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Characterization of the Phosphorylation of Protein Kinase C by Phosphoinositide-dependent Kinase-1

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Title: Characterization of the phosphorylation of protein kinase C by phosphoinositide-dependent kinase-1

Summary:

Phosphorylation of protein kinase Cs (PKCs) by phosphoinositide-dependent kinase I (PDK) is critical for PKC activity. In the nervous system of the marine mollusk *Aplysia*, there are only two major PKC isoforms, the calcium-activated PKC Apl I and the calcium-independent PKC Apl II, and both PKCs are persistently activated during intermediate memory. We monitored the PDK-dependent phosphorylation of PKC Apl I and PKC Apl II using phosphopeptide antibodies. PDK phosphorylation of PKCs was not sensitive to inhibitors of PI-3 kinase, PKC, nor to expression of a kinase-inactive PDK. Phosphorylation of a kinase inactive PKC was reduced, likely due to dephosphorylation. Localization of PDK-phosphorylated PKC Apl II using immunocytochemistry revealed an enrichment of phosphorylated PKC Apl II at the plasma membrane. These data suggest that increased PDK phosphorylation of PKC Apl II is important for persistent kinase activation. PKC phosphorylation at the PDK site is not affected by the presence of a kinase-inactive form of PDK. Using GFP (green fluorescent protein)-tagged PKC, which was microinjected into neurons, we determined that PKC was translocated to the juxta-membrane region by the PKC activator 4β-phorbol ester 12,13-dibutyrate (PDBu).

Titre: La caractérisation de la phosphorylation de protéine kinase C par la kinase phosphoinositide-dépendante

Résumé :

La phosphorylation des protéines kinases C (PKCs) par la kinase phosphoinositide-dépendante (KPD) est cruciale à l'activité de PKC. Dans le système nerveux du mollusque marin *Aplysia,* il y a seulement deux isoformes majeures de PKC, l'Apl I, qui est activée par le calcium, et l'Apl II, qui est indépendante du calcium. Les deux formes sont activées dans une façon persistante pendant la mémoire intermédiaire. Nous avons étudié la phosphorylation d'Apl I et Apl II en utilisant des anticorps contre des peptides phosphorylés. La phosphorylation de PKC par KPD n'était pas sensible aux inhibiteurs de la kinase phosphoinositol-3, PKC, ni à l'expression d'une kinase KPD inactive. La phosphorylation d'une kinase PKC inactive était réduite, possiblement dû à la déphosphorylation. En utilisant l'immunocytochimie pour examiner la distribution de Apl II, on a observé un enrichissement de Apl II phosphorylé sur la membrane cytoplasmique. Ces resultats suggèrent qu'une augmentation de la phosphorylation de PKC par PDK est importante pour l'activation persistante de la kinase. La phosphorylation de PKC au site PDK n'est pas affectée par la présence d'une forme inactive de PDK. En utilisant PKC marquée avec la protéine fluorescente verte, qui a été injecté dans les neurones sensorielles, nous avons determiné que le PKC est transloqué à la région juxta-membrane par l'activateur de PKC, 4β-phorbol ester 12,13-dibutyrate.

Acknowledgments

I would like to thank my supervisor, Wayne Sossin, for his insight, his open door, and his endless patience.

My time in the laboratory would surely not have been as enjoyable as it was were it not for the great group of people that make up the Sossin lab. To John, Arash, and Jonathan, thank you for taking the time to teach me. The three of you, Matt, and Xiaotang, made the lab a great place to work.

To my family, for their wisdom and their unwavering support, I thank you. Je vous aime.

To Tim, I simply would not have made it this far without your support. You are my rock.

List of Abbreviations

5-HT	serotonin or 5-hydroxytryptamine
aPKC	atypical protein kinase C
ATP	adenosine triphosphate
Bis	bisindolylmaleimide
BSA	bovine serum albumen
CFP	cyan fluorescent protein
cPKC	conventional protein kinase C
DAG	diacylglycerol
DiCPIP3	diC_{16}phosphatidylinositol 3,4,5-trisphosphate
ES	embryonic stem
GFP	green fluorescent protein
GST	glutathione-S-transferase
HA	hemagglutinin
ICC	immunocytochemistry
IGF-1	insulin-like growth factor-1
ITF	intermediate-term facilitation
ITP	intermediate-term potentiation
L-LTP	late-phase long-term potentiation
LTF	long-term facilitation
LTP	long-term potentiation
LY	LY294002
MAP-2	microtubule associated protein-2

MAPK	mitogen-activated protein kinase
MBP	maltose-binding protein
nPKC	novel protein kinase C
PBS	phosphate-buffered saline
PDBu	4β-phorbol ester 12,13-dibutyrate
PDGF	platelet-derived growth factor
PDK	phosphoinositide-dependent kinase-1
PDK2	phosphoinositide-dependent kinase-2
PH	pleckstrin homology
PI3K	phosphoinositide 3-kinase
PIF	phosphoinositide-dependent kinase-1 interacting fragment
PtdIns-3,4-P2	phosphatidylinositol (3,4)-bisphosphate
PtdIns-3,4,5-P3	phosphatidylinositol (3,4,5)-trisphosphate
PKA	protein kinase A
PKB	protein kinase B
PKC	protein kinase C
PKM	protein kinase M
PRK	protein kinase C related kinase
PS	phosphatidylserine
PV	peroxyvanadate
RACKs	receptors for activated C-kinases
RSK	90 kDa ribosomal S6 kinase
S6K1	p70 ribosomal S6 kinase

SGK	serum- and glucocorticoid-inducible protein kinase
STICKs	substrates that interact with C-kinases
STF	short-term facilitation
STP	short-term potentiation
TOR	target of rapamycin
YFP	yellow fluorescent protein
X/XO	xanthine/xanthine oxidase

Contributions of Author

Chapter 2

Pepio AM, Thibault GL, and Sossin WS (2002) Phosphoinositide dependent kinase (PDK) phosphorylation of protein kinase C (PKC) Apl II increases during intermediate facilitation in *Aplysia*. Manuscript submitted to J Biol Chem; under review.

My contribution to this paper included immunoprecipitation of phosphopeptide immunoreactivity, pulldown experiments using nervous system extracts (including fusion protein generation for these experiments), all immunocytochemistry, as well as the experiments involving LY294002 and bisindolylmaleimide. Dr. Wayne Sossin and I performed the experiments establishing correlation of autonomous kinase activity with phosphorylation. All other experiments were performed by Antonio Pepio. DNA constructs were generated by Tina Hueftlein. I collaborated in the authorship of this paper with Dr. Wayne Sossin.

Chapter 3

Translocation of PKC can be monitored using a green fluorescent protein tag

I performed the dissections, cultured the cells, and performed the translocation experiments. These cells were injected by Dr. John Dyer. The DNA constructs were generated by Xiaotang Fan.

Chapter 1 – Literature Review

The discovery of long-term potentiation

The notion that memory is mediated by the strengthening of synapses is at least five decades old. When D.O. Hebb first proposed, in 1949, that "when an axon of cell A... excites cell B and repeatedly or persistently takes part in firing it, some growth process or metabolic change takes place in one or both cells so that A's efficiency as one of the cells firing B is increased", the idea gained little notice. In 1973, however, Bliss and Lømo provided the first evidence that the strength of a particular synapse could be modified by a change in the input to that synapse. In their seminal series of experiments, they initially applied a test stimulus to the entorhinal cortex of an anaesthetized rabbit, and then measured the field potential response of the postsynaptic cells in the dentate fascia. Next, they applied a high frequency stimulus (20 Hz for 15 seconds) to the perforant pathway which connected the two regions. Then, they once again measured the field potential response to the initial test pulse. They observed an increase in the amplitude of the response of the post-synaptic cells. They termed this increase in responsiveness "long-term potentiation" (LTP). LTP has since been proposed as potential mechanism which could underlie memory formation

Aplysia as a model system for the study of plasticity

Many model systems have been developed in order to study both the mechanisms that underlie plasticity, as well as the impact of synaptic plasticity on the animal's behaviour. One animal that exhibits many characteristics desirable in a model system is the sea slug, *Aplysia Californica*. *Aplysia* have a simple nervous system, consisting of

twenty thousand neurons, several orders of magnitude less than that of the vertebrate nervous system. The small number of neurons, in addition to the fact that the connectivity of these neurons is invariant, means that the specific neurons responsible for a particular function can be identified (Kandel, 2001). For instance, a weak stimulus to the siphon will activate about eight sensory neurons, which in turn will cause six identified motor neurons innervating the gill to fire, resulting in withdrawal of the gill (Byrne, Castellucci, & Kandel, 1974). In addition, *Aplysia* have very large neurons (up to 1000 μm), which makes them conducive to manipulations such as injection of plasmid DNA and electrophysiological studies. *Aplysia* also exhibit well-characterized, measurable, and plastic behavioural responses to particular stimuli. These responses can be shaped through several types of both associative and nonassociative learning (Kandel, 1978).

Learning and synaptic facilitation in Aplysia

One behaviour in *Aplysia* that is amenable to modification through nonassociative learning is the gill-withdrawal reflex. This is a defensive reflex through which a weak tactile stimulus to the siphon induces withdrawal of the siphon as well as the gill. If, however, the animal is first subjected to a noxious stimulus, such as a shock to the tail, its response to subsequent weak stimuli to the siphon will be greater. This enhancement of the animal's defensive reflex is referred to as sensitization. While a single shock will induce sensitization which lasts only minutes, five spaced shocks can result in an enhanced response that persists for hours or longer. If the tactile stimulus is applied repetitively to the siphon in the absence of the noxious stimulus, habituation will

eventually occur. Habituation entails an attenuation of the response to the stimulus (Kandel, 1978).

Both habituation and sensitization are accompanied by changes at the synaptic level. During habituation, there is a decrease in the number of active zones, as well as a decrease in their size and in the number of vesicles per active zone. This is paralleled by a decrease in the number of synaptic varicosities. The opposite changes are observed following sensitization (Bailey & Chen, 1983; Bailey & Chen, 1988). This increase is accompanied by an increase in the number of transmitter quanta released by the sensory neuron (Dale, 1988). This enhancement of transmission at *Aplysia* synapses is referred to as facilitation, and can be mimicked by the application of serotonin (5-HT) to the neurons (Montarolo, 1986; Emptage, 1993).

There is mounting evidence that these changes in synaptic transmission underlie the changes in behavioural response. Following sensitization training, sensory neurons were found to exhibit an increase in excitability, and motor neurons were hyperpolarized relative to control neurons (Cleary, 1998). It has also been observed that the neurons which fire during sensitization training show greater facilitation than those that do not fire, and that the degree of facilitation correlates significantly with the enhancement of siphon withdrawal (Antonov, 1999; Antonov, 2001).

