phosphate analysis and also through mutagenesis based upon the predicted similarity between the patterns of sites in PKC and $p70^{S6kinase}$ (Keranen et al., 1995; Tsutakawa et al., 1995; Bornancin and Parker, 1997). It is located in a hydrophobic region of the C-terminal variable region 5 (V5) of PKC, 19 amino acids following the autophosphorylation site. Mutation of this hydrophobic site in PKCα provided evidence that phosphorylation plays a role in controlling the rate of priming site (497,638) phosphorylation in PKCα (Bornancin and Parker 1997). Parallel studies on $PKC\beta_{II}$ have shown that phosphorylation at the homologous site serine 660 (S660) affects Ca^{2-} affinity in PKCβ (Edwards and Newton, 1997). This effect is perhaps mediated by direct C2 domain contact with this C-terminal V5 domain when the catalytic domain is in its closed (i.e. phosphorylated) state. While there continues to be controversy over the order of these 3 phosphorylation events and their precise consequences, the maintainance of these sites in a phosphorylated state is crucial to preventing desensitization of cPKCs (Hansra et al., 1996).

ii) The activation loop cascade

Various elements of conservation within PKCs have led to the finding that phosphoinositide-dependent kinase 1 (PDK1) is responsible for PKC activation loop phosphoryaltion. PDK1 will phosphorylate both nPKCs and cPKCs *in vitro* (Chou et al., 1998; Le Good et al., 1998). Co-expression of PDK1 with PKCδ/α in mammalian cells can also induce PKC phosphorylation at activation loop sites. In intact cells, the effect of PDK1 is blocked by the PI 3-kinase inhibitor LY 294002, and this effect appears to be directed through PDK1 and not PKC (Le Good et al., 1998). Consistent with this, PIP_3 cooperates with the PKC

activator PDBu in stimulating PDK1 phosphorylation of PKCδ *in vitro*. The evidence suggests that PDK1 and PKC need to be corecruited to membranes through interaction with their respective allosteric activators in order for phosphorylation to be efficient. A broad role for PDK1, or a relative, in PKC phosphorylation is supported by the finding that all PKC isoforms as well as $p70^{S6kinase}$ can form complexes with PDK1 (Le Good et al., 1998; Khan et al., 2001; Pepio et al., 2001). Whether PDK1 itself is responsible for all PKC activation loop phosphorylations *in vivo* remains to be determined, although recent observations indicated a role for PDK1 in both vertebrate and invertebrate PKC phosphorylation in *in vivo* (Dutil et al., 1998; Pepio et al., 2001). The most compelling evidence for a physiological role for PDK1 comes from studies on the protein kinase C related kinases (PRKs). These kinases have been shown to be regulated by small G-proteins of the Rho class, through interactions at the N-terminal domains (Shibata et al., 1996; Flynn et al., 1998). It has been found that PRKs also interact with PDK1 via their PDK1-interacting fragment (PIF) region (Balendran et al., 1999) and they are phosphorylated in their activation loops by this kinase (Flynn et al., 2000). However, this effect is dependent on Rho activity since it permits PDK1 binding (Flynn et al., 2000). It is assumed that PDK1 requires its own activator PIP_3 for effective catalytic activity by binding to its PH domain; this would be consistent with the PI 3-kinase dependence of PKC activation loop phosphorylation in intact cells (Chou et al., 1998; Le Good et al., 1998). However it has recently been shown that PDK1 can phosphorylate the activation loop of Akt in the absence of its PH domain (Flippa et al., 2000). For PKC there is direct *in vivo* evidence for the need for PKC activation, since calphostin C, which selectively blocks the allosteric activation of PKC by DAG, inhibits serum-induced activation loop

phosphorylation, as does the PI 3-kinase inhibitor LY294002, which would affect PDK1 recruitment/activation (Parekh et al., 1999).

