NOTE TO USERS

Regulation of Phosphorylation of Protein Kinase C in Neurons

by

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Table of Contents

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Abstract

In Aplysia, persistent increases in synaptic strength are mediated by persistent activation of protein kinase C. In particular, the novel PKC Apl II is persistently active between 90 minutes and 2 hours after training. Although unknown, the most likely mechanism of persistent activation is phosphorylation. We characterized phosphorylation of PKC Apl II at a conserved hydrophobic site, serine 729, with a phosphospecific antibody. Phosphorylation of S729 increased and persisted 2 hours after serotonin treatment, but did not change after phorbol ester activation. Furthermore, a serine 729 to alanine mutation reduced the activity and stability of Apl II. We studied upstream regulation of S729 phosphorylation, and showed that it is affected by both PDK-1 and atypical PKC Apl III in SF9 cells but not by FRAP or PtdIns3K in neurons. Finally we characterized atypical PKC Apl III using immunoblotting, and used kinase assays to identify isoform specific inhibitors for future studies.

Abstract (Franąais)

Chez l'a*plysie*, les augmentations persistants de force synaptique sont effectuées par l'activité persistante de le kinase PKC. En particulier, la PKC Apl II est constamment actif entre 90 minutes et 2 heures à la suite de l'entrainment. Bien qu'inconnu, le mechanisme plus probable de l'activation persistante est la phosphorylisation. Ici, nous caractérisons la phosphorylisation de PKC Apl II au niveau de la serine 729, qui est un residue hydrophobique conservé, a l'aide d'un anticorps phosphospecifique. Phosphorylisation de S729 a été accru et persiste 2 heures après un traitement de serotonin, mais n'a pas change après activation des esters de phorbol. Aussi, un remplacement de la serine 729 par l'alanine reduit l'activité ainsi que la stabilité d'Apl II. Nous avons étudié la régulation de la phosphorylation du site S729 de la PKC Apl II. Nos résultats démontrent que la phosphorylation de ce site est contrôlée par la PDK1 et la PKC atypique Apl III dans les cellules Sf9 et non pas par la FRAP ou la PtdIns3K dans les neurones. En dernier lieu, nous avons caractérisé la PKC atypique Apl III par immunobuvardage etpar des essais kinases afin d'identifier des inhibiteurs spécifiques de cette protéine qui pourraient servir pour d'autres études.

Foreword

An understanding of memory is a core priority of the cognitive sciences because our memories have enormous and arguably absolute influence over our behaviour and consciousness. We make decisions based on previous experiences, experiences encoded as memories. Our intelligence, which is to say factual knowledge, rests in that which we have committed to memory. Even social behaviours or non-cognitive reflex behaviours depend on various forms of learning – committing to memory.

In our laboratory, we capitalize on the technical methodology emerging from the wave of progress enjoyed by molecular biology/biochemistry in the past two decades in our effort to dissect the formation of memories at its most reduced model form. The simplicity of this reduced model, the sensorimotor synaptic facilitation in the marine mollusk *Aplysia*, allows us to document biochemical changes in the system, confident that they are specific, relevant and contingent to the question of memory formation.

Chapter 1: PKC Apl II

Introduction

To eventually understand behavioural memory, it is helpful to first study reduced models of memory, neuronal synapses, since they form the elementary units of learning within the larger system of which they comprise. Memory at the cellular level can be studied without needing to know the nature or origin of the memory being encoded. For example the link between perhaps the most studied model of cellular memory - long term potentiation (LTP) - and its assumed physiological process, hippocampal spatial memory, to this day remains unproven (Lynch, 2004).

In the current understanding of cellular memory, the survival or extinction of a memory depends on the strength and number of connections between neurons encoding that memory. The strength of a synapse depends on both presynaptic factors such as the amount of neurotransmitter released, and postsynaptic factors such as the number of receptors present in the membrane.

A well studied system for cellular memory is the sensory to motor synapse in the marine mollusc *Aplysia californica*. This synapse participates in a defensive withdrawl reflex of the animal's gill in response to a disturbance of its siphon (Kandel, 1979). The strength of this reflex can be reduced by habituation training, and increased by sensitization training. A noxious stimulus to the tail sensory neuron causes sensitization through serotonergic interneurons that synapse on the presynaptic siphon sensory neuron. Serotonin causes an increase in synaptic strength called facilitation, and exogenous

application of 5-HT can simulate the interneuronal mediation that occurs during sensitization training (Mercer et al., 1991). Facilitation can be short term (STF), lasting under 30 minutes, intermediate term (ITF), lasting 2 hours or more, and long term (LTF), lasting more than 24 hours (Pinsker et al., 1973; Ghirardi et al., 1995). These three phases of memory are mechanistically independent; pharmacological studies have shown that LTF requires new protein synthesis and RNA synthesis, ITF often requires protein synthesis, and STF requires only modification of existing proteins (Montarolo et al., 1986; Müller & Carew, 1998).

Study of the *Aplysia* sensorimotor synapse has shed some light on the molecular basis of facilitation. Serotonin binds its receptor, stimulating the generation of second messengers which activate protein kinases. Protein kinases initiate pathways that ultimately modify and enhance the synapse. Indeed, covalent modification of proteins such as the phosphorylation of ion channels by cyclic AMP dependent protein kinase (PKA) is a mechanism known to underlie short term facilitation, and analagously CaMKII phosphorylates AMPA channels causing early phase LTP (Shuster et al., 1985; Bolshakov et al., 1997). Protein kinases are particularly interesting components of intracellular signalling because they represent a point of amplitude control within their pathway and they often have many targets. For example, neuronal Protein Kinase C (PKC) targets components of the cytoskeleton (Gruenbaum et al., 2003), vesicular release machinery (Nagy et al., 2002), and ion channels (Yang & Tsien, 1993).

The role of protein kinase C in memory signaling has received interest since the discovery of PKC. PKCs consist of an amino terminal regulatory subunit and a carboxy terminal catalytic subunit, joined by a "hinge" linker region. The regulatory subunit

contains a pseudosubstrate - a region that resembles the substrate sequence of PKC that cannot be phosphorylated but occupies the phosphate acceptor site in the kinase domain of the catalytic subunit. The mechanism of regulated, transient PKC activity requires cofactors and second messengers to bind the regulatory domain which frees the pseudosubstrate in deference to real substrate. Substrate binds the kinase domain, allowing its phosphorylation by transfer of inorganic phosphate from adenosine triphosphate (ATP).

The PKC family consists of three isotype families: conventional, novel and atypical (for review, see Newton, 2001). In mammals there are 10 unique identified PKCs: PKC alpha, PKC beta I, PKC beta II and PKC gamma are conventional PKCs (cPKC). PKC delta, PKC epsilon PKC theta and PKC eta are novel PKCs (nPKC). PKC zeta and PKC iota/lambda are atypical PKCs (aPKC). *Aplysia* have three identified PKCs: PKC Apl I, PKC Apl II and PKC Apl III – one from each family of conventional, novel and atypical PKCs, respectively.

The families differ primarily in the cofactor binding motifs within their regulatory subunit, and therefore, differ in the cofactors required for activity. Conventional PKC contains two C1 domains, which bind diacylglycerol (DAG), followed a C2 domain which binds Ca^{2+}. Novel PKC contains a "novel" C2 domain which does not bind calcium, followed by a C1 domain binding DAG. Atypical PKC has an "atypical" C1 domain that does not bind DAG, and no C2 domain. In the classical model of cPKC activation, a G protein coupled receptor activates phospholipase C, which hydrolyzes PIP_2 into IP_3 and diacylglycerol (DAG). IP_3 then signals the release of Ca^{2+} from intracellular stores within the endoplasmic reticulum. The binding of calcium to the C2

domain of conventional PKCs is believed to be permissive to the binding of DAG to the C1 domain (Bolsover et al., 2003), and because DAG is a membrane integrated lipid, cPKCs are recruited to the membrane. At the membrane, the relative concentration of the lipid phosphatidylserine (PS) is high and the interaction of PS with the regulatory domain provides sufficient energy to release the pseudosubstrate. Novel PKCs do not require Ca^{2+}, but they do require DAG and are also translocated to the membrane during activation. Additionally, phosphatidic acid generated by phospholipase D is believed to increase nPKC affinity to the membrane (Lopez-Andreo et al., 2003). Experimentally, non physiological, exogenously applied phorbol esters such as phorbol 12,13 dibutyrate (PDBu) and 12-O-tetradecanoylphorbol-13-acetate (TPA) mimic the effect of DAG and are potent activators of conventional and novel PKCs.

Of these various isoforms, several are brain or nervous system enriched, in particular PKC gamma, PKC epsilon and PKM zeta (Tanaka & Nishizuka, 1994; Hernandez et al., 2003) in rat brain and spinal cord. *Aplysia* ganglia contain all three of PKC Apl I, Apl II and Apl III. Although the details of neuronal PKC signaling are still being investigated, a broad role for PKC involvement in learning has been well established over the past 15 years. Initial experiments tracked the subcellular distribution of PKC, given that PKC translocation to the membrane is an indicator of PKC activity. For example, eyeblink classical conditioning caused the translocation of PKC from cytosol to membrane, and the redistribution of PKC from soma to dendrites in rabbit hippocampus (Olds et al., 1989); and PKC gamma is translocated to the membrane in mouse hippocampus following spatial discrimination training (Van der Zee et al., 1992). More directly, increases in PKC activity during memory formation have been measured.

Contextual fear conditioning in rats activates PKC isotypes from all three families (Young et al., 2002) and classical olfactory conditioning in honeybees activated PKC both transiently and long term (Grunbaum & Miller, 1998). Still others looked at training of behavioural tasks and showed that inhibition of PKC impaired memory as measured by task performance. For example, blocking PKC activity with pharmacological inhibitor chelerythrine impairs permance of a peck-avoidance task in chicks (Serrano et al., 1995). PKC beta knockout mice exhibit impaired cued and contextual fear conditioning (Weeber et al., 2000); and PKC gamma knockout mice have abnormal LTP and impaired spatial learning (Abeliovich et al., 1993). Inhibiting PKC also prevents use dependent LTD in cerebellar purkinje cells (Eto et al., 2002). Conversely, the activation of PKCs enhances memory both at the level of performance and of synaptic strength. For example, using 1-Oleoyl-2-acetyl-sn-glycerol (OAG) prolonged retention of spatial discrimination memory in a radial maze (Nogues et al., 1996), and activation of PKC with phorbol esters increased facilitation in *Aplysia* sensory neurons (Manseau et al., 1998) and induced hippocampal LTP (Colley et al., 1989), even in the presence of NMDA blockade (Kleschevnikov & Routtenberg, 2001).

Kinases are especially relevant for memory signalling because of their capacity to exist in a persistently active form. Since an intermediate or long term memory by definition must persist after the initial stimulus, persistent kinase activity is thought to be a molecular effector of the "remembered" stimulus. Alternatively some kinases may, as is the case with ERK/MAPK activating CREB (Bartsch et al., 1998), intiate translation required for long term facilitation and protein synthesis necessary for structural changes beyond 24 hours.

The dogma about persistent protein kinase activity focuses on the autoinhibiting regulatory domain of the enzyme, and its regulation of the kinase domain. If the regulatory subunit is removed, autoinhibition can be relieved and the enzyme becomes constitutively active. This removal may be an allosteric conformational relief, or may be a complete proteolytic loss of the regulatory subunit. Although transiently active Protein Kinase C is involved in short term *Aplysia* sensorimotor facilitation (Ghirardi et al., 1992), it is also involved in intermediate term facilitation. In a reduced *Aplysia* tail-shock preparation, maintainance of ITF after sensitization training requires persistent activity of PKC (Sutton et al., 2004).

Importantly, during the maintainance of ITF, PKC is persistently and autonomously active. This activity does not require the usual cofactors at the regulatory domain of PKC (Sossin & Schwartz, 1994). Problematically, *Aplysia* neurons contain a conventional, novel and atypical isoform of PKC. Isoform specific immunoprecipitation experiments have shown that persistent kinase activity induced by ITF protocols are depleted by an antibody specific to the novel PKC Apl II (Sossin, 1997). Furthermore, the enzyme has an intact regulatory subunit, because the regulatory domain specific calphostin C can inhibit the enzyme, albeit at higher concentrations than for the non-autonomous enzyme (Sossin 1997). Thus the mechanism of persistent activity of PKC is not known.

Persistent kinase activity may be due to phosphorylation. CaMKII, the kinase responsible for postsynaptic changes during LTP, becomes independent of its activator, Ca^{2+} through phosphorylation (Miller & Kennedy, 1986). This allows for persistent , Ca^{2+} autonomous activity thought to underlie LTP. Enzymes in the PKC family are also

regulated by upstream phosphorylation before they in turn phosphorylate other targets. Apl II has three highly conserved phosphorylation sites: an activation loop, the autophosphorylation turn site, and the hydrophobic site (S729).