Stages of plasticity

Plasticity in *Aplysia* can be subdivided into three phases. The first phase of plasticity is called short-term facilitation (STF). It is dependent on covalent modification (i.e. phosphorylation), and is independent of transcription or translation (Montarolo,

1986). The second phase is intermediate facilitation (ITF). It is longer-lasting and mechanistically distinct from STF. It, too, is dependent on kinase activity, but may require translation ITF is, however, independent of transcription (Sutton & Carew, 2000). The third phase is termed long-term facilitation (LTF). LTF is dependent on both transcription and translation (Montarolo, 1986). These three forms of facilitation are induced in a graded manner: a stimulus which is strong enough to induce STF may not be intense enough to induce ITF or LTF. Each of these has a counterpart in vertebrates, called short-term potentiation (STP), LTP, and late-phase LTP (L-LTP). Similarly, studies have demonstrated that memory is induced in three distinct phases: short-term, intermediate-, and long-term (Davis & Squire, 1984). As with facilitation, both potentiation and the phases of memory are dependent on phosphorylation, translation, and transcription, respectively, in a manner that parallels the analogous plastic changes.

Aplysia therefore provide a simple system for study, in which well-understood behaviours are controlled by identified neurons. Modifications of these behaviours are significantly correlated with morphological and functional modifications of the specific synapses involved in the behaviour. This provides a useful model system for investigating synaptic plasticity.

Evidence for the role of specific kinases in learning and memory

Application of serotonin (5-HT) to *Aplysia* ganglia results in facilitation. One to four pulses of 5-HT lasting 5 minutes each result in STF, which lasts less than 30 minutes. Five pulses result in ITF, which lasts up to 3 hours. This treatment can also result in LTF, which begins 10 to 15 hours after the 5-HT treatment, and can persist for

more than 24 hours (Mauelshagen, 1996). Each of these types of facilitation differs from the others not only in terms of time course but also mechanistically.

ITF can also be induced by other stimulation paradigms, and each of these is also mechanistically distinct. For instance, a short pulse (5 minutes) in conjunction with post-synaptic activity results in a form of ITF which is dependent on protein kinase C (PKC) but not on protein kinase A (PKA) nor on translation. Five pulses of 5-HT bring about ITF which requires protein synthesis for its induction, as well as PKA for its expression, but is independent of PKC (Sutton & Carew, 2000.)

Persistent kinase activation is a likely candidate for the expression of intermediate facilitation. Many lines of evidence suggest that some types of facilitation depend on PKC activation. Pharmacological activation of PKC through phorbol esters results in a long-lasting enhancement of the excitability of sensory neurons, as manifested by an increase in the number of spikes fired by these neurons in response to a depolarizing pulse (Manseau, 1998). PKC's role in facilitation appears to be most important in synapses that are depressed (Ghirardi, 1992). A dominant negative isoform of Apl II but not Apl I disrupts reversal of depression (Manseau, 2001). The role for PKC in memory also seems to extend to vertebrates. Mice in which the β isoform of PKC has been knocked out are deficient with respect to fear conditioning, and PKCγ isoform knockout mice have deficiencies in spatial and contextual learning. (Weeber, 2000; Abeliovich, 1993).

Autonomous PKC activity

Two hours following a 90-minute application of 5-HT to *Aplysia* ganglia, there is a significant increase in the level of autonomous PKC activity. This activity is thought to emanate from Apl II, and can be immunoprecipitated using antibodies to Apl II. The transition to an autonomous kinase is dependent on protein but not RNA synthesis. Although one mechanism by which PKC can become autonomously active is through generation of a catalytic fragment (also known as protein kinase M (PKM)), this is not thought to be the mechanism underlying this particular activity, since this autonomous kinase is inhibited by calphostin-C, a PKC inhibitor that targets the regulatory domain of PKC. However, autonomous PKC requires a higher dose of calphostin-C for its inhibition than does regulated activity, suggesting that the regulatory region has been modified (Sossin, 1997). Autonomous PKC activity has been shown to play an important role in memory in other systems. For instance, in *Drosophila*, the expression of a persistently active PKC zeta fragment, PKM zeta, enhanced memory (Drier, 2002).

The exact mechanism by which the kinase becomes autonomous, however, is unknown. One possible mechanism is through interaction with reactive oxidative species. Incubation of hippocampal slices with the superoxide-generating xanthine/xanthine oxidase (X/XO) system results in an increase in autonomous PKC activity (Knapp, 2002). Interestingly, the X/XO system induces L-LTP in parallel with autonomous PKC activity, and this L-LTP can be blocked by the inhibition of PKC, suggesting that the autonomous activity is important for potentiation (Knapp, 2002).

The PKC Family of Kinases

PKCs are a family of lipid-activated kinases. There are three broad classifications of PKC: classical PKCs (cPKCs), novel PKCs (nPKCs), and atypical PKCs (aPKCs). Classical PKCs are calcium- and phorbol-ester activated, while novel PKCs are calcium-independent, but depend on phorbol esters. The third class, atypical PKCs (aPKCs), are both calcium- and phorbol ester-independent (Newton, 1995).

Vertebrates have twelve known isoforms of PKC. Conventional PKC isoforms include PKC α, βI, βII, and γ; novel isoforms include PKC δ, ε, η, and θ. Vertebrates also express the atypical kinases PKC ξ and ι/λ. Two members of a novel subfamily, PKCμ (also known as protein kinase D in mouse) and PKCν, have recently been identified. These isoforms differ quite strikingly from those of the other three subfamilies of PKC in that they lack the typical pseudosubstrate site, and have a pleckstrin homology (PH) domain as well as two hydrophobic N-terminal domains which are absent in other isoforms (Hayashi, 1999). Subsequently, they are often referred to as "PKC-like" or "PKC-related" kinases (PRKs) (Brandlin, 2002).

Aplysia only express three PKC isoforms. PKC Apl I is a conventional PKC isoform most closely related to the vertebrate βI, and PKC Apl II is a novel isoform which is quite similar to the ε isoform in vertebrates (Sossin, 1993). *Aplysia* also express PKC ζ, an atypical form of PKC. Since only one isoform from each of these subfamilies is expressed, isoforms can be easily dissociated due to distinct activation requirements, making *Aplysia* a convenient model system for the study of PKCs.

PKCs contain four regions which are conserved between isoforms, referred to as C1 through C4. There are also five variable regions, V1 to V5, which are not conserved

between isoforms and are interspersed between the conserved regions. The C3 and C4 domains are located in the C-terminal catalytic region and constitute the ATP binding site and kinase domains, respectively. The N-terminal regulatory region consists of a pseudosubstrate, a C1 domain, and a C2 domain (Newton, 1995).

The C1 domain is a lipid-binding domain and consists of two tandem repeats of cysteine-rich zinc fingers. It is this domain which binds diacylglycerol (DAG) and is responsible for membrane penetration (Medkova, 1999). The C1 domain of aPKC has only one cysteine-rich domain, and cannot bind phorbol esters (Newton, 1997) The C2 domain of cPKCs binds phosphatidylserine (PS) in a calcium-dependent manner. This is the initial step of PKC translocation, and is required for C1 binding to the membrane. This domain contains several aspartic acid residues, which coordinate the calcium ion. The C2 domain of nPKCs is structurally similar to that of cPKCs but lacks these aspartic acid residues (Nalefski, 1996). Lipid binding of the C2 domain of Apl II is regulated by phosphorylation at a serine residue (serine 36 in Apl II). However, since this site is not strongly conserved, it is unclear whether this mechanism holds true for other nPKCs (Pepio, 2001). Atypical PKCs lack the C2 domain (Johannes, 1994).

PKC activation

Newly synthesized PKC is in a conformation such that its activation loop is exposed (Dutil, 2000). It associates with phosphoinositide-dependent kinase-1 (PDK), and PDK phosphorylates its activation loop. PDK dissociates from PKC, unmasking the C-terminus (LeGood, 1998; Gao, 2001). Conventional PKCs can then autophosphorylate at two sites in its C-terminus: the turn motif, which is thought to lie at the top of the

kinase domain; and the hydrophobic site, so named because it is surrounded by hydrophobic residues such as tyrosine and phenylalanine (Cenni, 2002). Phosphorylation at these two sites induces a conformational change in which the C-terminal becomes hidden within the enzyme. Thus, the conformational change protects the C-terminal sites from de-phosphorylation by phosphatases. The kinase will remain in this latent, mature conformation until acted upon by its allosteric activators (Dutil, 1998).

With respect to nPKCs, the regulation of this site is somewhat more controversial. Some groups have reported that it is a trans-phosphorylation, mediated by a complex which includes PKCζ, while others have reported that for PKCε, it is an auto-phosphorylation (Ziegler, 1999; Cenni, 2002). In addition, the site is downstream of TOR (target of rapamycin), and phosphorylation is inhibited by rapamycin (Ziegler, 1999). Finally, although phosphorylation at this site is thought to occur constitutively in cPKCs following phosphorylation by PDK, it appears to be regulated in nPKCs, at least in part, by DAG and phosphatidylinositol-3,4,5-triphosphate (PtdIns-3,4,5-P3) (Cenni, 2002).

PKC activation can follow activation of cell surface receptors, such as those for hormones, some neurotransmitters, or growth factors. A rise in phospholipase C activity downstream of these receptors leads to an increase in DAG levels in the membrane, as well as an increase in inositol triphosphate, which binds to its receptor on the endoplasmic reticulum and triggers release of calcium stores. The C2 domain of cPKCs binds PS in a calcium-dependent manner. The C1 domain can then bind DAG, resulting in a conformational change which removes the pseudosubstrate from the kinase core and results in PKC activation (Medkova, 1999).

Regulation of PKC through other phosphorylation sites

Apl II has a much higher requirement for PS than Apl I. Deletion of the C2 domain of Apl II lowers the kinase's requirement for PS, suggesting that this domain plays an inhibitory role (Sossin, 1996). The inhibitory effect of the C2 domain of Apl II is alleviated by phosphatidic acid, as well as by phosphorylation at a site within the C2 domain, serine 36 (Pepio, 1998; Pepio, 2001). This site is thought to be mediated by autophosphorylation, although *in vitro* it can also be phosphorylated *in trans.* Phosphorylation at this site increases in the presence of PKC activators, such as the phorbol ester analog 4β-phorbol ester 12,13-dibutyrate (PDBu) (Pepio, 2001). Apl II can be activated through activation of receptor tyrosine kinases, or through long-term treatments with 5-HT (Manseau, 2001).

The ser 613 site of Apl I may regulate the subcellular localization of the enzyme. Apl I that is phosphorylated at this site is retained in the cytosol. This may be due to reduced affinity for Ca^{2+}, since mutating the site to an alanine residue increases the affinity of Apl I for calcium; it may also be a result of specific binding by a cytosolic protein exclusively when Apl I is phosphorylated at this site (Nakhost, 1999).