The essence of these studies is that there is a cascade of kinases involving PI 3-kinase, PDK1 and various members of the PKC superfamily. The specificity of function is driven, at least in part, by effector/second messenger interactions with the target kinase. For PKC, the consequence of phosphorylation is an increase in catalytic activity, without bypassing the requirement for allosteric activators.

iii) Implications for PKC signaling

There are some interesting implications deriving from the pattern of phosphorylations acting on conventional and novel PKC isoforms. With respect to the "core" phosphorylations themselves, it would appear that they are capable of regulating the specific activity of the kinase. While this distinction exists between allosteric effectors and phosphorylation of PKCs, evidence suggests that phosphorylation occurs *in vivo* when PKC is in an active conformation. This is similar to Akt where its interaction with phosphoinositides through its regulatory PH domain permits phosphorylation by PDK1 (Alessi et al., 1997, 1998; Stephens et al., 1998). However, unlike Akt, when PKCs release their DAG activators, the "core" phosphorylations are not lost. It appears that the inactive, closed conformation of PKC is relatively resistant to phosphatases. Thus, DAG can acutely activate these PKC isoforms, but the extent of the activation is modulated by the phosphorylation state. This is most evident from work on another interesting type of phosphorylation sites that are modulatory but not critical to kinase activation. These sites are mechanisms for membrane

translocation/dissociation and have been shown to oppose the C2 domain mediated membrane association (Feng et al., 2000; Nakhost et al., 1999). Phorbol esters can translocate nPKCs to the membrane in cells, and for PKC Apl II this translocation appears to be partially dependent on C2 domain phosphorylation. While one might expect PDBu mediated translocation to depend only on the C1 domain, mutations in the C2 domain of cPKCs can decrease PDBu mediated translocation, suggesting that both domains are required for translocation (Medkova and Cho, 1998b; Oancea and Meyer, 1998). Moreover, the C2 domain is required for translocation of cPKCs by natural stimuli (Feng et al., 2000). Since there are few differences in lipid and/or phorbol ester-binding between the C1 domains of cPKCs and nPKCs (Pepio et al., 1998), it is likely that the C2 domain is also required for nPKC translocation *in vivo*. Deletions in the C2 domain can block PDBu binding and the presence of the C2 domain decreases the affinity of the C1 domain for PDBu in a PS-dependent fashion (Pepio and Sossin, 1998; Quest and Bell, 1994). Phosphorylation may not only increase lipid binding to the C2 domain, but may decrease C2 domain-mediated inhibition of the C1 domain.

An important consequence of this behavior of PKC is that the accumulation of phosphorylated PKC isoforms may serve to integrate information over time. As well, the phosphorylation of the C2 domain may not modulate the activity but may regulate the localization of active PKC isoforms to specific microdomains (Ng et al., 1999; Pepio and Sossin, 2001). For nPKCδ and nPKCε to become phosphorylated at their core sites, it is clear that i) DAG must be present and ii) PIP_3 must recruit/activate PDK1. Such a system might evolve this modulatory control to sense events which the cell would want to buffer

itself against. Thus, for example, a cell transiently exposed to an amino acid-depleted environment might shut down new protein synthesis rapidly, but would not be expected to commit itself immediately to apoptosis. Perhaps the role of PKCα phosphoryaltion in survival reflects such a protective mechanism (Whelan and Parker, 1998).

F) Receptor-Mediated Signal Transduction and Persistent PKC Activation

Upon stimulation of cell surface receptors, DAG is immediatedly produced from inositol phospholipids, most rapidly from PIP_2 hydrolysis, as a result of PLC activation. This DAG molecule disappears quickly, although the level of DAG often increases again with a relatively slow onset, and persists for minutes to hours. It has been shown that this second wave of DAG appearance results from the hydrolysis of PC, because of the fatty acid composition of this DAG molecule matches that of PC (Jarpe et al., 1994). This sustained elevation of DAG levels is frequently observed in response to various long-acting signals such as growth factors, cytokines, and phorbol esters. Mitogenic signals sometimes initiate only the second phase of DAG elevation and previous experiments have shown that sustained elevation of DAG levels for several hours is essential for long-term cellular responses such as growth and differentiation (Berry et al., 1990; Asaoka et al., 1991; Aihara et al., 1991; William et al., 1990). Together, DAG and IP_3 stimulated calcium release from the ER are sufficient to produce a transient activation of cPKCs in cells. The amplification of the DAG signal, however, requires the additional activation of a PC hydrolyzing enzyme such as PLD. The PLD production of PA from PC has been shown to require Ca^{2+}, and signals stemming from PIP_2, PI 3-kinase, the small G-proteins ARF and RhoA, and cPKCs (see Fig. 2). PIP_2 has been shown to have a direct effect on PLD activation presumably through direct