We previously characterized PKC Apl II phosphorylation at the activation loop site T561 (Pepio et al., 2002). In *Aplysia* sensorimotor ganglia preparations, 5-HT increased phosphorylation in the activation loop, and the phosphorylation persisted for 2 hours after treatment. Furthermore, autonomous kinase activity was correlated with T561 phosphorylation. Although the activation loop is regulated during 5-HT mediated facilitation, it may not be a critical phosphorylation. pT561 is a priming phosphorylation and may only be required for sequential phosphorylation of the other two nPKC phosphorylation sites during initial enzyme maturation. For example in conventional PKC beta II, activation loop phosphorylation is not a strict requirement for PKC activity, as the enzyme is still active even after a phosphatase has removed it (Keranen et al., 1995).

Furthermore, the hydrophobic site is more regulated than the activation loop, making it a good candidate mechanism to mediate 5-HT signaling. For example, in mammalian novel PKC delta, S662 is highly sensitive to serum in serum starved cells, presumably due to the concentration of growth factors present. Moreover, during LTD, phosphorylation of the hydrophobic site S657 in conventional PKC alpha is decreased (Thiels et al., 2000). However, the exact upstream control has not been fully elucidated. Notably, this site is homologous to hydrophobic sites in other PKCs and indeed, several other kinases including P70/S6K, Akt/PKB, and RSK-1 (Storz & Toker, 2002), may share common regulatory inputs with these other related sites.

In this thesis, I study the carboxy terminal hydrophobic phosphorylation site (S729) in *Aplysia* novel PKC Apl II. I show evidence for a possible role in cellular memory and describe S729 in overall enzyme function. Finally I look at trans control of S729 by several candidate regulators: PtdIns3 Kinase, FRAP, PDK-1 and atypical PKC Apl III.

Materials and Methods

Experimental animal system – Aplysia californica were anesthetized with $MgCl_2$ injection, sacrificed, and their pleural-pedal paired ganglia (for serotonin related experiments) or abdominal, cerebral and buccal ganglia (for all other experiments) were dissected out. The ganglia were desheathed under a dissecting microscope and recovered in resting media containing physiological salt and nutrients and buffered to physiological pH. Each experimental trial control vs. treatment was paired left vs. right side (randomly) from within the same animal.

Cytosolic vs membrane fractions – Nervous tissue samples were homogenized in a low salt homogenization buffer (10 mM $MgCl_2$, 50 mM Tris pH 7.5, 10% v/v Glycerol) containing protease inhibitors leupeptin, benzamidine and aproteinin. For phospho antibody experiments, phosphatase inhibitors microcystine, 50 μM NaF and 5 μM sodium pyrophosphate were added. Homogenates were then spun at 51000 rpm for 30 minutes. This process pellets membrane associated proteins, leaving soluble cytoplasmic proteins in the supernatant. The pellet was resuspended in homogenization buffer and samples were denatured with Laemlli buffer and 95° heat and run on 9% SDS-PAGE.

Antibodies – The peptide EFRGF[pS]FANPDamide was designed from the cloned sequence of the target protein. This peptide was dissolved, and conjugated to BSA-maleimide carrier at its carboxy terminal aspartic acid with a 1-Ethyl-3(3-dimethylaminopropyl)carbodiimide (EDC) catalyst, then injected into rabbits, in a Titermax Gold™ mixture. Rabbit sera was diluted 20% in PBS and purified on a Pierce Sulfolink™ column against the original peptide, then concentrated.

Immunoblotting – Unless otherwise stated, all western blots used the following standard protocol: 5% w/v skim milk powder in tris buffered saline (TBS), pre block 30 minutes, primary incubation 1 hour, 3x wash 10 min., secondary incubation 1 hour, 2x wash 10 min., 2x wash in TBS 10 min. Blotting was performed at room temperature. Phospho antibodies were pre-blocked with non-phospho peptide at a concentration of 25:1 molar ratio.

Quantitation of Immunoblotting – Western blot films were scanned and the bands were quantified using Image J software with uncalibrated OD. We define phosphoratio as changes in phosphospecific antibody staining relative to changes in overall protein levels, calculated as $\Delta(p1/p2) / \Delta(n1/n2)$ where p = phospho signal and n = non phospho signal. PKC translocation was measured by comparing the non phospho antibody signal between cytoplasm and membrane fractions, corrected by amount of protein loaded.

Heterologous overexpression system – Enzyme clones were transfected into an Invitrogen Bac-n-Blue® baculovirus vector, whose Lac-Z reporter will express blue only if homologous recombination successfully incorporated the cloned element. Blue viral plaques were picked, virus was titred to a sufficient concentration and used to infect SF9 insect cell lines. SF9 cells infected with a transfected viral stock will overexpress the cloned protein over a course of three days. Cells were homogenized for western analysis, or for enzyme purification.

Enzyme purification –PKC Apl II, Apl III and its various mutants were cloned with an added amino-terminal histidine chain tag that did not interfere with function. His-tagged proteins could be purified using Invitrogen's Probond® His-affinity resin columns. Proteins not his-tagged in this manner will not bind the column and will be

washed out. The enzymes were then eluted into a storage buffer (50mM Tris pH 7.5, 1mM $MgCl_2$, 100uM $ZnSO_4$, 10% v/v glycerol) and concentrated.

Kinase assays – Enzymatic activity of our kinases was assayed in an *in vitro* reaction, with all parameters as previously described in Sossin (1997). Briefly, PKC Apl II was incubated with 10 μM subsrate A-ε peptide, radioactive 32-ATP, PS/TPA or water, and PKC inhibitors or water. PKC Apl III used the ε peptide substrate. Reactions were performed at room temperature for 30 minutes. Each reaction was blotted onto P81 Whatman® filter paper, stopped in a 2% w/v cold ATP solution, washed 4x with 0.425% v/v phosphoric acid, and then counted in a scintillation counter for final levels of radioactivity incorporated into the substrate. Specific activity per immunoreactivity was calculated for assay reactions that were non-saturated.

Results

Antibody - We raised an antibody specific to the hydrophobic site containing phosphorylated serine 729 of novel *Aplysia* PKC II. We then affinity purified the antibody against its original phospho peptide and immunoblotted it to purified PKC expressed in the SF9-baculovirus system. The antibody recognized a single band at 100 KDa, the expected size of PKC Apl II, in both SF9 expressed and nervous system derived blots (Figure 1b). The antibody is phospho specific because it reacted only with purified PKC Apl II, even if pre-blocked 25:1 by the non-phospho peptide, and it did not recognize purified Apl II S729A which cannot be phosphorylated. Assuming the antibody staining correlated with phosphorylation at S729, we measured phosphorylation by quantitating western blot bands. An antibody to an unrelated, non-phosphorylated N terminal site of PKC Apl II controlled for phospho staining levels by measuring total protein levels (Figure 1a).

Activity – Each of the three priming phosphorylations contributes to PKC function or structure. Serine to alanine mutations of mammalian S662 in novel PKC delta reduce the activity of the kinase (Le Good et al., 1998). Mutations to the site in other isoforms, or at other sites of PKC, also impair its activity or structural stability. To test the function of *Aplysia* S729, we generated S729A and S729E mutations. Previous western blots showed that wild type PKC Apl II is significantly phosphorylated, whereas Apl II S729A is unphosphorylated (Figure 1). We expressed and purified these clones from SF9 cells and compared their kinase activity to wild type PKC using kinase assays.

The S729A mutation significantly reduced the specific activity of PKC (Figure 2a) in phorbol ester/lipid activated PKC. S729A PKC Apl II mutants also had a similar-fold reduction in activity in the absence of PDBU/TPA, indicating that the remaining activity – the autonomous activity – was attributable to the difference in S729 phosphorylation. Unpredictably, the S729E mutation also significantly reduced both the regulated and autonomous specific activity of PKC suggesting that the negative charge alone may be insufficient for functional effect.

Some PKC phosphorylations are required for enzyme stability. For example, dephospho PKC alpha is unstable and more sensitive to phosphatases. One factor affecting enzyme stability is temperature. To test if S729 phosphorylation stabilized PKC Apl II, we pre-incubated the enzymes at 5, 10 and 15 degrees higher temperatures for 10 minutes before repeating the kinase assay. Both the S729A and S729E mutants lost more activity at higher temperatures than wild type enzyme (Figure 2b).

5-HT – Exogenously applied serotonin simulates a sensitizing stimulus at the sensorimotor synapse in *Aplysia* (Mercer et al., 1991). In particular, exposing isolated pleural-pedal ganglia to 5-HT for 90 minutes induces persistent PKC activity (Sossin & Schwartz, 1994). Previously we found that serotonin phosphorylates the activation loop site of PKC and the phosphorylation persisted during ITF maintainance after 2 hours (Pepio et al., 2002). However in mammalian novel PKCs, the hydrophobic site is more regulated than the activation loop, and removing the activation loop phosphate does not remove mature PKC kinase activity (Keranen et al., 1995). Thus we tested whether serotonin also persistently affects S729 phosphorylation in neurons.

During regulated activation, nPKCs translocate to the membrane; 5-HT increases autonomous activity on the membrane but not the cytoplasm (Sossin & Schwartz, 1994) therefore if phosphorylation is a mechanism of persistent activity, it is predicted to be increased in membrane associated PKC. As expected, there was an average 40% increase in S729 phosphoratio ($n=10$, $p=0.05$ student's paired t-test) in the membrane fraction 2 hours after 5-HT treatment of ganglia (Figure 3a, b). Similarly, the phosphoratio of cytosolic PKC was not significantly increased ($P>0.40$, student's paired t-test). Normal S729 phosphorylation levels in cytoplasm vs. membrane are statistically the same, with an average 1% more phosphorylation in cytoplasm ($n=23$, $p>0.17$, student's paired t-test) (Figure 3c). We also examined the subcellular distribution of PKC. 5-HT did not translocate PKC to the membrane, similar to previous results (Pepio et al., 2002). In fact, 2 hours after 5-HT treatment, there was a small decrease in the amount of PKC on the membrane, compared to untreated (Figure 8), suggesting that 5-HT does not directly translocate PKC.

To compare the observed persistent 5-HT phosphorylation with the effect of regulated PKC activation, we applied 10 μM of the phorbol ester PDBu to ganglia and looked for changes in phosphorylation. There was no significant change in S729 phosphoratio, even immediately after treatment, in either cytosol ($n=4$, $p>0.8$, student's t-test) or membrane associated PKC ($n=4$, $p>0.17$, student's t-test) (Figure 3d,e). This further supports the notion that phosphorylation and translocation are separate processes and that S729 phosphorylation is not a result of transient nPKC activity. As expected, PDBu increased the percent of membrane associated PKC ($n=4$, $p=0.07$, student's t-test), an effect of phorbol esters' simulation of DAG binding at the membrane (Figure 8).

Regulation: inhibitors – 5-HT phosphorylation of S729 led to the question of how this phosphorylation was controlled, specifically, what factors transduce 5-HT signaling to PKC. One model could be a second kinase trans phosphorylates PKC at S729, while another possibility is that PKC itself autophosphorylates S729, and dephosphorylation is the regulatory step. To study autophosphorylation, we inhibited PKC Apl II by treating ganglia with classical pharmacological inhibitors of PKC. The inhibitors chelerythrine and bisindolylmaleimide (bis) both inhibit Apl II kinase activity measured in P-32 kinase assays (see Chapter 2: Figure 4,5). Chelerythrine did not significantly decrease phosphoratio at S729 in either membrane ($n=4$, $p>0.6$, student's t-test) or cytosolic fraction ($n=4$, $p>0.7$, student's t-test) (Figure 4c,d). Bis slightly increased membrane phosphoratio, but not significantly ($n=4$, $p>0.12$, student's t-test), and did not change cytosolic phosphoratio ($n=4$, $p>0.7$, student's t-test) (Fig 4a,b). Notably, bis translocated PKC to the membrane by an unknown mechanism, while chelerythrine did not (Fig 8). Bis increased the amount of PKC on the membrane by a three-fold average ($p=0.03$). This replicates our previous finding of bis translocation (Pepio et al., 2002).

Although pharmacological inhibitors did not noticeably reduce phosphorylation, PKC is at least partly phosphorylated at S729 in wild type enzyme, even in a heterologous system such as SF9 cells (Figure 1). Since this may happen soon after the protein is translated, inhibitors may be unable to stop it. Thus we examined a PKC Apl II K/R kinase dead mutant expressed in SF9 cells, which has no initial kinase activity. The K/R mutation did not completely abolish S729 phosphorylation (Figure 4e), however kinase dead PKC Apl II had average 65% decreased S729 phosphorylation ($n=9$, $p<0.0001$, student's paired t-test).