PKC regulation - summary

PKCs are therefore regulated by their allosteric activators, by oxidation, and by phosphorylation. While phosphorylation of the activation loop by PDK-1 is a critical step in maturation, its conformation is regulated by C-terminal sites. The subcellular localization of PKC is regulated by phosphorylation sites in the C-terminus of Apl I, the C2 domain of Apl II, as well as by the allosteric activators DAG and Ca^{2+}.

PDK

PDK was first identified as a protein kinase B (PKB) kinase. It is a member of the AGC family of kinases, and its kinase domain is highly homologous to that of PKC. It also has a PH domain, which acts as a lipid-binding module, selectively binding phosphatidylinositol-3,4-biphosphate (PtdIns-3,4-P_2) and PtdIns-3,4,5-P_3 (Alessi, 1997). Stimuli that induce tyrosine kinase activity very often lead to phosphoinositide 3-kinase (PI3K) activation, resulting in a rise in PtdIns-3,4-P_2 and PtdIns-3,4,5-P_3 levels (Stephens, 1998). PtdIns-3,4,5-P_3 recruits PDK to the membrane via this PH domain (Filippa, 2000).

PDK regulation

In addition to PKC, PDK has several substrates, including PKA, the aforementioned PKB, p70 ribosomal S6 kinase (S6K1), serum- and glucocorticoid-inducible protein kinase (SGK), and 90 kDa ribosomal S6 kinase (RSK) (Cheng, 1998; Pullen, 1998; Kobayashi, 1999; Park, 1999; Jensen, 1999). Its regulation is complex, and is in part mediated by the substrates themselves. For instance, PKB must be bound to the membrane via its PH domain in order that its activation loop may be exposed (Filippa, 2000), while S6K1 must first be phosphorylated by mitogen-activated protein kinase (MAPK) to unmask its activation loop (Alessi, 2000). For PKC, the pseudosubstrate is removed from the kinase core in the newly synthesized enzyme, and this is essential for PDK phosphorylation (Dutil, 2000). Thus, although PDK has a high level of basal activity, it can selectively activate different targets, based on their conformation (Toker & Newton, 2000).

PDK and the insulin pathway

It has been proposed that PDK is the missing link in the insulin pathway leading to PKB (Alessi, 2000). Insulin stimulation increases the ability of PDK to phosphorylate PKB, for instance, in certain expression systems such as NIH-3T3 cells. However, in other systems, such as 293 cells, insulin does not have this effect. It has been suggested that PDK has some basal activity, and that this activity can be increased by phosphorylation of PDK, through a mechanism dependent on insulin (Chen, 2001).

Insulin treatment of *Aplysia* ganglia results in a transient activation of Apl I, as well as persistent activation of Apl II. Apl II can also be activated by diC_{16}phosphatidyl-inositol 3,4,5-trisphosphate (DiCPIP3), a synthetic PI3K product. Furthermore, activation of Apl II can be blocked by wortmannin, a PI3K inhibitor. Although activation of Apl I is probably a result of PLCγ activation, which produces DAG and Ca^{2+} transiently, persistent Apl II activation may be due to a rise in PtdIns-3,4,5-P3 levels. One mechanism that might account for this would be activation of PDK by PtdIns-3,4,5-P3 (Sossin, 1996).

Dependence of PDK on lipids

The regulation of PDK is complex. In spite of its name, its dependence on PtdIns-3,4,5-P3 and PtdIns-3,4-P2 is controversial. PDK has a nanomolar affinity for these lipids, and one role that has been proposed for this binding is PDK translocation to the membrane via the PH domain (Stephens, 1998; Anderson, 1998; Filippa, 2000). Recruitment of PDK to the membrane may be important in bringing it in proximity to its substrates, many of which are membrane-bound in stimulated cells. In fact, artificially

myristolated PDK is constitutively active towards PKB. The subcellular localization of PDK under basal conditions is unclear. Many studies have found that PDK1 is mostly cytosolic in unstimulated cells, although two studies report that it is constitutively membrane-bound (Anderson, 1998; Currie, 1999; Andjelkovic, 1999). This may be due to its extremely high affinity for PtdIns-3,4,5-P3 – at extremely low concentrations, i.e. even in unstimulated cells, PtdIns-3,4,5-P3 may still be able to induce PDK translocation (Storz & Toker, 2002). Some studies have found that translocation to the membrane can be blocked with the PI3K inhibitor wortmannin (Anderson, 1998). However, in other studies, the use of insulin-like growth factor (IGF), platelet-derived growth factor (PDGF), and insulin, all of which result in an increase the production of PtdIns-3,4,5-P3, has not induced translocation (Currie, 1999). It is unclear why these studies have produced such disparate results: they do not seem to be a function of different expression systems, nor of the extent of serum starvation.

In addition to regulating translocation, it has been proposed that the PH domain of PDK plays an autoinhibitory role (Filippa, 2000). The evidence in support of this includes the finding that inhibition of PI3K activity reduces myristolated PDK's phosphorylation of myristolated PKB. In addition, phosphorylation of a PH-domain deleted PKB is enhanced by PtdIns-3,4,5-P3, an effect which clearly is not simply due to proximity to the substrate. Deletion of the PH domain of PDK-1 resulted in a four-fold increase in the rate of PKC phosphorylation (Filippa, 2000). In spite of the PH domain's strong affinity for PtdIns-3,4-P2 and PtdIns-3,4,5-P3, these may not alleviate the autoinhibition. A recent study found that phosphorylation of PKC was found to be unaffected by the presence or absence of phosphoinositides or PI3K inhibitors, while

PKB phosphorylation was abolished under the same conditions by PI3K inhibitors (Sonnenburg, 2001). In addition, other substrates lacking a PH domain (such as S6K) are phosphorylated by PDK in the absence of PtdIns-3,4,5-P3 (Pullen, 1998).

Although some studies find that the phosphorylation of PDK substrates such as PKB, SGK, and PKC is blocked by PI3K inhibitors (Alessi, 1997; Park, 1999, LeGood, 1998), others observe that phosphorylation of SGK and PKC, as well as S6K, is resistant to these inhibitors (Pullen, 1998; Sonnenburg, 2001; Park 1999).

PDK is also regulated by sphingosine. This lipid not only induces an increase in the level of PDK autophosphorylation *in vitro*, but also results in an increase in the amount of PKB phosphorylation by PDK. It is not yet known whether the increase in PKB phosphorylation is due to direct regulation of PDK, or to a substrate-binding effect (Storz & Toker, 2002).

Regulation of PDK by phosphorylation

PDK undergoes phosphorylation at five serine residues. Only one of these, a site within the activation loop located at serine 241 in human PDK, has been found to be required for PDK activity. This site is highly conserved in all known PDK homologues; the other serine residues are not (Casamayor, 1999). Since the site is phosphorylated in bacterial cells, but not phosphorylated in the kinase-dead mutant, it is thought to be an autophosphorylation. Phosphorylation at this site is not increased by IGF-1, nor by insulin, and is not affected by wortmannin (Casamayor, 1999; Pullen, 1998). One study, however, has found that PDK isolated from cells treated with insulin has a higher level of phosphorylation at serine 241 as well as greater catalytic activity towards PKB. In this

study, it is suggested that the differences between studies may lie in the expression systems – while the former studies were done in 293 cells, the latter were done in NIH-3T3 cells and rat adipose cells (Chen, 2001).

Although the other sites are not required for activation, since their mutation to alanine does not affect PDK activity, they may play a more subtle role in PDK function. Mouse PDK was found to be constitutively active upon mutation of the auto-phosphorylation site Thr516 to a negatively charged residue. Although auto-phosphorylation at this site was not important in the regulation of autokinase activity, it does result in phosphorylation of PKB to an extent equivalent to that seen with insulin stimulation. Phosphorylation at this site not affected by insulin, however (Wick, 2002).

PDK is also subject to phosphorylation at three tyrosine residues. Two of these have been found to affect PDK activity, and are phosphorylated by Src (Park, 2001). Peroxyvanadate (PV), a tyrosine phosphatase inhibitor, induces an increase in the level of phosphorylation at these sites, as well as translocation of PDK to the membrane. This PV-induced translocation is blocked by wortmannin, although PV's effect on tyrosine phosphorylation is wortmannin-insensitive (Grillo, 2000). Abl tyrosine kinase also phosphorylates this site, resulting in an increase in the activity of PDK towards PKB and SGK (Prasad, 2000)

Regulation of PDK by the PDK1-interacting fragment

The C-terminal region of PRK2 was found, through a yeast-two hybrid screen, to bind the kinase domain of PDK. This region, referred to as the PDK1-interacting fragment (PIF), contains a hydrophobic motif similar to that of PKBα, except that there is

an aspartic acid residue in the place of the PDK-phosphorylated serine residue. The hydrophobic motif of PKB is not well phosphorylated by PDK *in vitro* or in co-transfection experiments; as such, another kinase, named PDK2, was proposed to mediate this phosphorylation. However, interaction of PDK1 with PIF converts it from a kinase which could only phosphorylate PKB's activation loop to one which could phosphorylate both the activation loop and the hydrophobic site. PIF also converts PDK from a form that is not directly activated by PtdIns-3,4,5-P3 to one that is activated three-fold by PtdIns-3,4,5-P3 (Balendran, 1999).

Larger fragments of PRK2 however, which are generated by caspase-3, inhibit PDK phosphorylation of PKB at both the hydrophobic site as well as the activation loop. This fragment can also inhibit PDK phosphorylation of PKC δ and ζ, as well as induce a reduction in the level of PDK autophosphorylation (Hodgkinson, 2002).

Splice variants of PDK

A splice variant of PDK, PDK-1β, has recently been identified in mice. This isoform is exclusively expressed in the brain and testis, and is similarly autophosphorylated at serine residues. Although co-expression with constitutively active Src results in the same level of tyrosine phosphorylation as is seen with PDK, it is not well phosphorylated by peroxyvanadate, or insulin plus peroxyvanadate. Interestingly, the level of expression of this splice variant of PDK increases with age (Dong, 2002).

Indirect mechanisms of regulation by PDK

The role of PDK's phosphorylation of PKC is not fully understood. PDK phosphorylation of PKC is necessary for the maturation of PKC; however, it appears that,

at least for cPKCs, once the C-terminal sites have been phosphorylated the PDK site phosphorylation is no longer required. PDK binds PKC primarily through PKC's C-terminus, as well as through a weak interaction with C1. PDK's affinity for the C-terminus is increased by an order of magnitude when the terminus is phosphorylated. However, this region is masked following phosphorylation, and so at basal levels of stimulation, PDK likely interacts more strongly with the unphosphorylated kinase. Upon stimulation, PDK might be able to interact once again with the C-terminus. The strong interaction raises the possibility that active PKC might act as a sink for PDK (Gao, 2001).

PDK may also influence PKC at other levels of regulation. For instance, embryonic stem (ES) cell knockouts of PDK show measurably lower levels of PKC α, βI, δ, and ε protein. This may be due, in part, to lack of phosphorylation of the hydrophobic site. This site has been shown to be important for the protein's stability, and is only phosphorylated after PDK site phosphorylation (Edwards, 1997). However, mRNA levels of PKCδ were 50% lower in the knockout cells relative to control, suggesting that PDK may therefore play an additional role: stabilization of mRNA.