interactions, whereas the effects of PI 3-kinase are less well defined. Most likely this PLD stimulated activity is via production of PIP_3 and the subsequent recruitment of PH domain containing proteins such as GRP-1 but evidence to this end is somewhat lacking (Klarlund et al., 1997). As well, two important feedback loops exist in these signaling pathways. The first is through the feedback of PA onto PI 4-kinase and the second is by ARF/Rho feedback onto PI 5-kinase, both of which produce additional PIP_2 prolonging PLC substrate directly activating PLD (see Fig. 2) (Natarajan et al., 1996). Additionally, studies suggest that PLD is stimulated through direct interactions with ARF/RhoA. The effects of cPKCs are upstream of ARF effects and may stimulate PLD activity in this manner or through some unknown mechanism acting on PLD directly.

The second wave of DAG appearance results from the hydrolysis of PC raising the level of DAG often with a relatively slow onset. This DAG may be responsible in part for the persistent activation of PKC. Since nPKCs appear to be activated later in these pathways, it might be resonable to think that the late phase of DAG induces their activity. In addition to signaling cascades leading to PLD activation and PA production, the phosphorylation of the C2 domain of nPKCs may activate these PKC isoforms (see Figs. 1 and 2). Additionally, phosphorylation might serve to provide a mechanism for the localization of nPKCs that are persistently activated by the persistent PC-derived form of DAG. Despite the well-characterized mechanism of cPKC regulation, the activation of nPKC regulation appears to have delayed onset and requires more factors than the traditional allosteric activators, PS and DAG. Furthermore, experimental evidence suggests that the C1 protein domain of nPKC isoforms function in a similar fashion to those of nPKCs, whereas the C2 domain may be the

rate limiting step in nPKC activation. Understanding the role of these domains that comprise the kinase regulatory region of nPKCs may help to unravel the mechanisms of their regulation in the nervous system.

II. CONCLUSIONS

In the nervous system, PKCs play important roles in synaptic plasticity and learning. Activation of PKC is both necessary and sufficient to produce long-lasting changes in neuronal excitability, morphology, and connectivity. Furthermore, molecular modifications of PKC itself give rise to different properties of the enzyme known to be important during plasticity. Despite the well-characterized mechanism of conventional Ca^{2+}-dependent PKC regulation, a detailed analysis of Ca^{2+}-independent PKC regulation has not been presented.

This thesis has explored the role of the C2 domain of the Ca^{2+}-independent PKC Apl II in the *Aplysia* nervous system. The results presented are consistent with a modulatory role of the C2 domain in regulating PKC Apl II. The C2 domain has been shown to interact with the C1 domain to regulate activator binding in a lipid-dependent manner. Additionally, C2 domain-mediated membrane binding in PKC Apl II is regulated by phosphorylation. Determining whether membrane binding of other C2 domains is regulated by phosphorylation may help to unravel the mechanisms of Ca^{2+}-independent PKC activation.