Regulation: related pathways - Having shown the potential for trans phosphorylation, we next examined candidate regulators of hydrophobic site phosphorylation. The serine/threonine kinase PDK-1 targets a variety of proteins for phosphorylation, including PKC. PDK's most prominent target is the activation loop motif at T561, although its interaction with the hydrophobic site is inconclusive. Notably, PDK-1 may phosphorylate the analagous hydrophobic site in related kinases AKT, P70/S6K and RSK (Williams et al., 2000). To test the effect of PDK-1, we heterologously coexpressed PKC Apl II with PDK-1 using the SF9/baculovirus system (Figure 5a). Unexpectedly, PDK-1 actually reduced phosphorylation of S729 in wild type PKC Apl II by an average 42% (n=4, p=0.06, student's paired t-test) (Figure 4a,b). Interestingly when we coexpressed PDK-1 with the K/R kinase dead PKC construct, it increased S729 phosphorylation by an average 277% (n=5, $p<0.01$, student's paired t-test) (Fig 4a,b). The apparently opposite effects of PDK-1 suggested it was likely not acting as the direct kinase to S729.

In mammalian preparations, atypical PKC iota/zeta was identified in a screen of interactors targeting PKC delta S662 (Ziegler et al., 1999). Given that PDK-1 had a significant influence of S729 phosphorylation, we next tested the effect of atypical PKC Apl III with a similar coexpression experiment if SF9 cells (Figure 5c). There was no change in S729 phosphoratio in wild type PKC Apl II (Figure 5d). Both wild type Apl III (n=6, $p<0.01$, student's paired t-test) and kinase dead Apl III (n=4 $p<0.05$, student's paired t-test) significantly reduced S729 phosphoratio in kinase dead Apl II (Figure 5d). PKC Apl III is clearly not a kinase to PKC Apl II at the hydrophobic site. These results

also suggested that PKC Apl III was interfering with S729 phosphorylation of PKC Apl II.

Rapamycin – The hydrophobic site is highly conserved between protein kinases, and its regulation in P70/S6 kinase has been characterized. Rapamycin, an inhibitor of the mTOR translational upregulation pathway, blocks phosphorylation of the homologous hydrophobic site in P70/S6 kinase, T399. It has also been shown that rapamycin blocks S662 phosphorylation in mammalian PKC delta and epsilon. Therefore we tested rapamycin on dissected *Aplysia* ganglia (Figure 6a). Rapamycin did not significantly affect PKC Apl II S729 phosphoratio in either membrane ($n=5$, $p>0.7$, student's paired t-test) or cytosolic fractions ($n=5$, $p>0.3$, student's paired t-test) (Figure 6b), nor did it alter the subcellular distribution of PKC (Figure 8).

LY – Given the significant effect of PDK-1 on S729 phosphorylation, this signaling pathway may be traced further upstream to the PtdIns3K pathway, which is an activator of PDK-1. Conventional calcium dependent PKCs are not phosphorylated at their hydrophobic site if PtdIns3K is blocked by inhibitor LY294002 (LY) (Ziegler et al., 1999). LY also prevents the serum induced increase in phosphorylation in PKC delta and epsilon (Parekh et al., 1999). We examined whether inhibiting PtdIns3K in neurons would reduce PKC Apl II phosphorylation (Fig 7a). However, there was no significant change in S729 phosphoratio on the membrane ($n=4$, $p>0.2$, student's paired t-test) or the cytoplasm ($n=4$, $p>0.6$, student's paired t-test) after LY294002 treatment of ganglia (Figure 7b). LY treatment did not affect PKC subcellular distribution (Figure 8).

Figure 1

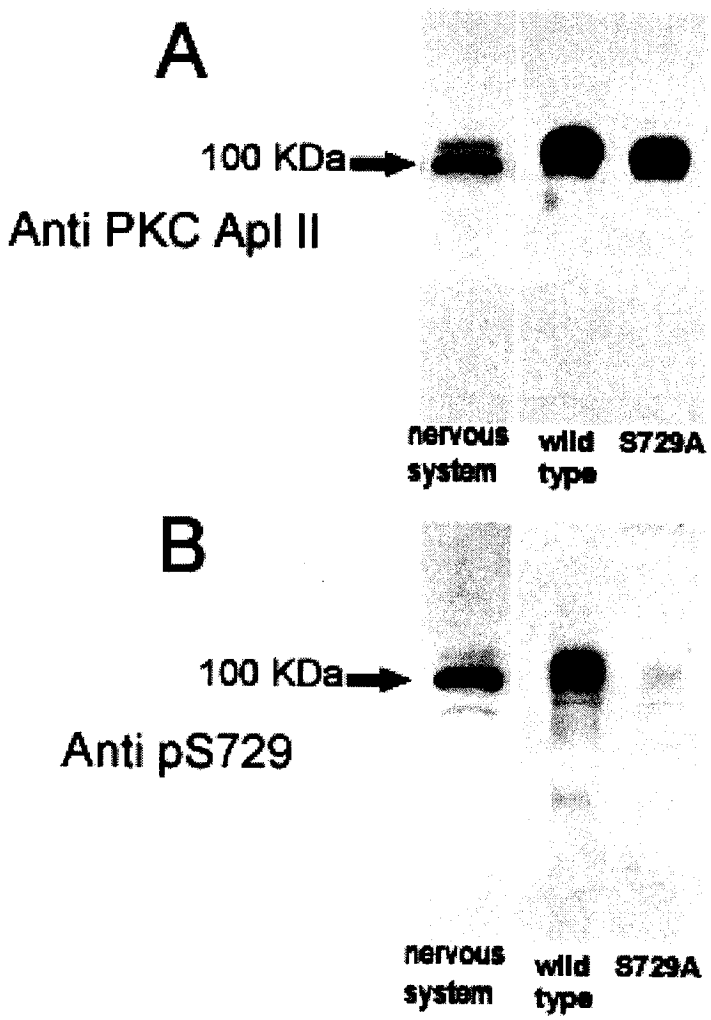
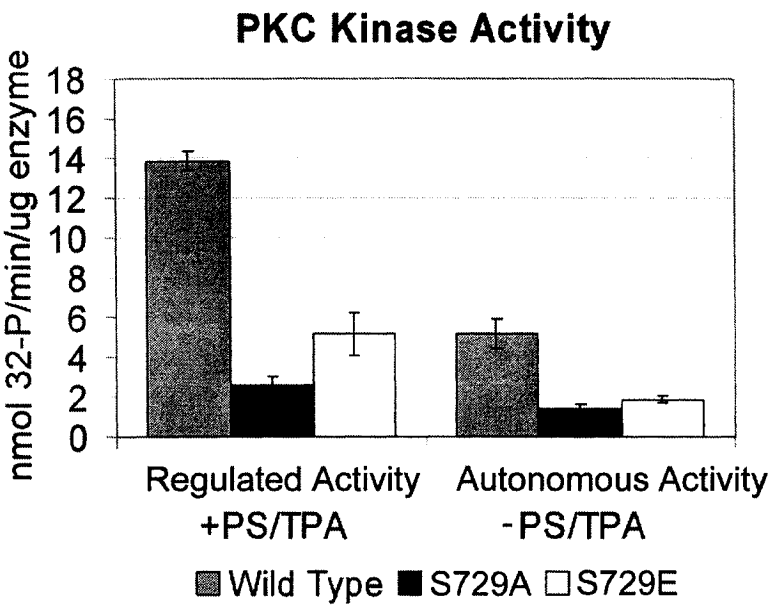

A
100 KDa
Anti PKC Apl II
nervous system
wild type
S729A
B
100 KDa
Anti pS729
nervous system
wild type
S729A

Figure 2

A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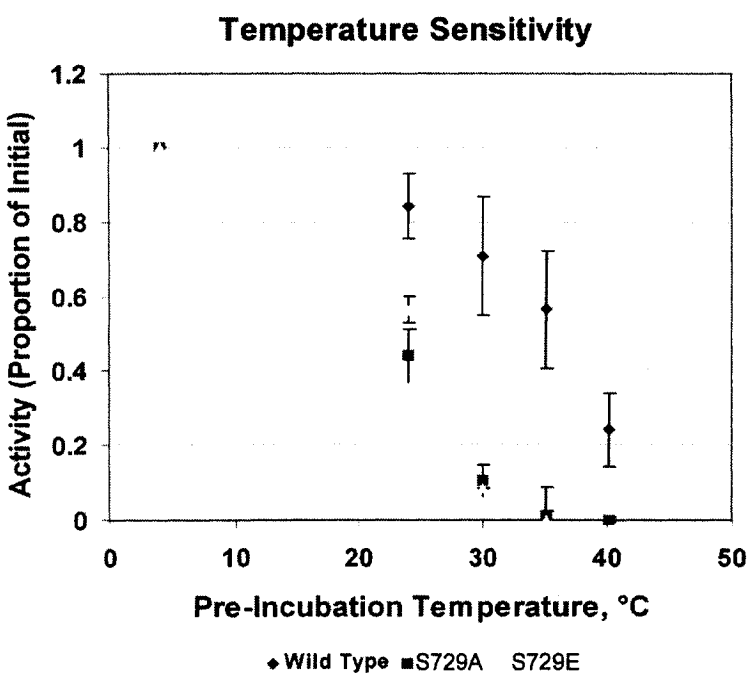
nmol 32-P/min/ug enzyme
18
16
14
12
10
8
6
4
2
0
Regulated Activity
+PS/TPA
Autonomous Activity
-PS/TPA
Wild Type
S729A
S729E

B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Activity (Proportion of Initial)
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1
0.8
0.6
0.4
0.2
0
0
10
20
30
40
50
Pre-Incubation Temperature, °C
Wild Type
S729A
S729E

Figure 3

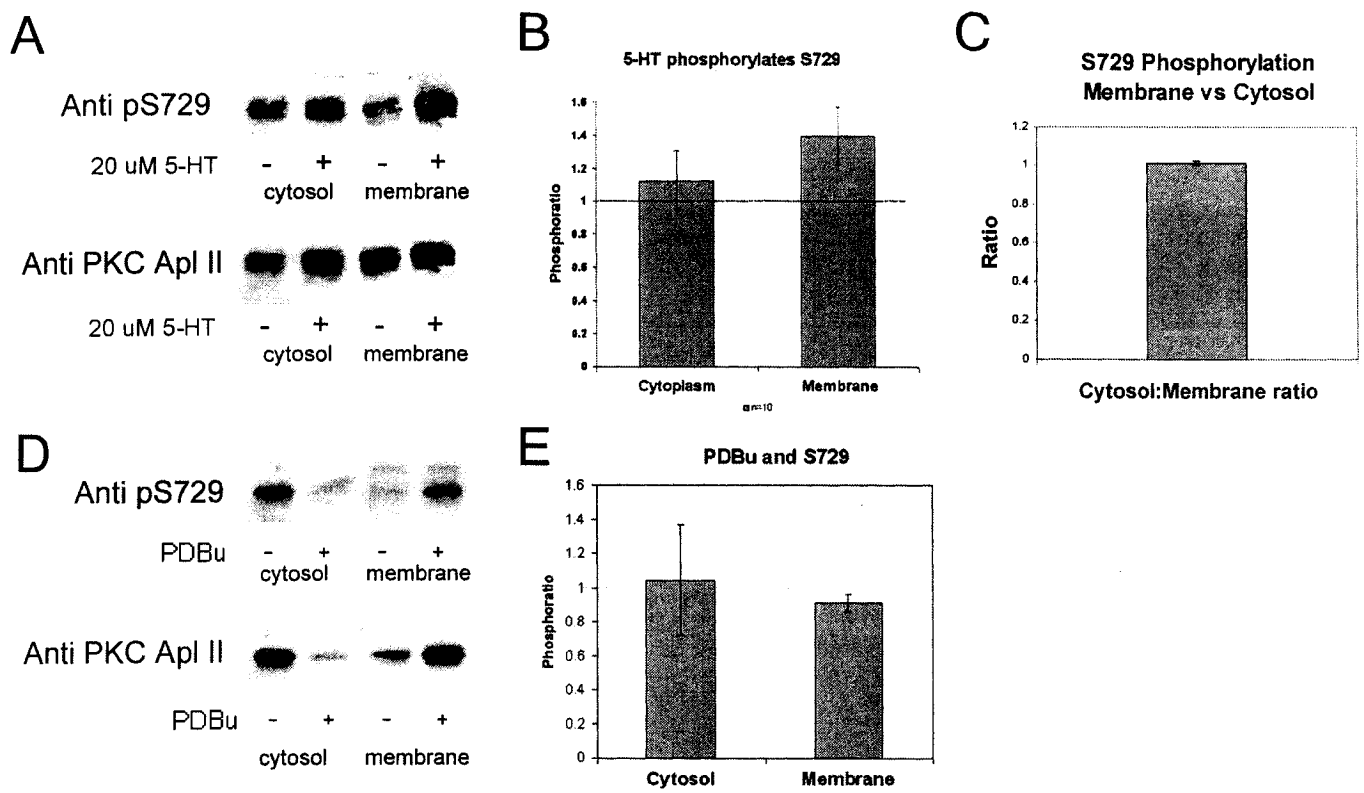
A
Anti pS729
20 uM 5-HT
- + - +
cytosol membrane
Anti PKC Apl II
20 uM 5-HT
- + - +
cytosol membrane
B
5-HT phosphorylates S729
Phosphoratio
Cytoplasm
Membrane
C
S729 Phosphorylation
Membrane vs Cytosol
Ratio
Cytosol:Membrane ratio
D
Anti pS729
PDBu
- + - +
cytosol membrane
Anti PKC Apl II
PDBu
- + - +
cytosol membrane
E
PDBu and S729
Phosphoratio
Cytosol
Membrane