The role of PDK in the regulation of PKB also appears to extend beyond simply phosphorylation. PDK can translocate PKB whose PH domain has been deleted to the cytoplasm. Co-expression of a kinase-dead form of PDK potentiates the phosphorylation of PKB following insulin treatment, probably by preventing its dephosphorylation (Filippa, 2000).

PDK summary

PDK therefore plays a crucial role in the regulation of several kinases. In part, it is regulated by the state of its substrates; it may also be regulated by its PH domain and PtdIns-3,4,5-P3. It is also regulated by phosphorylation, with the ser241 site being critical, and other serine/threonine and tyrosine phosphorylation sites playing a modulatory role. PDK's ability to phosphorylation certain sites, such as the C-terminal of PKB is conferred by an interaction with the protein fragment PIF, whereas interaction with other protein fragments inhibits phosphorylation by PDK. Beyond its direct role in regulating PKC by phosphorylation, it may also regulate levels of PKC mRNA.

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Preface to chapter 2

There are two phorbol-ester activated PKC isoforms in the nervous system of *Aplysia*: Apl I and Apl II. PKCs play an important role in learning and synaptic plasticity in *Aplysia*, and both isoforms are activated by behavioural sensitization, as well as application of serotonin to the ganglia. There are three levels of regulation of PKC activity: allosteric regulation, control of subcellular localization, and regulation through phosphorylation. Phosphorylation of PKC by PDK is the initial step in kinase maturation, and is required so that PKC can autophosphorylate at two sites on its C-terminus. Once the C-terminal autophosphorylations have been achieved, PDK site phosphorylation is not required for the activity of conventional PKCs. Beyond its role in the maturation of PKC, the regulation of this phosphorylation, as well as its role in the mature kinase, are not well understood, particularly for novel PKCs.

Aplysia PDK has been cloned in our laboratory (Khan, 2001). It is highly homologous to human PDK, with respect to the PH domain and the kinase domain, but has an N-terminal region which is not highly conserved. Our goal was to examine how PDK phosphorylation of PKC was regulated, and whether this phosphorylation might be affect PKC function in the context of intermediate memory. Using a GST-PDK fusion protein, we showed that PDK binds to PKC Apl II from nervous system extracts. PDK was also shown to phosphorylate PKC *in vitro*.

The PH domain of PDK binds PtdIns-3,4,5-P3 with high affinity, but the importance of this binding has not been established. A study performed in 293 cells have found that inhibition of PtdIns-3,4,5-P3 production through the use of the PI3K inhibitor LY294002 inhibits phosphorylation of nPKC, while another study done in NIH-3T3 cells

found that this inhibitor had no effect on phosphorylation of cPKCs (LeGood, 1998; Sonnenburg, 2001). Through the use of phosphospecific antibodies, we monitored nPKC phosphorylation at the PDK site in *Aplysia* neurons and have found that it was unaffected by LY294002. Nor was PKC phosphorylation at the PDK site affected by the PKC inhibitor bisindolylmaleimide, although it did induce an increase in the amount of PKC in the pellet fraction.

Treatment of *Aplysia* ganglia with serotonin induces changes in neuronal excitability similar to those seen with behavioural training. This treatment was found to induce an increase in the amount of PKC Apl II on the membrane that is phosphorylated at the PDK site. This treatment is also known to result in an increase in autonomous kinase activity on the membrane, which is attributable to autonomous Apl II activity. We have found a strong correlation ($p>0.01$) between the amount of autonomous kinase activity measured and the relative phosphorylation of Apl II at the PDK site.

In addition, through immunocytochemistry, we determined that PDK which is phosphorylated at the PDK site was enriched in the plasma membrane. The level of phosphorylation of PKC was unaffected by a kinase-dead GFP-tagged PDK construct.

Phosphoinositide dependent kinase (PDK) phosphorylation of protein kinase C (PKC) Apl II increases during intermediate facilitation in *Aplysia*

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Running Title: **Phosphorylation of PKC at the PDK site in *Aplysia* neurons**

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Summary

Phosphorylation of protein kinase Cs (PKCs) by phosphoinositide-dependent kinase I (PDK) is critical for PKC activity. In the nervous system of the marine mollusk *Aplysia*, there are only two major PKC isoforms, the calcium-activated PKC Apl I and the calcium-independent PKC Apl II and both PKCs are persistently activated during intermediate memory. We monitored the PDK dependent phosphorylation of PKC Apl I and PKC Apl II using phosphopeptide antibodies. During persistent activation of PKCs in *Aplysia* neurons, there is a significant increase in the amount of PDK-phosphorylated PKC Apl II in the particulate fraction, but no increase in the amount of PKC Apl I phosphorylated by PDK. PDK phosphorylation of PKCs was not sensitive to inhibitors of PI-3 kinase, PKC, or expression of a kinase-inactive PDK. Localization of PDK phosphorylated PKC Apl II using immunocytochemistry revealed an enrichment of phosphorylated PKC Apl II at the plasma membrane. These data suggest that increased PDK phosphorylation of PKC Apl II is important for persistent kinase activation.

Introduction

In the marine mollusk, *Aplysia californica*, PKCs are important for both short- and intermediate-term changes in synaptic strength between sensory and motor neurons that accompanies behavioral sensitization (1). Following behavioral sensitization, or prolonged treatment of ganglia with serotonin (5-HT), the kinase activity in the particulate fraction of both Ca2+-activated conventional PKC Apl I and Ca2+-independent novel PKC Apl II are increased (2,3). Persistent PKC activity is required for the maintenance of synaptic facilitation under some conditions (4). The mechanisms for the persistent activation of the PKCs are not well defined. Both initial PKC activation and protein translation are required for persistent activation of the PKCs (2). However, there are differences in the persistent activation of the two isoforms. The increase in PKC Apl II activity appears to be mainly due to autonomous activation of the kinase, while the increase in PKC Apl I appears to be mainly due to increased levels of regulated PKC Apl I on the membrane (2,3). There is increased phosphorylation of PKC Apl I at a conserved autophosphorylation site during intermediate memory, however the phosphorylated kinase is located exclusively in the cytoplasm and presumably does not contribute to the increase in particulate kinase activity (5). Increased phosphorylation of PKC has been postulated to underlie persistent activation of PKCs during vertebrate learning models (6-8) and PKC activity is regulated by phosphorylation (9).

All PKCs require phosphorylation at the activation loop site by phosphoinositide-dependent kinase 1 (PDK) for full catalytic activity (10). Ca2+-activated PKCs are phosphorylated by PDK soon after translation (11-13). After PDK-dependent

phosphorylation, Ca2+-activated PKCs undergo two major autophosphorylations that are required for the stability and folding of the enzyme, a process termed maturation (9). Once these sites are phosphorylated, the PDK site can become de-phosphorylated without affecting the stability of the enzyme (12). Since PDK docks at a C-terminal site that is no longer easily accessible in the mature enzyme, classical PKCs are not easily re-phosphorylated (14). Thus, while over-expression of PDK or PDK dominant negatives affect the proportion of PKC found in the mature fraction, it does not affect the amount of mature PKC phosphorylated at the PDK site (12), presumably due to the lack of rephosphorylation.

PDK phosphorylation of novel PKCs has also been examined, although a detailed model for their phosphorylation has not been proposed. In contrast to Ca2+-activated PKCs (Sonnenburg et al., 2001), PDK phosphorylation of PKCδ is regulated both by PKC autophosphorylation and by PI-3 kinase activity (15). Regulation of phosphorylation of other kinases by PDK appears to be dependent on substrate conformation and subcellular localization as opposed to activation of PDK itself (16-18).

PDK has an N-terminal kinase domain and a C-terminal pleckstrin homology (PH) domain that binds phosphoinositides including phosphatidylinositol (3,4,5) triphosphate (PtdIns(3,4,5)P3) (19-21), although kinase activity does not require PtdIns(3,4,5)P3 binding (22). We have cloned PDK from *Aplysia* and both the kinase domain and the PH domain are highly homologous to both vertebrate and invertebrate PDKs, suggesting that this is a PDK orthologue (23)

To examine the role of PDK in the regulation of PKC in *Aplysia*, we raised phosphopeptide antibodies to the PDK site in the classical PKC Apl I and the novel PKC Apl II. We then used the antibodies to characterize PDK phosphorylation of PKCs in the *Aplysia* nervous system. Our results demonstrate differences in PDK regulation of classical and novel PKCs in the nervous system and suggest an important role for PDK phosphorylation of PKC Apl II during intermediate facilitation.

Experimental Procedures

Isolation of Nervous System. Aplysia californica (50-250 g) were obtained from Marine Specimens Unlimited at Pacific Palisades, California, and maintained in an aquarium for at least 3 days before experimentation. The animals were first placed in a bath of isotonic $MgCl_2$/artificial sea water (1:1, vol/vol) and then anesthetized by injection of isotonic $MgCl_2$. Pleural and pedal ganglia were isolated from the animal and pinned to silicone plastic in ice-cold dissecting medium (2,24). The ganglia were then desheathed in order to facilitate penetration of pharmacological agents and incubated in resting medium (2,24) for 3 h at 15°C to minimize effects of dissection. In some experiments bag cell clusters were isolated from abdominal ganglia and treated in a similar fashion.

Antibodies. Peptides containing the conserved PDK site in PKC Apl I and Apl II (Fig. 1) with the serine converted to phosphoserine were synthesized (Quality Controlled Biochemicals, Hopkington, MA). The peptides were conjugated to BSA maleimide (Pierce, Rockford IL) via the endogenous cysteine, and injected into rabbits three times at four week intervals. In order to reduce the fraction of antibody that might recognize the non-phosphorylated peptide, the serum from animals was passed over an affinity column of the non-phosphorylated form of the immunizing peptide coupled to Sulfolink (Pierce). This process was repeated twice. After each passage, specifically retained antibodies were eluted from the column. The final flow through was then passed over an affinity column of the immunizing phosphopeptide coupled to Sulfolink and retained antibodies were eluted and concentrated in a Centriplus-10. Antibodies to total PKC Apl I, total

PKC Apl II, and PDK have been described (23,25).

Experiments in SF9 Cells. High titer stocks of baculovirus encoding PDK, PDK (K-N) PKC Apl I, PKC Apl II and PKC Apl II K-R (23,26,27) were used at an MOI of 5 for each virus. Supernatant and membrane fractionation was as described (27).

GST pulldowns. The GST-PDK and GST-PDK K-N constructs have been described (23). We expressed these constructs in bacteria and purified the GST fusion proteins on glutathione beads. The beads were then incubated with supernatants of SF9 cell extracts either infected with baculovirus encoding PKC Apl I or PKC Apl II. The beads were incubated overnight and then washed three times with PBS. The beads were then eluted with sample buffer and separated on SDS-gels. For experiments in ganglia, 250 μg of soluble nervous system extract (5) was incubated for four hours with beads and then washed three times with homogenization buffer before elution of the beads with sample buffer.