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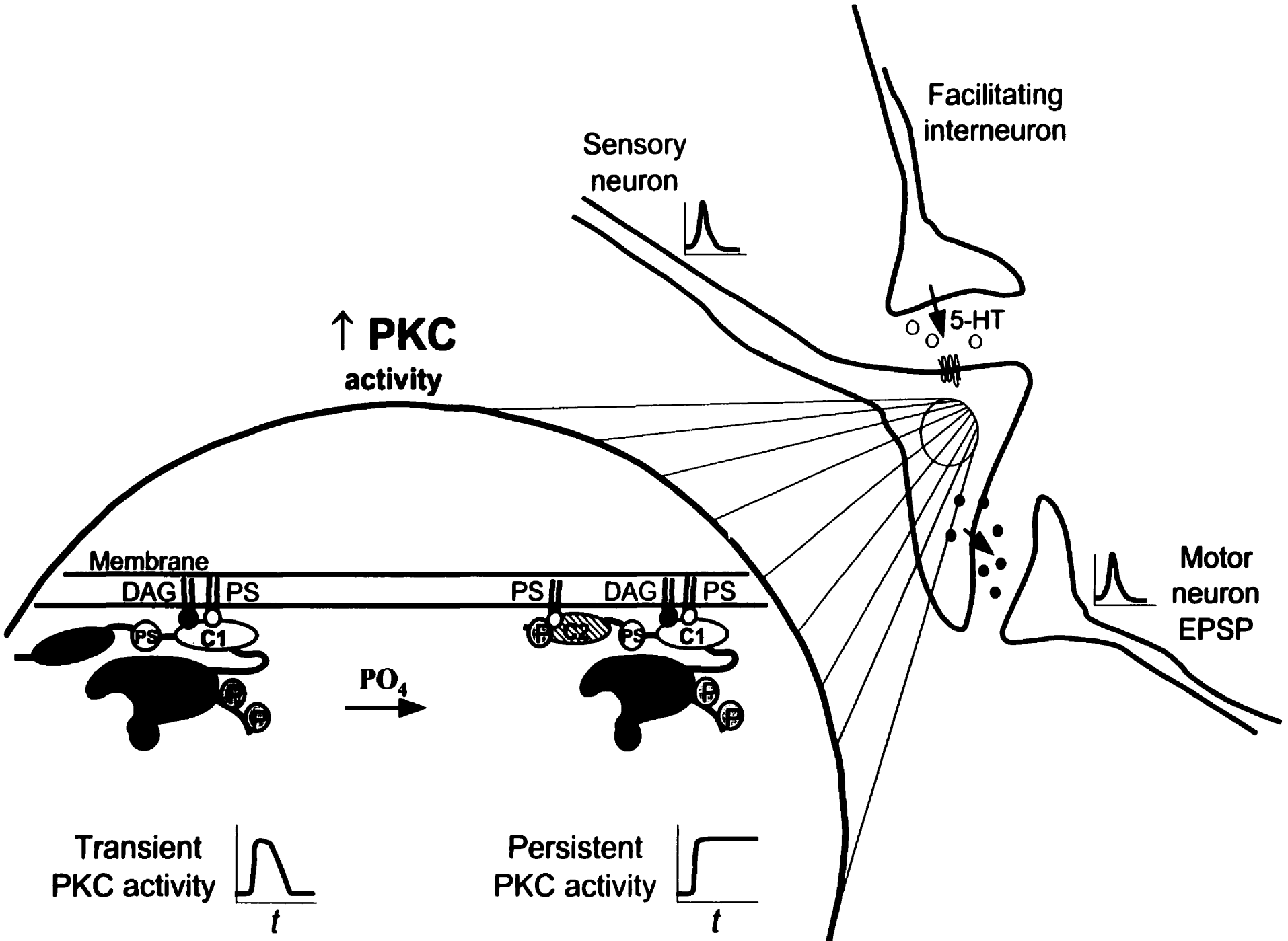
Facilitating interneuron
Sensory neuron
↑ PKC activity
5-HT
Membrane
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Motor neuron EPSP
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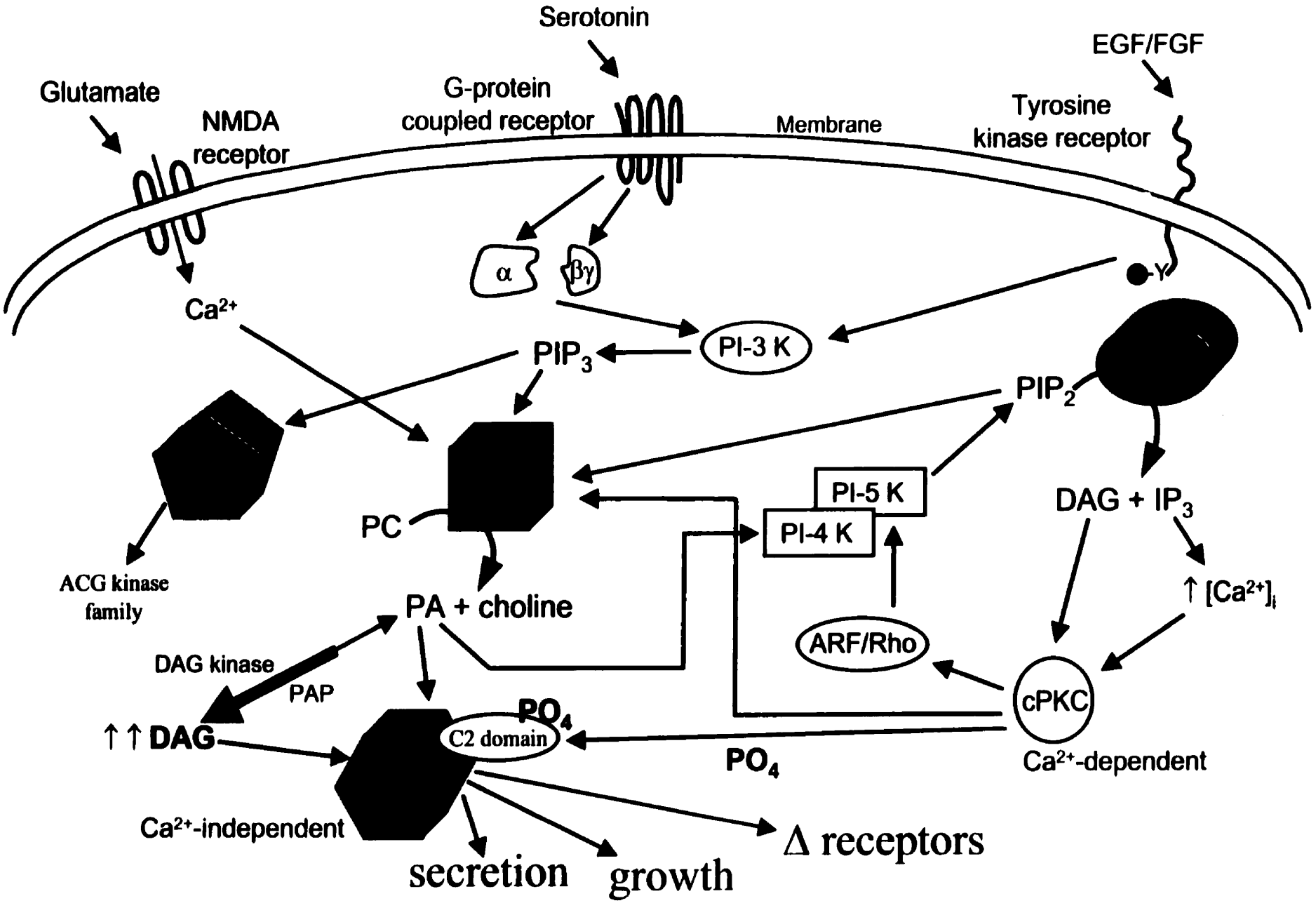
Serotonin
EGF/FGF
Glutamate
NMDA receptor
G-protein coupled receptor
Membrane
Tyrosine kinase receptor
α
βγ
γ
Ca2+
PI-3 K
PIP3
PIP2
PC
PI-5 K
PI-4 K
DAG + IP3
ACG kinase family
PA + choline
↑ [Ca2+]i
ARF/Rho
DAG kinase
PAP
cPKC
PO4
↑↑ DAG
C2 domain
PO4
Ca2+-dependent
Ca2+-independent
Δ receptors
secretion
growth