Figure 4

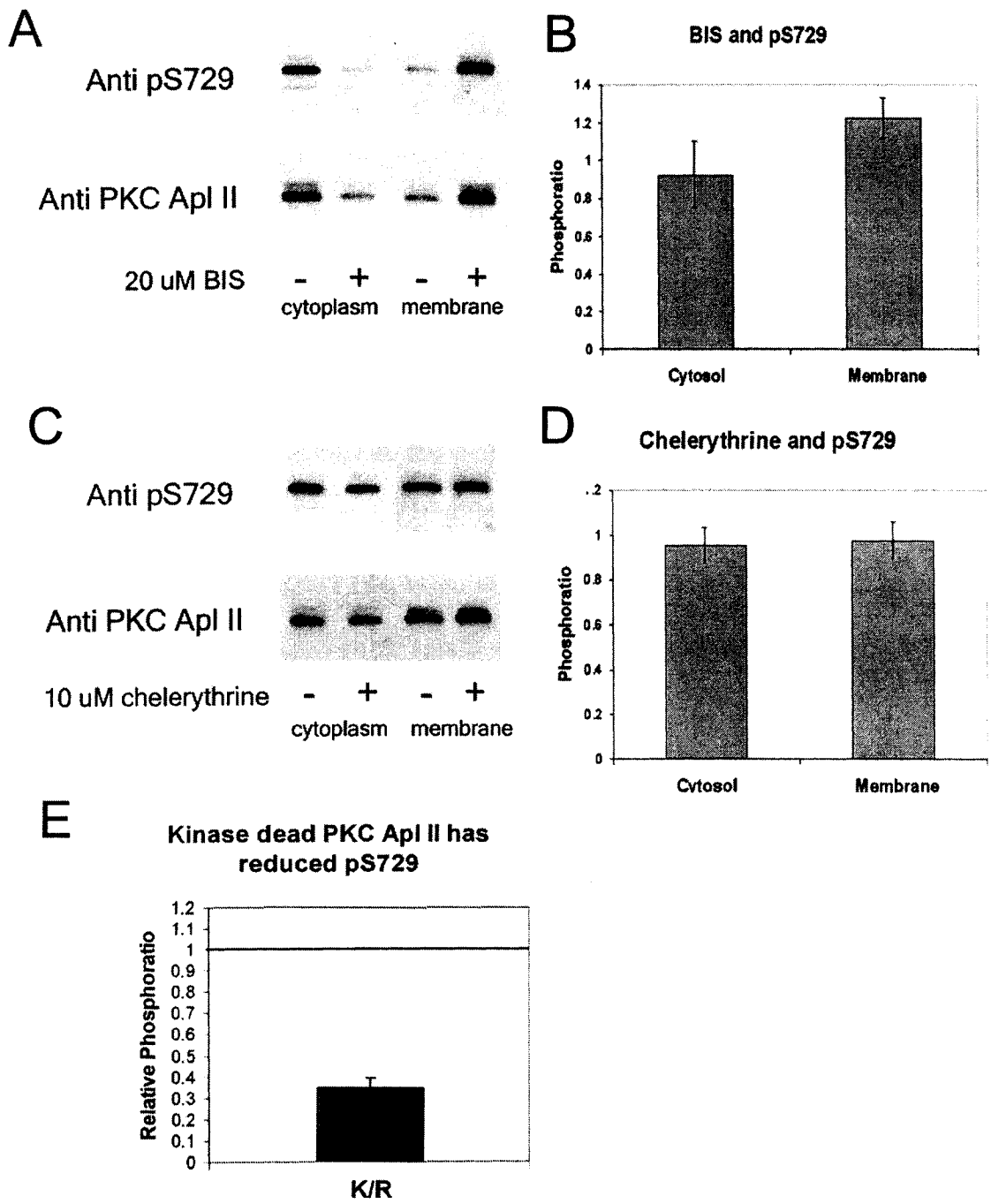
A
Anti pS729
Anti PKC Apl II
20 uM BIS
- + - +
cytoplasm membrane
B
BIS and pS729
Phosphoratio
1.4
1.2
1
0.8
0.6
0.4
0.2
0
Cytosol
Membrane
C
Anti pS729
Anti PKC Apl II
10 uM chelerythrine
- + - +
cytoplasm membrane
D
Chelerythrine and pS729
Phosphoratio
1
0.8
0.6
0.4
0.2
0
Cytosol
Membrane
E
Kinase dead PKC Apl II has reduced pS729
Relative Phosphoratio
1.2
1.1
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
K/R

Figure 5

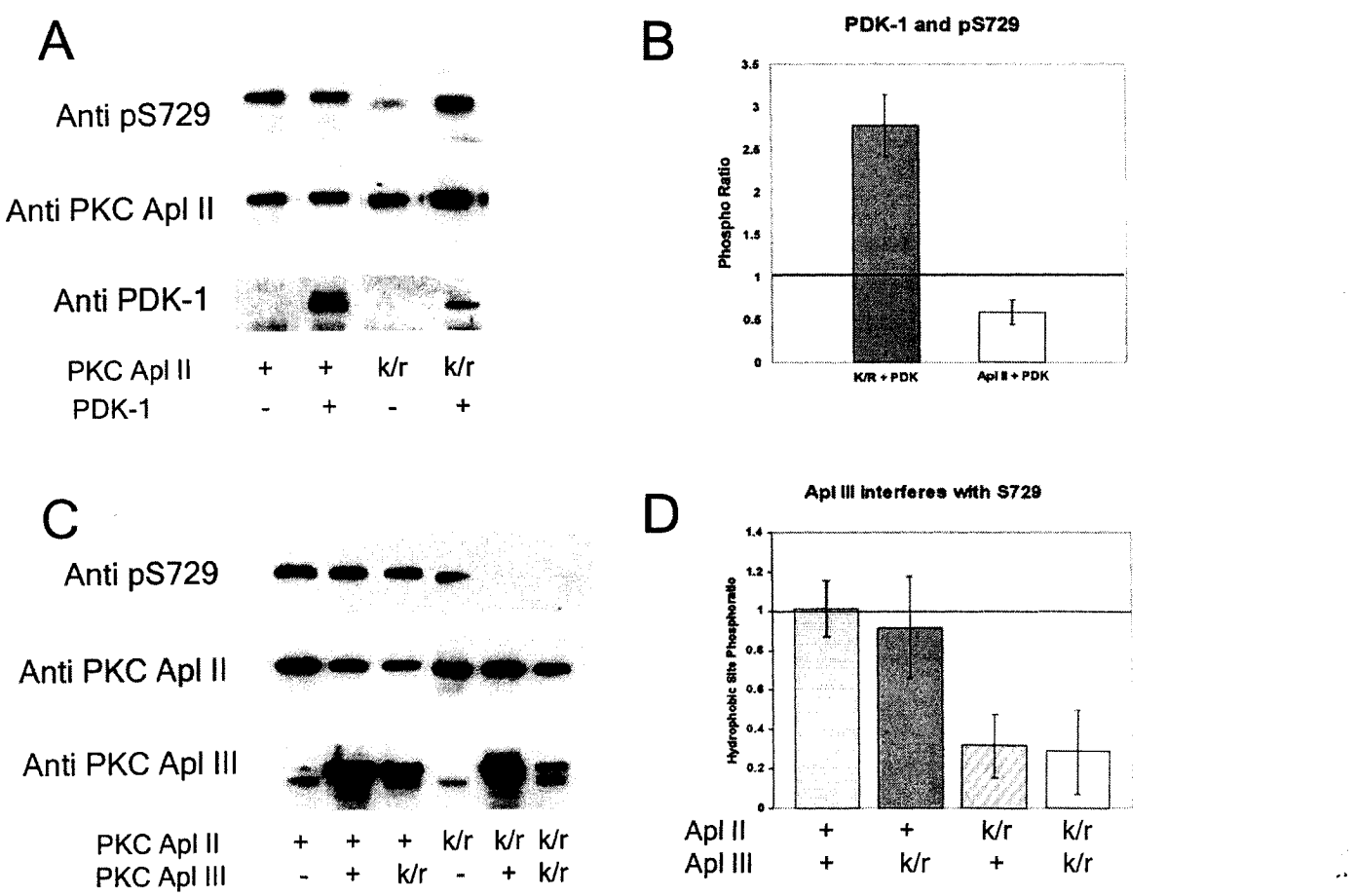
A
Anti pS729
Anti PKC Apl II
Anti PDK-1
PKC Apl II + + k/r k/r
PDK-1 - + - +
B
PDK-1 and pS729
Phospho Ratio
3.5
3
2.5
2
1.5
1
0.5
0
K/R + PDK
Apl II + PDK
C
Anti pS729
Anti PKC Apl II
Anti PKC Apl III
PKC Apl II + + + k/r k/r k/r
PKC Apl III - + k/r - + k/r
D
Apl III interferes with S729
Hydrophobic Site Phosphoratio
1.4
1.2
1
0.8
0.6
0.4
0.2
0
Apl II + + k/r k/r
Apl III + k/r + k/r

Figure 6

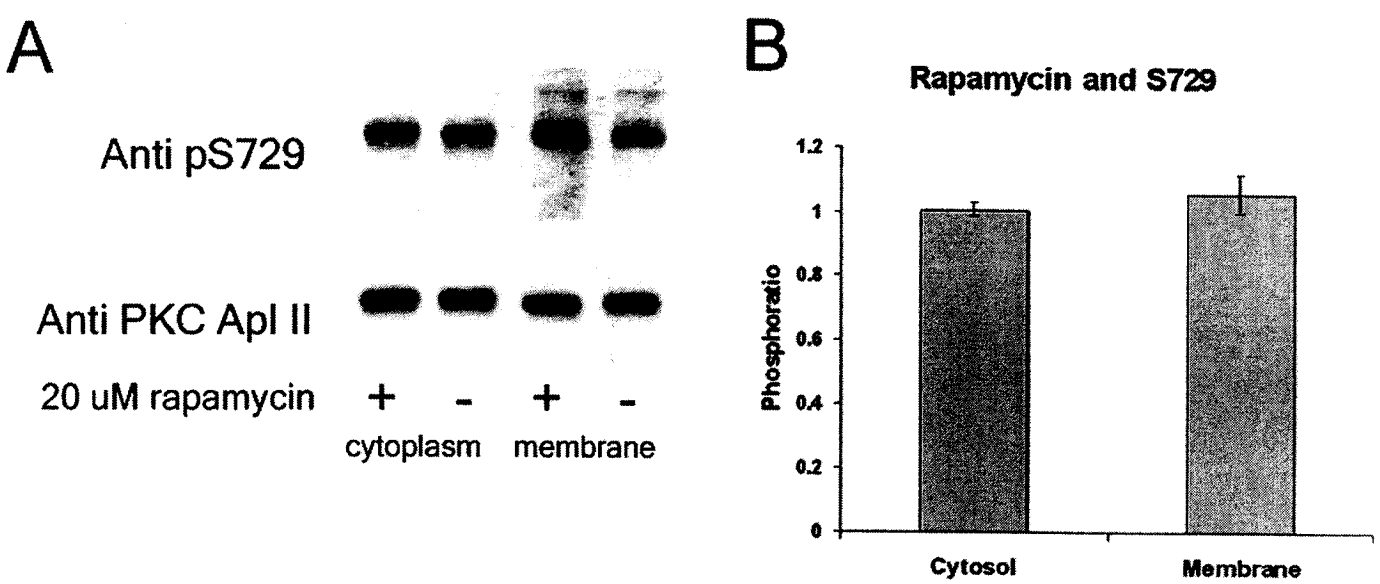
A
Anti pS729
Anti PKC Apl II
20 uM rapamycin
+ - + -
cytoplasm membrane
B
Rapamycin and S729
Phosphoratio
1.2
1
0.8
0.6
0.4
0.2
0
Cytosol
Membrane