Constructs. The fusion protein used to generate non-phosphorylated PKC Apl I was made by inserting a BamHI fragment of PKC Apl I into pMALC (New England Biolabs, Beverly, MA) cut with BamHI. The fusion protein starts with the sequence GSLSF from the hinge region and includes the native carboxyl-tail. The fusion protein used to generate non-phosphorylated PKC Apl II was made by first cutting out a fragment encoding the catalytic fragment from PKC Apl II with Nhe I and EcoR I. The ends of this fragment were filled in with Klenow and ligated to the pGEX3.1 vector that had been cut with

EcoRI and the ends filled in with Klenow. The sequence of PKC Apl II starts with ASNEH and includes the native carboxy-tail. The PDK and PDK (KN) constructs used to make baculovirus have been described (23). To generate an expression construct for *Aplysia* neurons we cut out PDK (K-N) from the BB4 vector with Xho and Kpn and inserted it into the GFP-PKC Apl II C2 pNEX-3 vector (27) cut with Xho and Kpn. This replaced the C2 domain of PKC with the PDK sequence to generate an EGFP-PDK fusion protein starting with SRPGG from *Aplysia* PDK. This removes the first 130 amino acids of *Aplysia* PDK; this sequence is not conserved over evolution and does not contain any region of the kinase or PH domain.

Experiments in Ganglia. The protocol for inducing intermediate facilitation was identical to that described (2,3,5). Autonomous kinase activity on the membranes was measured as described (3). Immunoprecipitations were done as described (25).

Immunoblotting. Western blots were performed as described (28) with the antibody to phosphorylated PKC Apl II at 1 μg/ml, the antibody to phosphorylated PKC Apl I at 1 μg/ml, the antibody to total PKC Apl I at 2.5 μg/ml, and the antibody to total PKC Apl II at 1 μg/ml. The phosphopeptide antibodies were pre-blocked with the non-phosphorylated peptide at a molar ratio of 50-1. Goat anti-rabbit, horseradish peroxidase-conjugated secondary antibody (Pierce, Rockford, IL) was used at 0.5 μg/ml. Results were visualized by enhanced chemiluminescence (Renaissance Plus, NEN-Dupont Boston, MA). Immunoblots 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/). We calibrated our data with the uncalibrated OD feature of NIH image which transforms the data using the formula [y=log10(255/(255-x))] where x is the pixel value (0-254). Control experiments demonstrated that after this calibration, values were linear with respect to amount of protein over a wide range of values.

Quantitation of Immunoblots For each blot probed with both phosphopeptide specific and total antibodies a phospho-ratio was calculated (immunoreactivity of anti-phosphopeptide antibody compared to immunoreactivity of total antibody). This ratio, while not comparable between different blots, was useful for determining differences in phospho-ratio between supernatant and pellet, or between treatments. When examining treatments, (e.g. 5-HT, Bis, etc.), the percentage change was calculated (((experimental phospho-ratio - control phospho-ratio)/control phosphoratio)*100). When comparing supernatant to pellet a simple ratio was calculated (pellet phosphoratio/supernatant phospho-ratio). Statistical tests involved paired two-tailed Student's-t tests between control and experimental conditions or when appropriate unpaired two-tailed Student's t-tests. To determine if the phospho-ratio for pellet/supernatant was different than 1, a one sample Student's t-test was performed. To correlate phospho-ratio and autonomous activity, for each blot/kinase assay the maximum value for the phospho-ratio or autonomous activity was set to 1 and other values calculated relative to the maximum value. This allows comparison of multiple experiments on the same axis.

Injection of sensory neurons with GFP-PDK(K-N). The protocol was identical to that previously described (27) with the following modification. Cells were plated on poly L-lysine plates with 20% hemolymph to increase process outgrowth. Cells were left for three to five days to allow adequate expression before fixation.

Immunocytochemistry Cultures were rinsed with artificial seawater, and then fixed for 30 minutes in 4% paraformaldehyde in phosphate-buffered saline (PBS) with 30% sucrose. Cells were then permeabilized in 0.1% Triton X-100 in PBS with 30% sucrose for 10 minutes, and washed three times with PBS. Free aldehydes were quenched with a 15 minute incubation in 50 mM ammonium chloride in PBS. Non-specific antibody binding was inhibited by incubating with a blocking solution (10% Normal Goat Serum, 0.5% Triton X-100, in PBS) for 1 hour. Cultures were incubated overnight with the primary antibody. For the chicken/rabbit PKC co-staining,1:600 chicken anti-PKC and 1:1000 rabbit anti-PKC in blocking solution was used. The cultures were washed 4 x 10 minutes with PBS, and were then incubated with secondary antibody in blocking solution (1:150 CY2 anti-chicken, 1:400 CY3 anti-rabbit) for 1 to 2 hours. For all other experiments, the rabbit phosphopeptide antibody was preabsorbed using the nonphosphorylated form of the peptide at 1 μg/μl for 30 min prior to use, and then was added to the cultures in a 1:475 dilution in blocking solution. Cultures were washed 4 x 10 minutes each in PBS, and then incubated with secondary antibody in blocking solution (1:400 CY3 anti-rabbit, 1:200 CY5 anti-chicken) for 1 to 2 hours. Finally, the cells were washed 4 x 10 minutes with PBS, and the coverslips were mounted onto slides, using Mowiol as the mounting medium. Cultures were viewed on a Zeiss LSM 510 with Axiovert 200M inverted

microscope.

Quantitation of Immunocytochemistry. All quantitation was done on LSM 510 software. The mean pixel intensity of boxed plasma membrane areas (2-3/cell) and boxed cytoplasmic areas (2-3/cell) were quantitated. *Aplysia* neurons have highly infolded membranes due to their large size and thus, even in a confocal section (approximately 15 um) the plasma membrane is not well defined. We used a box (approx. 1-2 um wide) as a rough definition of plasma-membrane associated area. Efforts were made to use confocal images for quantitation from the mid-point of cells for standardizing between different cells. Occasionally, pigment granules could be seen in the sensory neuron cell bodies that were visible using the secondary antibody used for the total PKC Apl II antibody (CY5). Areas including these granules were not used in the quantitation. For each cell the average pixel intensity was calculated for cytosol and membrane areas for both total and anti-phosphopeptide antibodies. A phospho-ratio was then calculated for supernatant and membrane and then the membrane/cytosol phosphate ratio for each cell was calculated. To examine the effect of cells injected with GFP-PDK (K-N), we compared the average of the mean cytosolic intensity, the mean membrane intensity and the phospho-ratio for the expressing cells and non-expressing cells on the same cover slip to calculate an effect of GFP-PDK (K-N) for each experiment.

Results:

Characterization of phospho-specific antibodies to the PDK site.

The threonine that is phosphorylated by PDK in vertebrate PKCs is well conserved in the *Aplysia* PKCs (Fig. 1A). We thus raised antibodies to phosphopeptides containing phosphothreonine in this position for both PKC Apl I and PKC Apl II. We biased the peptides used for immunization to the divergent region N-terminal of the PDK site in order to make antibodies that are specific for individual PKC isoforms and which do not recognize other PDK-phosphorylated proteins (Fig. 1A). The resulting antibodies were highly specific for PKC recognizing single bands from nervous system extracts that co-migrated with the bands recognized by our well characterized antibodies to total PKC Apl I or PKC Apl II (2,25) (Fig. 1B). To evaluate the specificity of the antibodies for phosphorylated PKC, we blotted bacterially expressed PKC catalytic domain as a control since there is no PDK in bacteria and thus, the bacterially expressed PKC will not be phosphorylated at the PDK site. This demonstrated that the antibodies are highly specific for phosphorylated PKCs as they do not react with PKC expressed in bacteria (Fig. 1B). The bands recognized by the phosphopeptide antibodies are solely due to immunoreactivity to PKC as they can be completely immunoprecipitated by antibodies to total PKC (Fig. 1C).

A kinase dead PKC has less stable phosphorylation at the PDK site

Phosphorylation at the PDK-site is not due to autophosphorylation, since a kinase-inactive PKC (27) was well phosphorylated at the PDK site when expressed in SF9 cells (Fig. 1D). However, the overall level of phosphorylation at the PDK site in the kinase

inactive PKC was less than that for the wild-type kinase expressed in SF9 cells (Fig. 1D). This decrease may be due to increased de-phosphorylation of the kinase inactive PKC Apl II. Indeed, phosphorylation at the PDK site was much more sensitive to phosphatases in the homogenization buffer for the kinase inactive PKC Apl II K-R than for wild-type PKC Apl II (Fig. 1E; quantitated in Fig. 1F). This is consistent with previous evidence from classical PKCs that PKC autophosphorylation is required to cause a conformational change that protects the PDK site from de-phosphorylation (11).

PDK binding to PKCs

In many cases PDK binds to its substrates. To evaluate whether PDK could bind to PKC Apl II we first examined whether GST-PDK could pull-down SF9 cell expressed PKCs. PKC Apl II was pulled down by the GST-PDK fusion protein, but were not pulled down by GST alone (Fig. 2). Interestingly, the PKC phosphorylated at the PDK site was not bound as well as unphosphorylated PKC (Fig. 2A; quantitated in Fig 2B). Similar results have been observed for PKC βII where PDK immunoprecipitated de-phosphorylated PKC better than phosphorylated PKC (14). One explanation for this result is that PDK binds to the carboxy-terminal region of PKCs and that this binding site is not accessible in the mature folded form of the kinase (14). Thus, a higher proportion of PKC Apl II that was unphosphorylated might be in an immature, unfolded state and so more likely to have its carboxy-terminal region accessible for binding to PDK. Interestingly, when we repeated these experiments using endogenous PKC Apl II from the nervous system the proportion of phosphorylated and total PKC Apl II bound to GST-PDK was similar (Fig 2C; quantitated in Fig 2D). This may be due to the much lower proportion of immature

unfolded kinase in the mature nervous system, compared to when PKC Apl II is overexpressed in the baculovirus system.

PDK phosphorylation of PKC Apl II, but not Apl I during intermediate facilitation

The mechanism underlying persistent activation of PKCs is not known, but increased phosphorylation of PKCs by PDK is one possibility. We thus examined whether PDK phosphorylation of PKCs was modulated during the persistent activation of the PKCs stimulated by prolonged treatment with the facilitating transmitter, 5-HT (Sossin et al, 1994). Indeed, there was a significant increase in the percentage of PKC Apl II phosphorylated at the PDK site in the pellet fraction two hours following a 90 minute treatment of *Aplysia* ganglia with 5-HT (Fig. 3A, B; $p < 0.01$). There was no change in the percentage of soluble PKC Apl II phosphorylated at the PDK site or in the percentage of soluble or particulate PKC Apl I phosphorylated at the PDK site(Fig. 3A, 3B). As observed previously (2), there was a significant increase in the percentage of PKC Apl I in the particulate fraction (Fig. 3A, C; $p < 0.02$). While there was no significant difference in the percentage of total PKC Apl II in the particulate fraction, the percentage of PDK-phosphorylated PKC Apl II in the particulate fraction did increase (Fig. 3A, 3C).