Figure 7

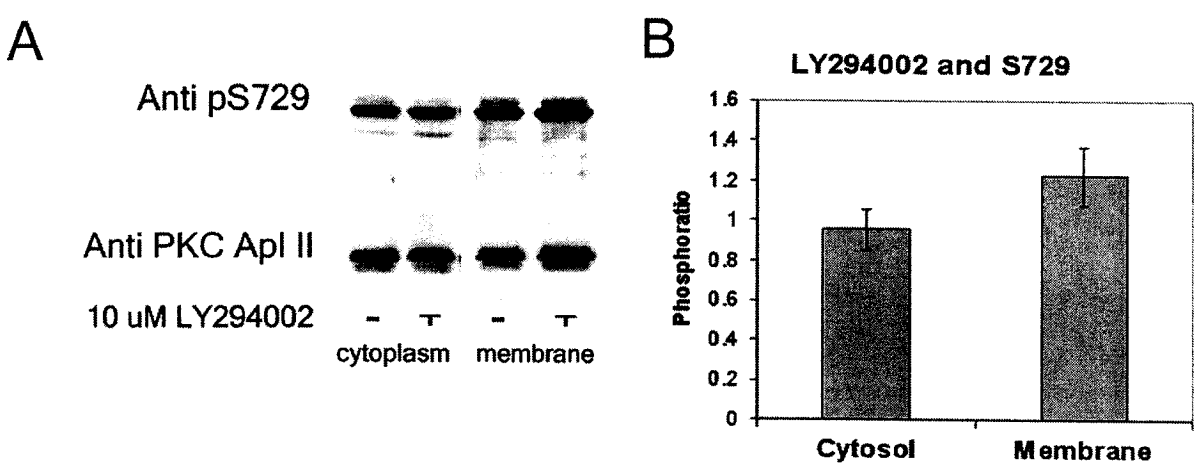
A
Anti pS729
Anti PKC Apl II
10 uM LY294002
- + - +
cytoplasm membrane
B
LY294002 and S729
Phosphoratio
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
Cytosol
Membrane

Figure 8

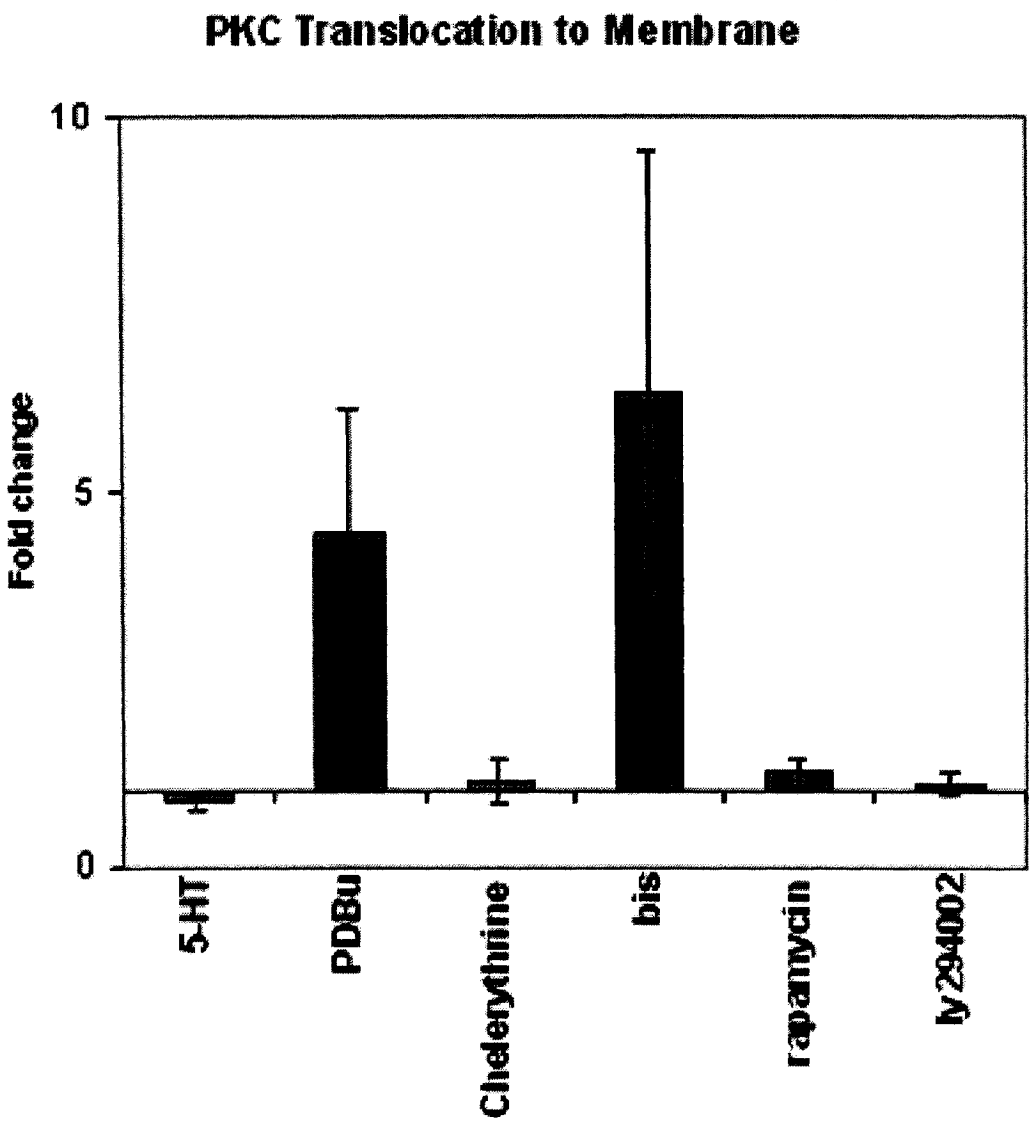
PKC Translocation to Membrane
Fold change
10
5
0
5-HT
PDBu
Chelerythrine
bis
rapamycin
ly294002

Figure Legends

Figure 1. Characterization of phosphospecific antibody. **A**, known anti PKC antibody detects both phosphorylated and dephosphorylated PKC Apl II as a single band at 100 KDa. Left: nervous system PKC is recognized. Middle, Right: SF9 cell expressed clones of PKC are recognized, regardless of phosphorylation state. **B**, phospho specific antibody to pS729 in the hydrophobic motif of PKC Apl II. Left: the same 100 KDa band is recognized in the nervous system, with no background crossreaction to other proteins. PKC Apl II is already phosphorylated at S729 without experimental manipulation. Middle, Right: Wild type clone of PKC is recognized in SF9 cell homogenates, but mutant clone with S729A mutation (which cannot be phosphorylated) is not recognized by the phosphospecific antibody. By inference, dephospho S729 should also not be recognized by the phosphospecific antibody.

Figure 2. Serotonin induces persistent phosphorylation of S729 in neuronal PKC. **A**, representative western blots show increased phosphorylation in the membrane fraction of pleural-pedal ganglia homogenates 2 hours after 90 minutes 20 μM serotonin treatment, relative to unchanged overall levels of protein. B, quantation of phosphoratio for serotonin treatment shows membrane, but not cytosolic increases. C, representative western blot of ganglia treated with 1uM PDBu for 1 hour shows that transient, regulated activation of PKC Apl II does not affect S729 phosphorylation. However the phorbol ester does translocate PKC to the membrane. D, quantitation of phosphoratio.

Figure 3. Effect of PKC inhibitors on S729 phosphorylation in neurons. A, representative western blot of Aplysia ganglia PKC shows no change in phosphorylation after treatment with 20 μM bisindolylmaleimide for 1 hour, relative to overall PKC levels. PKC is translocated to the membrane fraction by bis treatment. B, quantation of bis phosphoratio. C, representative western blot shows no change in phoshporylation after treatment with 10 μM chelerythrine for 1 hour, relative to PKC levels. D, quantiation of chelerythrine phosphoratio. E, quantiation of phosphoratio from kinase dead mutant PKC Apl II K/R from SF9 cell homogenates. S729 is incompletely dephosphorylated, though Apl II K/R has no kinase activity.

Figure 4. Coexpression of candidate kinases affects S729 phosphorylation in SF9 cell PKC Apl II. A, representative western blots of SF9 cell expressed PKC Apl II, coexpressed with PDK-1. Wild type Apl II phosphorylation is decreased by PDK, but kinase dead Apl II is increased by PDK. B, quantitation of phosphoratio for PDK coexpression. C, representative western blots of SF9 cell expressed PKC Apl II, coexpressed with atypical PKC Apl III. Wild type Apl II phosphorylation is unaffected by Apl III, but kinase dead Apl II is decreased by Apl III and by kinase dead Apl III. D, quantitation of phosphoratio for Apl III coexpression.

Figure 6. Rapamycin does not block S729 phosphorylation in neurons. A, representative western blots of *Aplysia* ganglia PKC after 1 hour of treatment with 20 μM rapamycin shows no changes in S729 phosphorylation relative to overall protein levels. B, quantitation of phosphoratio for rapamycin treatment.

Figure 7. LY294002 does not block S729 phosphorylation in neurons. A, representative western blots of *Aplysia* ganglia PKC after 1 hour of treatment with 10 μM LY294002 shows no changes in S729 phosphorylation relative to overall protein levels. B, quantiation of phosphoratio for LY treatment.

Figure 8. Effect of pharmacological manipulations on the subcellular distribution of PKC Apl II, calculated using non-phosphospecific control antibody and corrected by gel loading factor. PDBu and bis both translocate PKC Apl II to the membrane fraction.

Discussion

5-HT persistently phosphorylates S729 – We previously showed 5-HT persistently phosphorylates PKC Apl II at the activation loop, T561 and that this phosphorylation correlates with membrane associated activity autonomous of classical activating cofactors (Pepio et al., 2002). Here we show 5-HT also persistently phosphorylates the hydrophobic site during the same time frame as maintainance of intermediate facilitation. Conversely, phorbol ester PDBu did not increase phosphorylation of S729. PDBu is known a potent activator of conventional and novel PKCs, and it causes presynaptic potentiation in mammalian synaptic preparations (Hori et al., 1999) and enhances the slowly inactivating calcium current in *Aplysia* sensory neurons (Braha et al., 1993). Thus 5-HT mediated hydrophobic site phosphorylation is not due solely to increased DAG caused by transient activation.

Functional effect of phosphorylation –Phosphorylation of some kinases, such as CaMKII, is the mechanism of persistent activity; CaMKII phosphorylation at T286 makes the kinase calcium independent (Miller & Kennedy, 1986), and is required for LTP (Giese et al., 1998). An analogous mechanism may occur in PKC; The S729A mutation reduced kinase activity, similar to an S662A mutation in PKC delta found to reduce activity to 10% (Le Good et al., 1998). The greater stability of phospho S729 likely contributes to greater resistance against degradation over time and susceptibility to phosphatases. Indeed, detailed analysis of the crystal structure of nPKC theta has revealed that its hydrophobic site is phoshporylated and contribute to maintaining structural conformation (Xu et al., 2004).

The reduced activity and stability of the S729E mutant may indicate that negative charge alone is insufficient to supplement actual phosphorylation. Another possibility may be that neither mutant ever properly folds after it is first translated, immediately precluding proper activity. In this case, it would be helpful to attempt to perform a kinase assay on wild type PKC Apl II that has dephospho S729 after phosphatase treatment. Unfortunately, phosphatase PP2A, specific for the hydrophobic motif, could not remove phosphate from wild type Apl II even at high concentrations in the presence of PKC activators (data not shown).

Autophosphorylation vs. Trans phosphorylation – In mammalian PKC alpha, delta and epsilon, the hydrophobic site is highly regulated, particularly by the addition of serum to serum-starved cells (Parekh et al., 1999). However, when phosphatase treated *in vitro*, novel PKC epsilon becomes phosphorylated again at its hydrophobic site in the absence of any exogenous factors, suggesting the capacity for autophosphorylation (Cenni et al., 2002) similar to that seen in the hydrophobic site of conventional PKC Beta II (Behn-Krappa, A., 1999). Recently, the crystal structure of nPKC theta expressed in bacteria lacking upstream kinases reveals the hydrophobic site is already occupied (Xu et al., 2004). To address autophosphorylation, we inhibited PKC Apl II itself with chelerythrine and bis. Neither reduced phosphorylation of S729 although bis translocated PKC to the membrane fraction, indicating an inhibitory mechanism distinct from that of chelerythrine. This result agrees with the bis insensitivity of PKC delta S662/epsilon S729 phosphorylation (Parekh et al., 1999). However it is possible that S729 is already phosphorylated near saturation prior to adding the inhibitor. If so, PKC would be structurally stable and able to resist significant changes by phosphatases. Our kinase

dead PKC clone expressed in SF9 cells is incapable of phosphorylating any substrate. The kinase dead K/R mutant was incompletely dephosphorylated at S729, indicating that the hydrophobic site can be trans phosphorylated by other kinases. SF9 cells contain common endogenous kinases, although the heterologously infected protein is always highly overexpressed relative to endogenous proteins. Literature on hydrophobic phosphorylation of similarly studied nPKC kinase dead mutants is contradictory, with some groups finding no phosphorylation in a K/W mutant (Cenni et al., 2002) and others showing partial phosphorylation in a K/R mutant (Rybin et al., 2003). Due to the quantity of phosphorylation lost in our K/R kinase dead PKC, autophosphorylation plays some role at S729. It remains inconclusive whether autophosphorylation, upstream trans regulation, or both, act *in vivo* or during persistent activity.