In control ganglia, PKC Apl II that was not phosphorylated by PDK was mainly in the pellet fraction (i.e. the ratio of PDK phosphorylation was higher in the supernatant) (Fig. 3D). After 5- HT treatment, however, there was a shift in this ratio towards PDK phosphorylation in the pellet (Fig. 3C, D). In contrast, PKC Apl I had approximately equal phosphorylation at the PDK site in supernatant and pellet before and after 5-HT

treatment (Fig. 3C, D).

PDK phosphorylation is correlated with autonomous enzymatic activity

Autonomous kinase activity is also increased by 5-HT during intermediate facilitation and this activity derives from PKC Apl II (3). In a separate series of experiments, we determined both the ratio of phosphorylation of PKC Apl II at the PDK site and autonomous kinase activity from the pellets of ganglia treated with 5-HT (Fig. 4). There was a very good positive correlation between the relative levels of autonomous activity and the relative phosphoratio ($r2=0.58$, $p<0.01$, $n=16$). This result suggests a relationship between PDK phosphorylation and autonomous activity of PKC Apl II.

Neither inhibitors of PKC nor inhibitors of PI-3 kinase affect PDK phosphorylation

To explore mechanisms that regulate the ability of PDK to phosphorylate PKC in the *Aplysia* nervous system, we examined whether PDK phosphorylation of PKC was regulated by either PKC activity or by PI-3 kinase activity as has been noted for Ca2+-independent PKCs in vertebrates (15). Neither inhibitors of PKC nor PI-3 kinase affected the level of phosphorylation at the PDK site (Fig. 5). Inhibitors of PKC phosphorylation did lead to an increased level of PKC on the membrane for both PKC Apl I and PKC Apl II consistent with PKC activity being required for the release of PKC from the particulate fraction (5,29,30) (Fig. 5 A, B). We also examined whether insulin, which increases the activity and amount of PKC Apl II on membranes of bag cell neurons in a wortmannin-sensitive manner (31), led to increases in PDK phosphorylation of PKC Apl II. No significant changes were seen in the levels of PKC Apl II phosphorylated at the PDK site

after insulin treatment (12 +/- 20%; change in phospho-ratio of pellet fraction by insulin, n=7, S.E.M, p>0.5, two-tailed students t-test).

Immunocytochemical localization of phospho-PKC Apl II.

The antibodies to phosphorylated PKC also worked well in immunocytochemistry of cultured *Aplysia* neurons. To compare the localization of phosphorylated and non-phosphorylated PKC, we raised an antibody to total PKC Apl II in chickens. This antibody had similar specificity as the previously generated rabbit antibody raised to the same epitope and was indistinguishable in immunocytochemistry (Fig. 6A-B). Using the chicken antibody, we were able to label PKC Apl II phosphorylated at the PDK site and total PKC Apl II in the same cells. Comparing the two staining patterns revealed a significant increase in the relative immunoreactivity of phospho-PKC Apl II on or near the plasma membrane of cell bodies (see Fig. 6 C, D; quantitated in Fig.6G). No difference between the antibodies was seen in axons, varicosities or growth cones (Fig. 6E-F). To determine if we could control phosphorylation of PKC by PDKs using a dominant-negative strategy, we examined the ratio of staining for phospho-PKC and total PKC in neurons that were injected with a plasmid encoding a kinase inactive PDK. The PDK was amino-terminally tagged with GFP to monitor its level of expression. There was no significant change in the ratio of PDKphosphorylated PKC Apl II to total PKC Apl II in the cytoplasm or membrane-associated regions between cells expressing or not expressing the catalytically inactive PDK (Fig. 7). Thus, at the levels of expression we can attain in neurons, kinase inactive PDK does not appear to act as a dominant negative to block PKC phosphorylation by PDK.

Discussion

We generated phosphopeptide antibodies to the conserved PDK site in PKC Apl I and PKC Apl II. These antibodies are specific for PKCs phosphorylated at the PDK site as they do not recognize PKCs expressed in bacteria (where no PDK-like activity is present). Furthermore the bands recognized by the phosphopeptide antibodies can be completely immunoprecipitated by other antibodies to PKC. The major result from this study is that phosphorylation at the PDK site of PKC Apl II is increased after prolonged 5-HT treatment and is correlated with autonomous PKC Apl II activity. In contrast, the increased activation of PKC Apl I seen at this time is not correlated with increased phosphorylation at the PDK site. We also localized PDK phosphorylated PKC Apl II using immunocytochemistry and documented an enrichment of phosphorylated PKC Apl II at or near the plasma membrane.

Phosphorylation of Ca2+-independent or novel PKCs at the PDK site appear to be more regulated than that of conventional PKCs. In vitro phosphorylation of the novel PKCδ by PDK requires PtdIns(3,4,5)P3 and phorbol esters (15) while in-vitro phosphorylation of Ca2+-activated PKCs does not (12). Initial phosphorylation of PKCδ in vitro may resemble re-phosphorylation of conventional PKCs since PKCδis partially active when expressed in bacteria even though the PDK site is not phosphorylated (15). Thus, PKCδcan partially fold even in the absence of PDK phosphorylation (32). PKC Apl II resembles PKCδ more than PKCδ, however there has been little characterization of PKCδ phosphorylation by PDK. In contrast to PKCδ, there is a high basal level of PKC Apl II phosphorylation at the PDK site and this basal level was not sensitive to PI-3 kinase

inhibitors or PKC inhibitors. Thus, it is possible that phosphorylation of PKC Apl II at the PDK site may more closely resemble the constitutive phosphorylation of conventional PKCs (15). An important caveat is that we are examining PKC in primary nervous system tissue where there is probably considerable activation of most signal transduction pathways during the period of time before we gain experimental control of the ganglia. Thus, our results show that continued presence of phosphorylation of PKC at the PDK site does not require PI-3 kinase or PKC activity, but our results do not address how the initial phosphorylation of PKC Apl II at the PDK site is regulated in the nervous system.

PDK phosphorylation of PKC Apl II does appear to be more regulated than PDK phosphorylation of PKC Apl I. First, PKC Apl II that is not phosphorylated at the PDK site has a distinct subcellular localization. Using biochemical techniques, it is enriched in the pellet fraction (Fig. 3D) and using immunocytochemical techniques it is partially excluded from the plasma membrane (Fig. 6). These results suggest that either de-phosphorylation of PKC Apl II at the PDK site affects PKC localization, or that a substantial pool of endogenous PKC Apl II is not phosphorylated at the PDK site during maturation. This appears distinct from PKC Apl I where PKC Apl I is not differentially found in the pellet based on its phosphorylation at the PDK site (Fig. 3D). Second, there is increased phosphorylation of PKC Apl II, but not PKC Apl I in the pellet fraction during intermediate facilitation (Fig. 3B). There was no significant change in levels of total PKC Apl II in the pellet, suggesting that this increase was due to increased phosphorylation of the PDK site. The mechanisms that regulate this phosphorylation are unclear. Notably, a percentage of particulate PKC Apl II is autonomously active at this

time (3), and phosphorylation at the PDK site may be enhanced by the absence of a pseudosubstrate in the active loop. This idea is supported by the strong correlation between autonomous kinase activity and the percentage of PKC phosphorylated at the PDK site (Fig. 4). At this point it is not clear if the increased PDK phosphorylation is important in causing autonomous activation of the kinase, or a consequence of the autonomous activation. Unfortunately, since the kinase-dead PDK did not act as a dominant negative and since there are no specific inhibitors of PDK, we can not at this time determine if PDK is required for activation of PKC during intermediate facilitation.

Using immunocytochemistry we found that PKC Apl II phosphorylated at the PDK site is enriched near or on the plasma membrane. This suggests that the non-phosphorylated pool of PKC is excluded from the plasma membrane. This is apparently inconsistent with our finding that there is a pool of particulate PKC that is not phosphorylated (Fig. 3D). The particulate pool of PKC not phosphorylated at the PDK site may be associated with the cytoskeleton or an internal membrane fraction and thus may be measured in our immunocytochemical measurements as cytosolic. Indeed, a large percentage of particulate PKC Apl II in *Aplysia* is triton-insoluble (33).

While PDK phosphorylation of PKCs has been well studied in vitro and in cell lines, this is the first characterization of PDK phosphorylation in neurons. There were important differences in the PDK phosphorylation of the classical PKC Apl I and the novel PKC Apl II in *Aplysia* neurons. These differences could be explained by postulating that classical PKCs are only phosphorylated at the PDK site during initial formation of the

kinase, while novel PKCs may be regulated by re-phosphorylation of this site during the kinase life cycle. Phorbol esters stimulate de-phosphorylation of classical PKCs that directly leads to degradation of the kinase (34). In contrast, novel PKCs may be de-phosphorylated and stored as an inactive pool of enzyme that can be re-activated at a later time. Indeed, novel PKCs are less down-regulated by phorbol esters in many cell types (35-39), including *Aplysia* neurons (40)

This is also the first time PDK phosphorylation has been imaged by immunocytochemistry. The relative increase in staining at membranes for PKC Apl II phosphorylated at the PDK site would be consistent with increased PDK phosphorylation of activated PKC. PDK phosphorylation of PKC Apl II may play a pivotal role in its activation during intermediate facilitation. The activation of PKC Apl II at this time requires translation (3). Understanding what regulates PDK phosphorylation should provide clues to the important proteins whose translation needs to be regulated to persistently activate PKC Apl II during intermediate facilitation.

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Abbreviations: protein kinase C (PKC); phosphoinositide-dependent protein kinase (PDK); phosphatidylinositol (3,4,5) triphosphate (PtdIns(3,4,5)P3); serotonin or 5-hydroxytryptamine (5-HT); phosphate buffered saline (PBS), Bisindolylmaleimide (Bis); LY294002 (LY); green fluorescent protein (GFP); glutathione-S-transferase (GST); maltose binding protein (MBP).

Figure Legends

Figure 1. Characterization of phosphopeptide antibodies. A) Sequences of the PDK phosphorylation site for the *Aplysia* PKCs and their closest vertebrate homologue. The peptide used to raise the antibodies is in bold (pT stands for phosphothreonine). Note that while the sequence carboxy-terminal to the PDK site is absolutely conserved, the amino-terminal sequence is unique for each isoform. B) Bacterially expressed GST-PKC Apl II catalytic subunit fusion protein or MBP-Apl I catalytic subunit fusion protein and nervous system extracts were separated on a 10% SDS-polyacrylamide gel, transferred to nitrocellulose and probed with either the phosphopeptide antibody to the PDK site in PKC Apl II (Anti-Thr 561-P) or the phosphopeptide antibody to the PDK site in PKC Apl I (anti-Thr 478). The blots were then stripped and re-probed with either antibodies to the catalytic subunit of PKC Apl II or PKC Apl I. C) Immunoprecipitation of phosphopeptide immunoreactivity. Aplysia soluble nervous system extract (350 μg/tube) were immunoprecipitated with 20 μl of serum (preimmune or the specific antibodies to PKC Apl I and PKC Apl II (25). The immuoprecipitates and voids (1/20 of total) were separated on 9% SDS-polyacrylamide gels, transferred to nitrocellulose and probed with either the phosphopeptide antibody to the PDK site in PKC Apl II (Anti-Thr 561-P) or the phosphopeptide antibody to the PDK site in PKC Apl I (anti-Thr 478). The blots were then stripped and re-probed with either antibodies to the catalytic subunit of PKC Apl II or PKC Apl I. D) Comparison of level of phosphorylaytion and of total protein of PKC Apl II and PKC Apl II (K-R). E) Comparison of PKC Apl II and PKC Apl II (K-R) either using normal procedures (+), or homogenizing in the absence of phosphatase inhibitors (-). F) Percent change in PDK phosphorylation of PKC Apl II or PKC Apl II

(K-R) when homogenized in the absence of phosphatase inhibitors in the homogenization solution. (n=5, *, $p<0.01$ two-tailed Student's t-test between PKC Apl II and PKC Apl II K-R). Error bars are S.E.M.

Fig. 2. PDK binds to PKC Apl II. A) SF9 cell cytosolic extracts from cells expressing PKC Apl II were incubated with glutathione beads and GST or GST-PDK. The beads were washed and then eluted with sample buffer. Ten percent of the initial homogenate (Start), 10% of the unbound material (void) and the eluates from the GST-PDK beads and the GST beads were separated on a 9% SDS-polyacrylamide gel, transferred to nitrocellulose and first Ponceau-S stained to visualize the levels of GST fusion proteins. These are the single bands seen on the blots since the GST fusion proteins are added in excess. Next the blot was probed with the antibody to the PDK site in PKC Apl II (Anti-Thr 561-P), stripped, and re-probed with the total antibody to PKC Apl II. B) The percentage of the total PKC Apl II and the percentage of the PKC Apl II phosphorylated at the PDK site (Apl II T-561-P) bound to GST-PDK beads was quantitated. (N=8, $p<0.01$ two tailed Student's paired t-test between PKC Apl II and PKC Apl II T-561-P). Error bars are S.E.M. C) Nervous system soluble extracts (250 μg) were incubated with glutathione beads and GST or GST-PDK. The beads were washed and then eluted with sample buffer. Five percent of the unbound material (void) and the eluate from the GST-PDK beads and the GST beads were transferred to nitrocellulose and probed with the antibody to the PDK site in PKC Apl II (Anti-Thr 561-P), stripped, and re-probed with the total antibody to PKC Apl II. D) The percentage of the total PKC Apl II and the percentage of the PKC Apl II phosphorylated at the PDK site (Apl II T-

561-P) bound to GST-PDK beads was quantitated. (N=4, $p>0.5$ two tailed Student's paired t-test between PKC Apl II and PKC Apl II T-561-P). Error bars are S.E.M).

Fig. 3. PDK phosphorylation by facilitating stimuli. A) Paired pedal-pleural ganglia were treated with resting medium (-) or with 20 μM 5-HT (+) for 90 min, washed with resting medium and then incubated in resting medium for 2 hours. Ganglia were homogenized, separated into supernatant and pellet fractions, separated on 9% SDS-PAGE gels, transferred to nitrocellulose and probed with the phosphopeptide antibody to PKC Apl I (anti-Thr478-P). The blots were stripped and re-probed with the phosphopeptide antibody to PKC Apl II (Anti-Thr-561-P), stripped again and re-probed with the antibodies to total PKC Apl I and total PKC Apl II simultaneously. Samples of GST-Apl II catalytic subunit and MBP-Apl I catalytic unit were also separated on the gel to ensure the phospho-specificity of the antibodies. Results from two separate animals are shown (a and b). B) The phospho-ratio (See methods) was calculated for the supernatant, pellet and total fractions for PKC Apl II and PKC Apl I. The percentage change in this ratio by 5-HT was then measured (n=12, *, $p<0.05$ students paired t-test between phosphoratio in control and 5-HT treated ganglia). C) The proportion of kinase found on the pellet was calculated for each antibody. Then, the percentage change in this proportion caused by 5-HT was calculated. (n=12, *, $p<0.05$, Student's paired t-test between proportions on pellet in control and proportions on pellet in 5-HT). D) The phospho-ratio in supernatant and pellet was compared (Pellet/Sup) for PKC Apl II and PKC Apl I, before and after 5-HT. (n-12, *, $p<0.05$ one sided t-test compared to value of 1, **, $p<0.05$, Students paired t-test between control and 5-HT treated ganglia). Error bars are S.E.M.

Figure 4. Correlation of autonomous kinase activity and phosphorylation at the PDK site. Pleural-pedal ganglia treated with control or serotonin were assayed for both autonomous activity and phosphorylation at the PDK site. Autonomous activity (pmol/min/mg) and phosphoratio were first calculated. For each experiment, the maximum value for each measure was set to 1 and the relative values or autonomous activity and phospho-ratio calculated. Results are from two separate experiments, n=8/experiment). The correlation between the two values is highly significant ($r2=0.58$, $p<0.01$).

Figure 5. Effects of inhibitors on basal PDK phosphorylation of PKC site. A) Ganglia were treated with either resting medium or resting medium + 1 μM Bisindolylmaleimide (Bis) for 1 hour and then homogenized and separated into supernatant and pellet fractions. These were separated on SDS-polyacrylamide gels and blotted with the antibody to the phosphopeptide antibody to the PDK site of PKC Apl II (anti-Thr 561-P). The blot was stripped and blotted with the antibody to total PKC Apl II. B) The blot was stripped and blotted with the antibody to the phosphopeptide antibody to the PDK site of PKC Apl I (anti-Thr 478-P) and then stripped and blotted with the antibody to total PKC Apl I. C) The effect of Bis on the total phospho-ratio was determined. D) The proportion of kinase found on the membrane was calculated for each probed antibody and then the percentage change in this proportion caused by 5-HT was calculated (n=4,*, $p<0.05$ two-tailed Students paired t-test between control and Bis-treated ganglia). E-H; same as A-D but with 1 μM LY294002 instead on Bis. Error bars are S.E.M.

Figure 6. Immunocytochemistry of PKC phosphorylated at the PDK site. A cultured *Aplysia* neuron double stained with an antibody to PKC Apl II raised in rabbits (A) and an antibody to PKC Apl II raised in chickens (B). A cultured *Aplysia* sensory neuron double stained with a phosphopeptide antibody to the PDK site of PKC Apl II raised in rabbits (C) or the total antibody to PKC Apl II raised in chickens (D). An axon and giant growth cone of an *Aplysia* neuron double stained with a phosphopeptide antibody to the PDK site of PKC Apl II raised in rabbits (E) or the total antibody to PKC Apl II raised in chickens (F). N represents the nucleus, GC represents a growth cone, scale bars are all 10 □M. G) Quantitation of the membrane/cytosol phospho ratio (see methods). * $p<0.05$, one-sample t-test against 1, n=12. Error bars are S.E.M.

Figure 7. Effect of PDK (K-N) expression in *Aplysia* neurons. A cultured sensory neuron was injected with a plasmid encoding PDK (K-N) tagged with GFP at its 5' end (arrow). This neuron was adjoined by a neuron that was not expressing PDK (K-N) (arrowhead). The neurons were double-stained with a phosphopeptide antibody to the PDK site of PKC Apl II raised in rabbits (A) or the total antibody to PKC Apl II raised in chickens (B). The GFP signal was seen only in the injected neuron (C). D) Quantitation of the membrane/cytosol phospho-ratio of cells either expressing (GFP-PDK K-N) or not expressing (Control, same as Fig. 5G) * $p<0.05$, students one-sample t-test against 100%, n=12. Both groups had a membrane/cytosol ratio significantly different than 1, but there was no effect of expressing GFP-PDK (K-N). E) In each experiment (n=3) the percentage change of the average phospho-ratio for all the GFP expressing cells (n=1-4) to the average phospho-ratio for all the non-expressing cells (n=2-7) was calculated for both the

cytosol and the membrane regions. Scale bars are 10 μM. Error bars are S.E.M.