PDK-1, PKC Apl III - In conventional PKCs, PDK-1 phosphorylates the activation loop and cPKC autophosphorylates its hydrophobic site (Behn-Krappa & Newton, 1999). The kinase for the novel PKC hydrophobic site is not known, although PDK-1 has been reported to phosphorylate the hydrophobic sites of several related kinases (Williams et al., 2000). We show that heterologously overexpressed PDK-1 dramatically increased phosphorylation of S729 in kinase dead PKC Apl II, but paradoxically decreased phosphorylation in wild type Apl II. This decrease suggests that PDK-1 is not the kinase directly phosphorylating the hydrophobic site. One possible explanation is that PDK interacts with the site, physically blocking it in an antagonistic manner. Under this model, kinases would have difficulty accessing S729, accounting for the reduced phosphorylation in wild type. The K/R kinase dead PKC is incompletely folded and therefore much more susceptible to phosphatases than wild type. In the case

of the K/R mutant, PDK may in fact block access of phosphatases from further reducing the already attenuated S729 phosphorylation in the PKC population. The net result would be an apparent increase in phosphorylation.

atypical PKC zeta was isolated from a GST interaction screen using the hydrophobic-containing carboxy terminal of PKC alpha and further, it has been shown that expressing persistently active atypical PKC zeta increased novel PKC hydrophobic phosphorylation (Ziegler et al., 1999). In Apl II, however, atypical PKC Apl III further blocked phosphorylation of S729 in coexpressed kinase dead PKC Apl II. This detracts from the theory that atypical PKC is the kinase to novel PKC S729. However, it does appear to inhibit phosphorylation. Atypical PKC Apl III has a similar hydrophobic motif with a glutamic acid (E) residue as its equivalent S729. Apl III may in fact be competing with Apl II for PDK-1. If Apl III is overexpressed relative to low endogenous levels of PDK in SF9 cells, the effect of PDK's protection against phosphatases would be relieved and the result would be a perceived decrease in S729 phosphorylation. The interactions of PDK, Apl III and Apl II when overexpressed in SF9 cells may not necessarily occur under normal physiological conditions in neurons.

LY294002 - PtdIns 3 Kinase inhibitor LY294002 blocks phosphorylation of mammalian PKC delta S662 and epsilon S729 (Parekh et al., 1999), although it did not do so in PKC Apl II. Perhaps this is not entirely surprising, given that we previously showed LY294002 does not affect T561 phosphorylation of Apl II (Pepio et al., 2002). The effect of PDK-1, if dependent on PDK activity, should have been modulated by LY given that PtdIns3K is an upstream activator of PDK. However since PDK-1 appears to

be both increasing and decreasing PKC Apl II S729 phosphorylation, it is likely doing so without the influence of PtdIns3K.

Rapamycin - More interesting, it has been shown that rapamycin inhibition of mTOR signaling blocks phosphorylation of PKC delta and epsilon S662 in HEK cells (Parekh et al., 1999). As a caveat, this regulation of PKC delta/epsilon was observed in serum starved cells. One cannot serum starve primary neuron cultures. Therefore a precisely analogous method of activation demonstrating the involvement of these pathways is not available.

FRAP, the *Aplysia* homologue of mTOR, does regulate the hydrophobic site of P70/S6K in *Aplysia* and furthermore, this phosphorylation is effected by 5-HT treatment implicating signaling by memory inducing pathways (Khan et al., 2001). FRAP inhibition by rapamycin conversely does not affect S729 in PKC Apl II, despite the fact that phosphorylation at both sites are increased by 5-HT.

Chapter 2: PKC Apl III

Introduction

Atypical PKC is a calcium independent and DAG/phorbol ester independent isoform of PKC present in the nervous system. Atypical PKC has the same bipartite division of regulatory and catalytic domains seen in conventional and novel PKC, including a pseudosubstrate region, but it lacks a C2 domain and has a single atypical C1 domain. aPKCs are believed to be regulated only by phosphatidylserine at the regulatory domain, and by phosphorylation at the catalytic domain. Interestingly, the equivalent of the hydrophobic serine S729 in aPKC is a glutamic acid (E) phospho mimic. However, regulation of the activation loop phosphorylation by PDK-1 has been demonstrated in vertebrate systems (LeGood et al., 1998).

In mammals, persistently active atypical PKC zeta is necessary and sufficient for the maintainence of LTP (Ling et al., 2002), and it enhances odor avoidance task performance in transgenic *Drosophila* (Drier et al., 2002). The mechanism of persistent activation is a truncated PKM zeta that lacks an autoinhibiting regulatory subunit. However unlike many truncated PKMs, PKM zeta is not generated by proteolysis. It is translated from a separate mRNA, transcribed from an alternate internal promoter between the regulatory and catalytic sequences of PKC zeta (Hernandez et al., 2003). Whereas novel PKCs likely have a presynaptic role in facilitation, targeting release related proteins, atypical PKCs may play a postsynaptic role. In mammals, PKM zeta mRNA is dendritically located (Muslimov et al., 2004). In *Aplysia*, facilitation has been

found to have a postsynaptic, glutamate dependent component (Roberts & Glanzman, 2003)

A homologue of PKC Zeta was previously cloned in our lab from *Aplysia* using degenerate PCR and 5' RACE. Notably, during cloning, a population of nervous system derived PKC zeta cDNA contained spliced inserts of 9 and 16 amino acid length. These splice inserts were found in the hinge region between the regulatory and catalytic domain sequence. To our knowledge, these nervous system enriched splice inserts are unique among PKCs and their function is not known.

In this thesis, I characterize atypical PKC Apl III, using antibodies raised against the amino terminal, carboxy terminal, and 16 amino acid hinge insert splice. Futher I attempt to determine whether a truncated, persistently active PKM Apl III is found in *Aplysia*. The characterization of an atypical *Aplysia* PKC is relevant because all three isoform families (conventional, novel, atypical) are now represented in this model organism. Further analysis of isoform specific contributions to PKC mediated sensoriomotor facilitation is now possible.

Materials and Methods

Immunoprecipitation – Immunoprecipitations were performed with rabbit sera of the original antibody, preconjugated to Sepharose® CL-4B for 1 hour at 4°C. *Aplysia* ganglia homogenates were incubated with an equal volume of beads, overnight at 4°C. The supernatant was kept as void, and the remaining mixture was washed 3x 5 min in

PBS. Void and beads were denatured in Laemlli buffer at 95°C and run on 9% SDS PAGE.

Results

We raised three antibodies to the atypical PKC Apl III: an amino terminal antibody, a carboxy terminal antibody and an antibody to the 16 amino acid splice insert of the hinge region. Each antibody was affinity purified against the original peptide and blotted against *Aplysia* tissue extracts (Figure 9) to determine the distribution of Apl III in the nervous system, buccal muscle, gill, penis and hepatopancreas. Our cloned sequence predicted the molecular weight of full length PKC Apl III to be approximately 65.5 Kda. The sequence predicted a PKM Apl III of approximately 41 Kda. (Appendix B)

The amino terminal antibody recognized the predicted 65.5 Kda full length PKC Apl III in all tissues except hepatopancreas (Figure 1a, first lane). Notably, the nervous system band ran 3-4 KDa higher than other tissues. The 9 amino acid splice insert, ECKVEQYRE (1183 daltons) and the 16 amino acid splice insert, TAKVEGSSTKDGNQLN (1648) daltons together account for this size difference, which suggests the nervous system specific splice inserts found in Apl III mRNA translate to longer, nervous system specific protein. The amino terminal antibody also detected a band at 54 KDa, but its molecular weight was too large to qualify as a PKM Apl III. Furthermore, because a PKM by definition does not have a regulatory subunit, no protein recognized by the amino terminal antibody can be a PKM, regardless of its size.

The carboxy terminal antibody should recognize both PKC Apl III and PKM Apl III (Figure 1b). There was a 65.5 Kda band present in all tissues except hepatopancreas, corresponding to full length PKC Apl III. Again, the nervous system band ran at a slightly higher molecular weight than all other tissues (Figure 1b, first lane), suggesting the presence of the nervous system specific splice insert. The carboxy terminal antibody recognized a second band at 54 Kda, but since the amino terminal antibody recognized the same band we ruled out the possibility that it was PKM Apl III.

The splice antibody recognized full length PKC containing the extra 3 KDa splice insert, but not the 65.5 KDa PKC Apl III (Figure 1c). All tissues expressed the splice insert Apl III, but it was most abundant in the nervous system. Therefore, the splice insert is nervous system enriched, but not nervous system specific. The splice antibody recognized a lower molecular weight band at 45 KDa. However, a PKM by definition contains a carboxy terminal catalytic subunit. Since the carboxy terminal antibody did not detect such a 45 KDa protein, we ruled out the possibility that it was PKM Apl III.

The appearance of two unidentified bands (45 and 54 KDa) was perplexing. How could both carboxy and amino terminal antibodies recognize the same shorter PKC, and if so, where was the protein shortened? To verify the identity of legitmate Apl III bands, we next performed immunoprecipitations. Nervous system homogenates were immunoprecipitated with all three sera and blotted with each antibody (Figure 2).

All sera depleted the 68.5 KDa band, particularly the splice (Figure 2, Lane 2, Lane 5) and carboxy terminal (Lane 4 & 7). Conversely, all antibodies recognized the same 68.5 KDa band in the immunoprecipitate. Importantly, the carboxy terminal serum

did not deplete any lower molecular weight bands compared to pre-immune serum (Lane 1 & 8) suggesting that none of the lower bands were PKM Apl III.

Though PKM Apl III was absent in untreated neurons, it may be generated from existing PKC Apl III. PKM was originally discovered as a proteolyzed fragment of full length PKC (Sacktor et al., 1993). Since proteolysis in neurons is commonly mediated by calcium influx, we next treated neurons with the calcium ionophore ionomycin to see if Apl III could be cleaved (Figure 3). The carboxy terminal antibody, which should detect the catalytic subunit of a truncated PKM, did not detect any additional lower molecular weight bands after ionomycin treatment either in the presence (Lane 1) or absence (Lane 2) of protease inhibitors leupeptin, aprotinin and benzamidine.

Inhibitors – To test the involvement of the PKC Apl III isoform in facilitation, we returned to past studies of PKC in facilitation. Previous work which showed that PKC is required for sensorimotor facilitation (e.g., Ghirardi et al., 1992; Sutton & Carew, 2000; Sutton et al., 2004) often relied on pharmacological inhibitors of PKC, whose isoform specificity was not known. Therefore, the identity of the isoform(s) of PKC mediating facilitation in these experiments was not known. We now had both PKC clones (Apl II and Apl III) purified. To directly characterize the isoform specificity of classical PKC inhibitors, we performed kinase assays comparing PKC Apl II with PKC Apl III.

The inhibitor bisindolylmaleimide blocked PKC Apl II activity more effectively than atypical PKC Apl III (Figure 4, representative experiment). The inhibitor chelerythrine blocked PKC Apl II and PKC Apl III activity with equal efficacy, but inhibited artificially cloned PKM Apl III markedly better than either (Figure 5, representative experiment). Lastly, the synthetic Apl III inhibitor, based on the

pseudosubstrate sequence of the enzyme, was tested. This inhibitor had a weak affinity for PKC Apl III during kinase assays relative to the epsilon peptide substrate; however, it did inhibit PKC Apl III and not PKC Apl II (Figure 6, representative experiment).

Figure 1

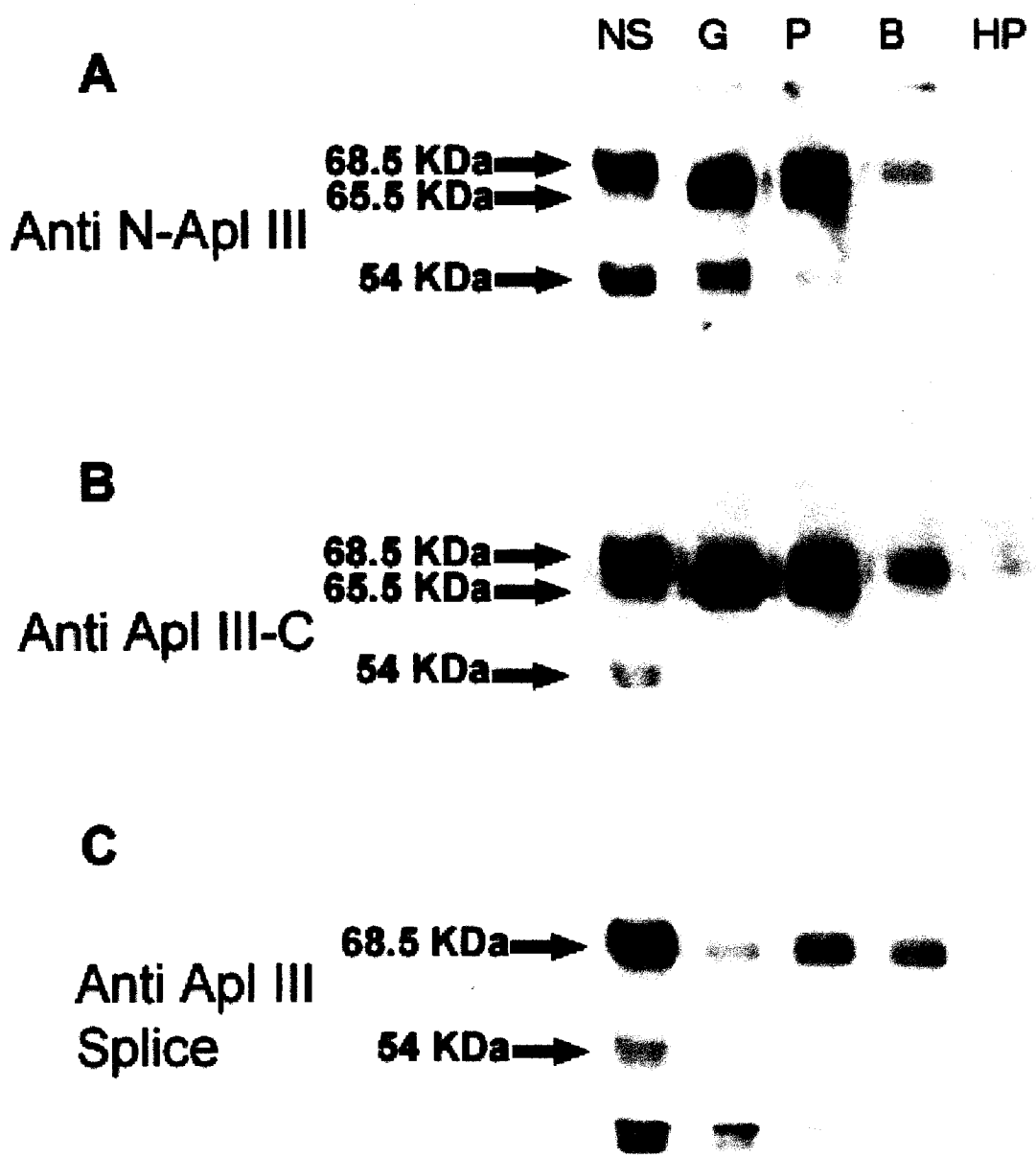
NS
G
P
B
HP
A
68.5 KDa
65.5 KDa
Anti N-Apl III
54 KDa
B
68.5 KDa
65.5 KDa
Anti Apl III-C
54 KDa
C
68.5 KDa
Anti Apl III
Splice
54 KDa

Figure 2

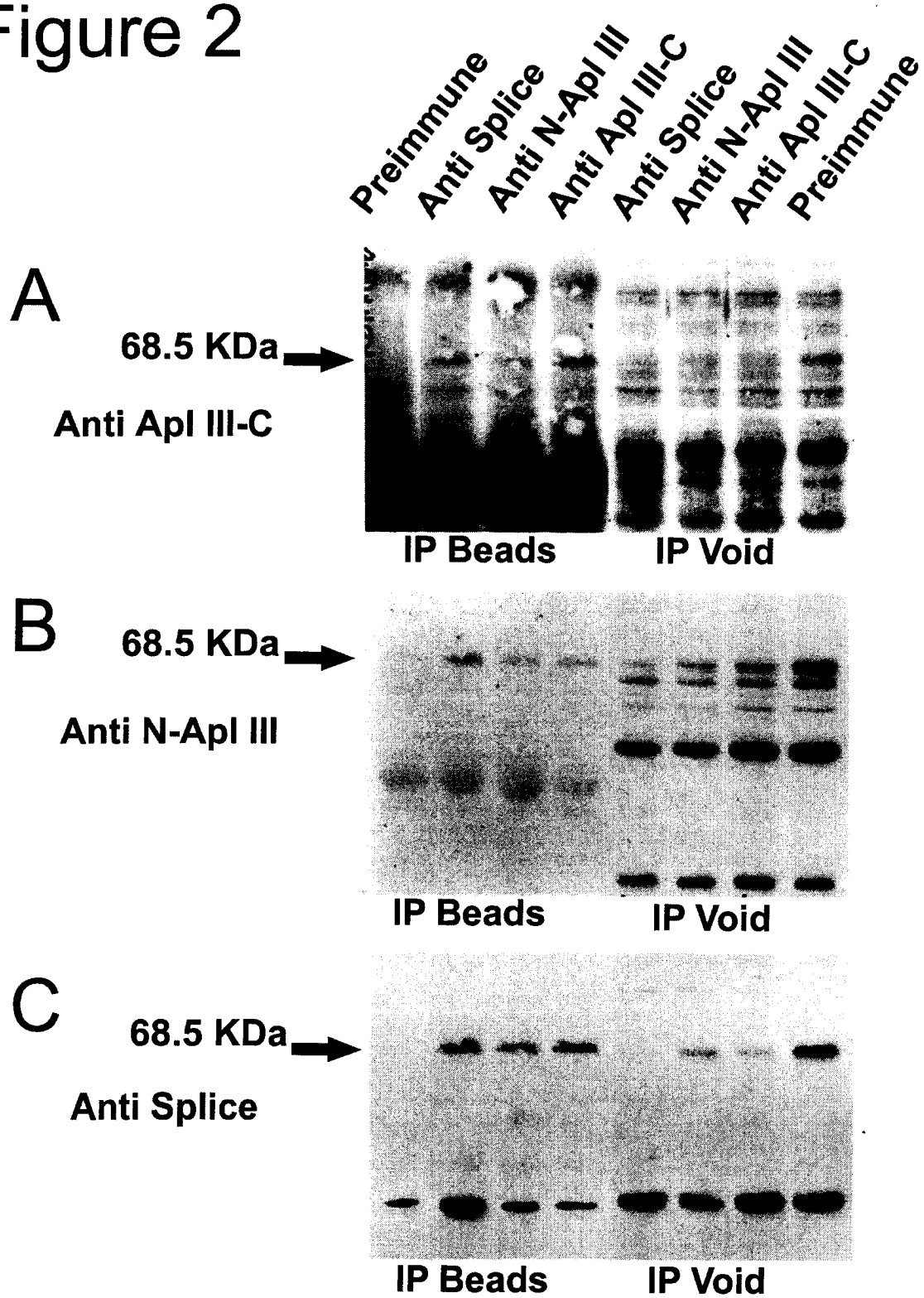
Preimmune
Anti Splice
Anti N-Apl III
Anti Apl III-C
Anti Splice
Anti N-Apl III
Anti Apl III-C
Preimmune
A
68.5 KDa
Anti Apl III-C
IP Beads
IP Void
B
68.5 KDa
Anti N-Apl III
IP Beads
IP Void
C
68.5 KDa
Anti Splice
IP Beads
IP Void

Figure 3

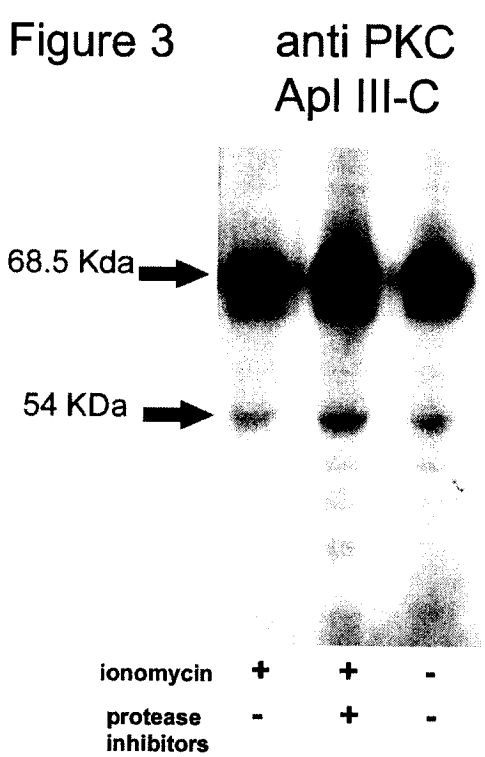
anti PKC
Apl III-C
68.5 Kda
54 KDa
ionomycin + + -
protease inhibitors - + -

Figure 4

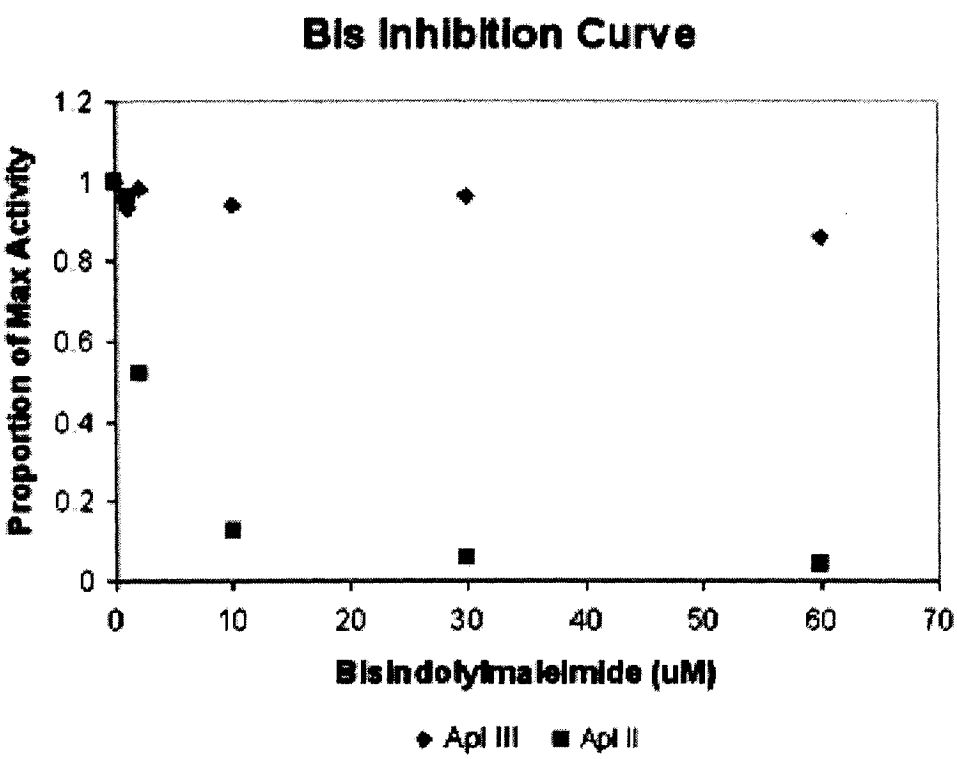
Bis Inhibition Curve
Proportion of Max Activity
1.2
1
0.8
0.6
0.4
0.2
0
0
10
20
30
40
50
60
70
Bisindolylmaleimide (uM)
Apl III
Apl II

Figure 5

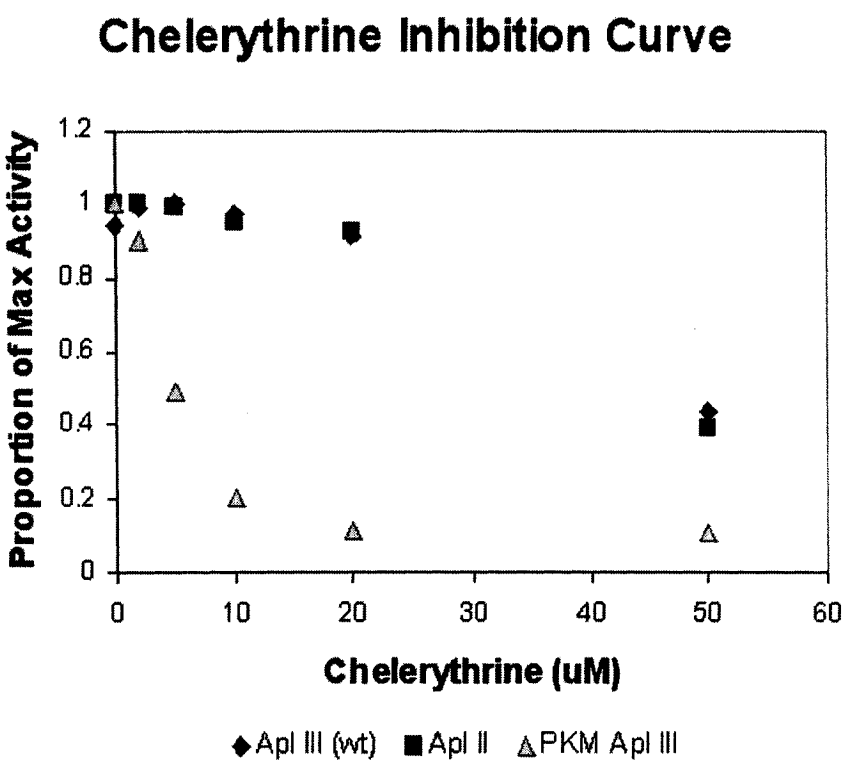
Chelerythrine Inhibition Curve
Proportion of Max Activity
1.2
1
0.8
0.6
0.4
0.2
0
0
10
20
30
40
50
60
Chelerythrine (uM)
Apl III (wt)
Apl II
PKM Apl III

Figure 6

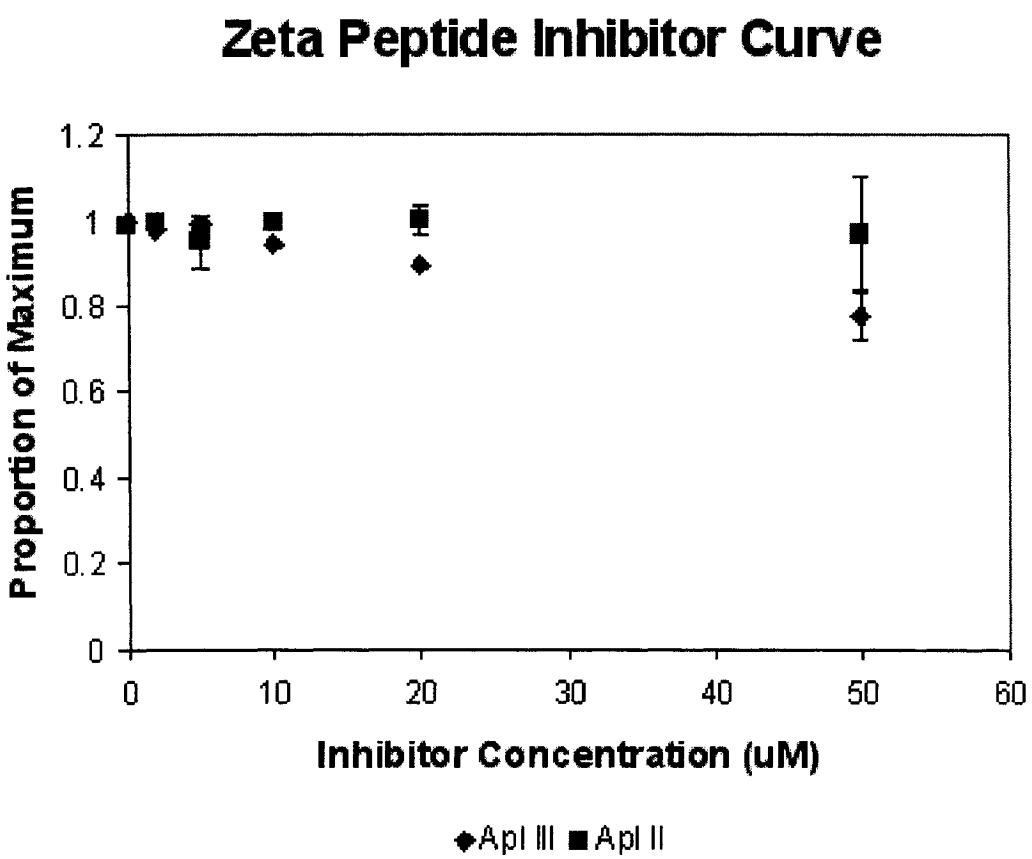
Zeta Peptide Inhibitor Curve
Proportion of Maximum
1.2
1
0.8
0.6
0.4
0.2
0
0
10
20
30
40
50
60
Inhibitor Concentration (uM)
◆Apl III ■Apl II

Figure Legend

Figure 1. Characterization of PKC Apl III antibodies against *Aplysia* tissue homogenates. NS – nervous system, G – gill, P – penis, B – buccal muscle, HP – hepatopancreas. A, Amino terminal antibody detects a predicted full length atypical PKC Apl III in all tissues except hepatopancreas. Notably, nervous system Apl III is slightly larger, confirming the presence of inserted amino acids suggested from neuronal mRNA sequence inserts. B, Carboxy terminal antibody detects full length PKC Apl III, with the slightly larger Apl III primarly found in nervous tissue. C, Splice specific antibody detects only the larger variant of full length Apl III, confirming that the larger size is a result of the inserted splice sequence.

Figure 2. Immunoprecipitation using antisera of the three Apl III antibodies, probed with purified antibody. A, ip blot probed with carboxy terminal antibody shows that only full length PKC Apl III is depleted relative to preimmune serum, and is pulled down successfully by each of the three sera. B, ip blot probed with amino terminal antibody shows only full length PKC is depleted and pulled down. C, ip blot probed with splice antibody. Full length PKC is depleted and pulled down; splice antibody serum depletes and pulls down a 45 KDa protein, but this is only recognized by the splice antibody and is not detected by the carboxy terminal antibody in the ip or in Figure 1.

Figure 3. Effect of ionomycin treatment on neuronal PKC Apl III. Lane 1, 5 μM ionomycin treatment for 20 minutes, plus 0.1 mM $CaCl_2$ in the absence of protease

inhibitors. Lane 2, 5 μM ionomycin treatment for 20 minutes, plus 0.1 mM $CaCl_2$ in the presence of protease inhibitors. Lane 3, untreated ganglia in the presence of protease inhibitors and EGTA.

Figure 4. Representative inhibitor concentration curve. Bisindolylmaleimide inhibits novel PKC Apl II activity, but not atypical PKC Apl III activity, in a kinase assay using purified, SF9 expressed PKC clones.

Figure 5. Representative inhibitor concentration curve. Chelerythrine inhibits both nPKC Apl II and aPKC Apl III activity with equal efficacy in a kinase assay. However, the artificially generated PKM Apl III from a catalytic subunit only construct is highly inhibited.

Figure 6. Representative inhibitor concentration curve. Apl III inhibitor peptide SIYRRGARRWRKL inhibits aPKC Apl III activity slightly at higher concentrations, indicating a poor affinity relative to the substrate epsilon peptide ERMRPRKRQGSVRRRV. However, the activity of PKC Apl II is not affected by the inhibitor.

Discussion

PKM Apl III – Antibodies against atypical PKC Apl III were unable to detect the presence of a truncated PKC in *Aplysia* nervous system homogenates. There were no recognized bands running at the molecular weight predicted by the sequence of the catalytic subunit of PKC Apl III and in particular, no bands were recognized by the carboxy terminal antibody that were not also recognized by the amino terminal antibody. Amino terminal immunoreactivity suggested that in all cases, the regulatory subunit was either present, or the antibody was cross-reacting with a background unrelated protein. Further evidence was provided by the immunopreciptation series of experiments, which showed that only the full length PKC Apl III was precipitated by antisera against the enzyme. Although this does not rule out some other mechanism of persistent, autonomous kinase activity associated with maintainance of cellular memory, it is unlikely that such activity is mediated by PKM in *Aplysia*. Moreover, even the exogenous initiation of calcium mediated proteolysis by ionomycin treatment in high calcium buffer failed to generate a PKM Apl III. This is surprising, given most hinge regions are quite labile to proteolysis, and PKM was in fact generated accidentally through proteolytic cleavage at the hinge region during the initial discovery of PKC (Inoue et al., 1977; Kishimoto et al., 1989).

Splice Insert – The slightly larger molecular weight of nervous system PKC Apl III relative to the majority of Apl III found in other control tissues corroborates the nervous system enrichment of the adjacent 9 and 16 amino acid splice inserts. Furthermore it suggests that the difference in mRNA size is translated as a difference in

the size of the final protein. The lower levels of splice insert Apl III in non-nervous tissues may be due to a smaller non-nervous splice insert mRNA population. A small mRNA population may explain why no splice insert cDNA was found in the reverse transcription of the non-nervous mRNA. Another possibility is that axons and nerve terminals also enervate contractile tissues. Preperations of these tissues may contain traces of immunoreactive PKC species from the axon and its terminals.

The location of the splice inserts, at the hinge region between the regulatory and catalytic subunits of PKC Apl III, positions it at an ideal location for the generation of a PKM Apl III. To date, no PKM Apl III could be identified in *Aplysia*, and the functional effect of the splice inserts, if any, remains elusive. Nonetheless PKM has been implicated in a variant of ITF that is site specific (Sutton et al., 2004). This PKM was generated by direct application of calpain, which may be tried in the future on PKC Apl III.

Inhibitors – Preliminary kinase assays on pharmacological inhibitors of PKCs demonstrate that the classical agent bisindolylmaleimide-1 is an inhibitor specific to novel PKCs, but not atypical PKC. This is useful insofar as previous studies that blocked PKC with bis may now only claim that novel PKC activity was blocked, but not atypical PKC activity. The compound chelerythrine inhibits novel and atypical PKCs with equal efficacy. However, its markedly greater inhibition of purified PKM Apl III clone is consistent with the fact that chelerythrine competes with the pseudosubstrate at the substrate binding site. Since PKM contains no pseudosubstrate - normally located in the regulatory subunit – chelerythrine may bind the catalytic pocket uncontested. Finally, the PKC Apl III inhibitor peptide showed specific inhibition for atypical PKC activity at

higher concentrations. It may be used in future experiments for isoform selective inhibition.

Conclusion

Protein kinases, including novel and atypical isotypes of Protein Kinase C, play a role in maintainance of memory at the neuronal level. Although novel PKC is regulated by phosphorylation, the nature of this regulation differs between *Aplysia* neuronal nPKC Apl II and mammalian non neuronal nPKC epsilon/delta. The kinase of the hydrophobic site of nPKC, S729, is not known. Candidate kinases from other systems, mTOR/FRAP and aPKC zeta/iota, do not phosphorylate PKC Apl II. In fact, the only manipulation that affected S729 phosphorylation in neurons was treatment with serotonin - the neurotransmitter released during sensorimotor facilitation. However, effects on phosphorylation of the nPKC hydrophobic site are found in heterologous cells from clones of PDK-1 and aPKC Apl III.

A role for atypical PKC Apl III in cellular memory remains unproven. Interestingly, an alternatively spliced form of PKC Apl III is much more abundant in the nervous system. This suggests that the 9 and 16 amino acids of the longer form are selected for a nervous specific role. Further characterization of the regulation of PKC Apl III is required, particularly to identify a mechanism of persistent activity and in doing so, ascribe a role in memory.

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Appendix A: List of Abbreviations

Bis: Bisindolylmaleimide-1 (also known as BIM-1)

CaMKII: Calcium/Calmodulin dependent kinase

DAG: Diacylglycerol

HEK: Human embryonic kidney (cell line)

5-HT: Serotonin

ITF: Intermediate term facilitation

LTF: Long term facilitation

LTP: Long term potentiation

PCR: Polymerase Chain Reaction

PDK: Phosphoinositide dependent kinase 1

PI3K/PtdIns3K: Phosphoinositide 3 Kinase

PDBu: Phorbol 12,13 Dibutyrate

PKA: Protein kinase A/Cyclic AMP dependent Protein Kinase

PKC: Protein kinase C/Calcium dependent Protein Kinase

5' RACE: 5' Rapid amplification of cDNA ends

STF: Short term facilitation

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February 18, 2004

The McGill University Animal Care Committee certifies that

Travis Lim has successfully completed the

Basic Level
of the
Theory Training Course on Animal Use for Research and Teaching
on
February 18, 2004.

The training includes the following topics:

- **Basic Level:** Regulations & Procedures, Ethics, Basic Animal Care, Occupational Health & Safety

- **Wildlife:** Basic principles for working with wildlife in the laboratory and in the field.

Please note that this certificate does NOT include practical training, which is obtained by successfully completing an Animal Methodology Workshop where another certificate is issued.

Certification is valid for 5 years, starting on the date indicated above.

Deanna Collin
Animal Care Training Coordinator, animalcare@mcgill.ca

(Confirmation of training can be obtained by request to the above email address)

Note: Trainee must keep this certificate as other institutions may request it as evidence of training

McGill University
Environmental Safety

THIS IS TO CERTIFY THAT

Travis Lim

HAS SUCCESSFULLY COMPLETED A BASIC

LABORATORY COURSE IN

RADIATION SAFETY

January 2004

C

Radiation Safety Officer
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