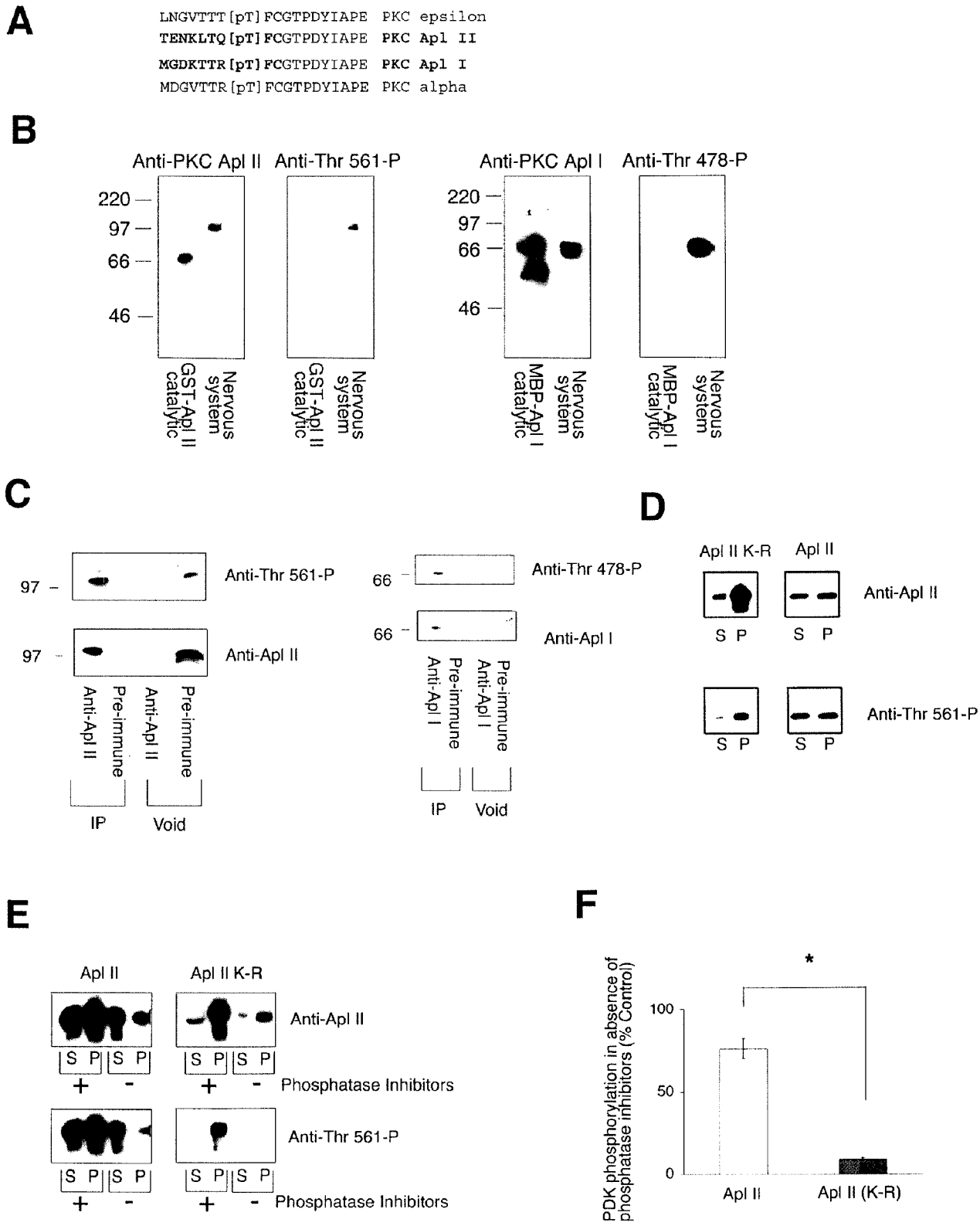

A
LNGVTTT[pT]FCGTPDYIAPE PKC epsilon
TENKLTQ[pT]FCGTPDYIAPE PKC Apl II
MGDKTTR[pT]FCGTPDYIAPE PKC Apl I
MDGVTTR[pT]FCGTPDYIAPE PKC alpha
B
Anti-PKC Apl II
Anti-Thr 561-P
Anti-PKC Apl I
Anti-Thr 478-P
220
97
66
46
GST-Apl II catalytic
Nervous system
MBP-Apl I catalytic
C
Anti-Thr 561-P
Anti-Apl II
Anti-Thr 478-P
Anti-Apl I
Pre-immune
IP
Void
D
Apl II K-R
Apl II
S P
E
Phosphatase Inhibitors
F
PDK phosphorylation in absence of phosphatase inhibitors (% Control)
100
50
0
*
Apl II
Apl II (K-R)

Figure 1

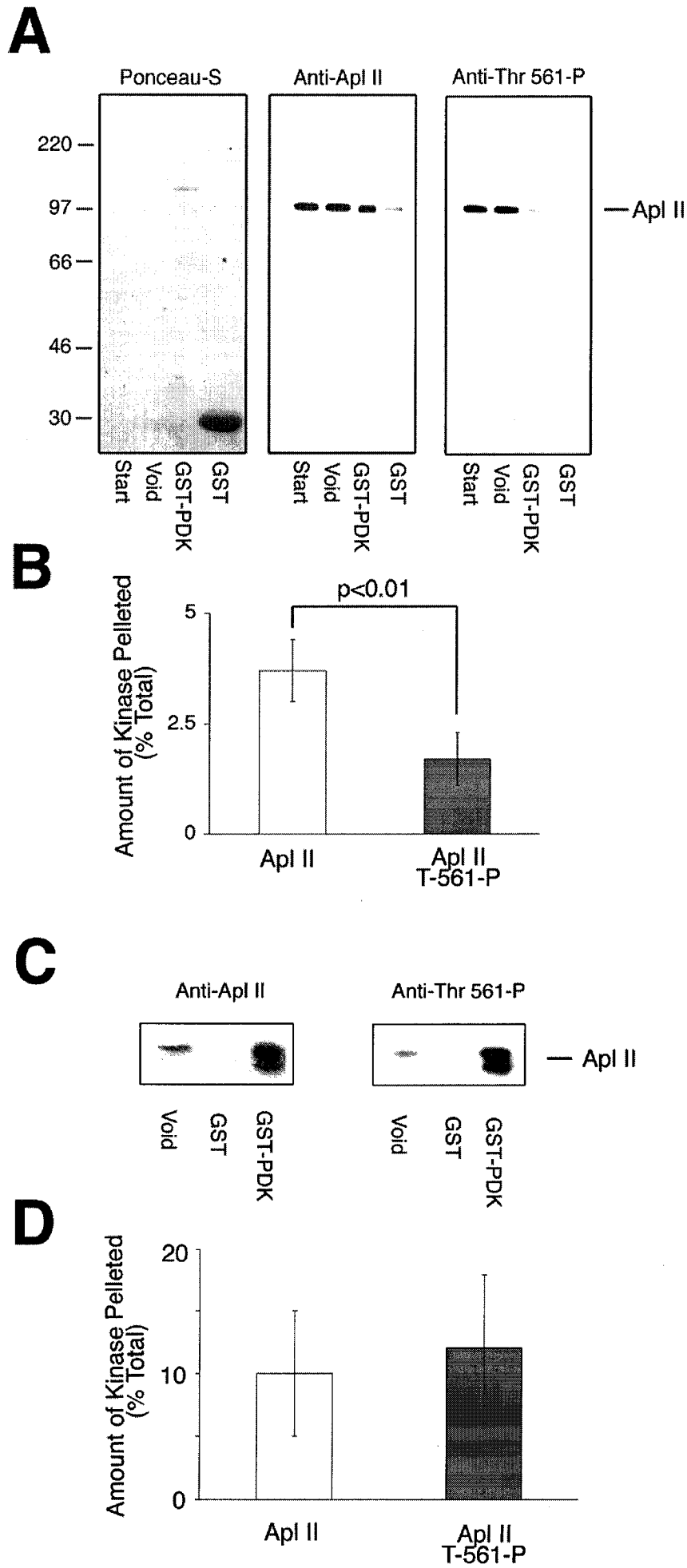

A
Ponceau-S
Anti-Apl II
Anti-Thr 561-P
220
97
66
46
30
Apl II
Start
Void
GST-PDK
GST
B
p<0.01
Amount of Kinase Pelleted (% Total)
5
2.5
0
Apl II
Apl II T-561-P
C
Anti-Apl II
Anti-Thr 561-P
Apl II
Void
GST
GST-PDK
D
Amount of Kinase Pelleted (% Total)
20
10
0
Apl II
Apl II T-561-P

Figure 2

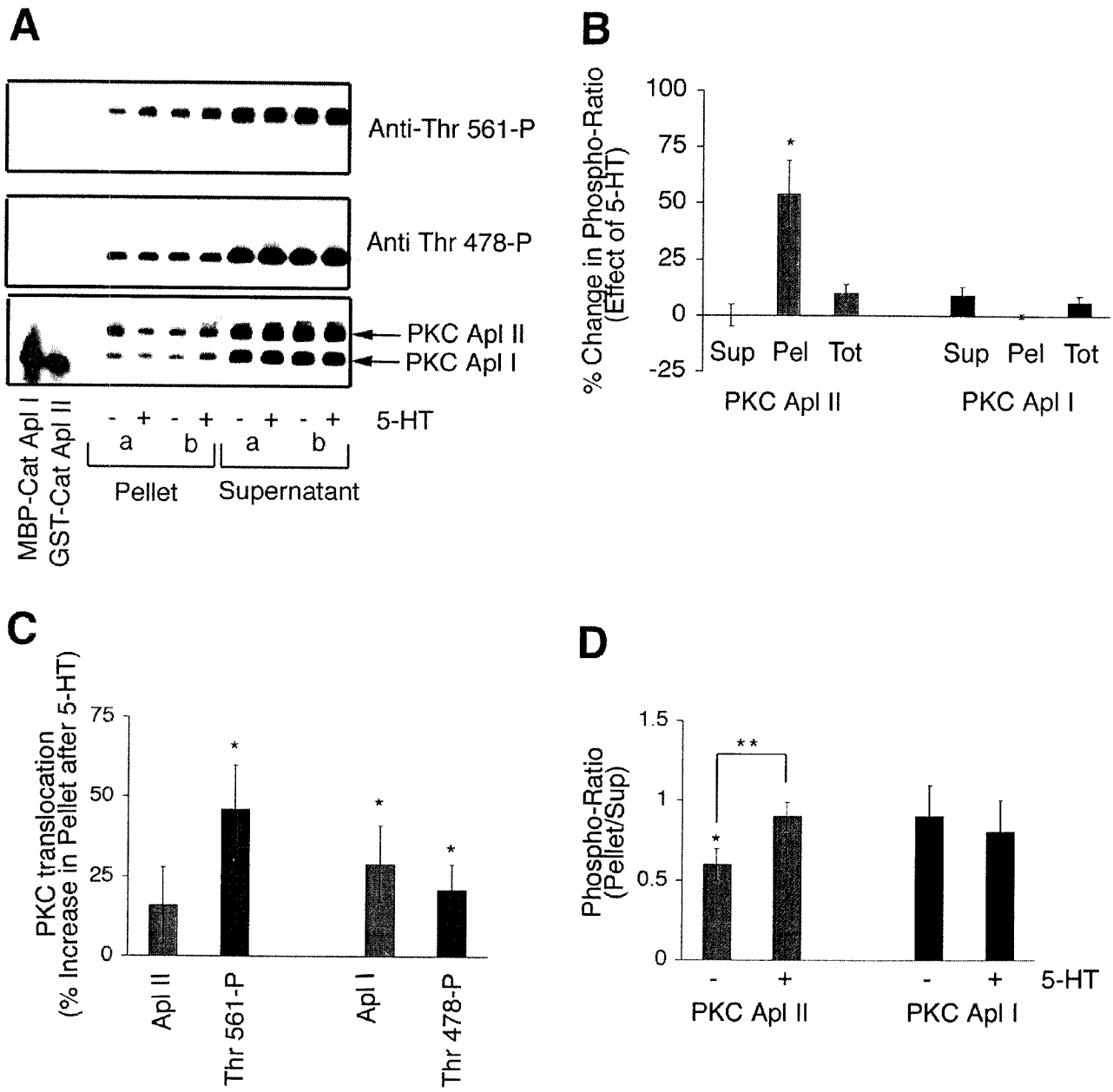

A
Anti-Thr 561-P
Anti Thr 478-P
PKC Apl II
PKC Apl I
MBP-Cat Apl I
GST-Cat Apl II
- + - + - + - + 5-HT
a b a b
Pellet Supernatant
B
% Change in Phospho-Ratio (Effect of 5-HT)
100
75
50
25
0
-25
Sup Pel Tot Sup Pel Tot
PKC Apl II
PKC Apl I
C
PKC translocation (% Increase in Pellet after 5-HT)
75
50
25
0
Apl II
Thr 561-P
Apl I
Thr 478-P
D
Phospho-Ratio (Pellet/Sup)
1.5
1
0.5
0
- + - + 5-HT
PKC Apl II
PKC Apl I

Figure 3

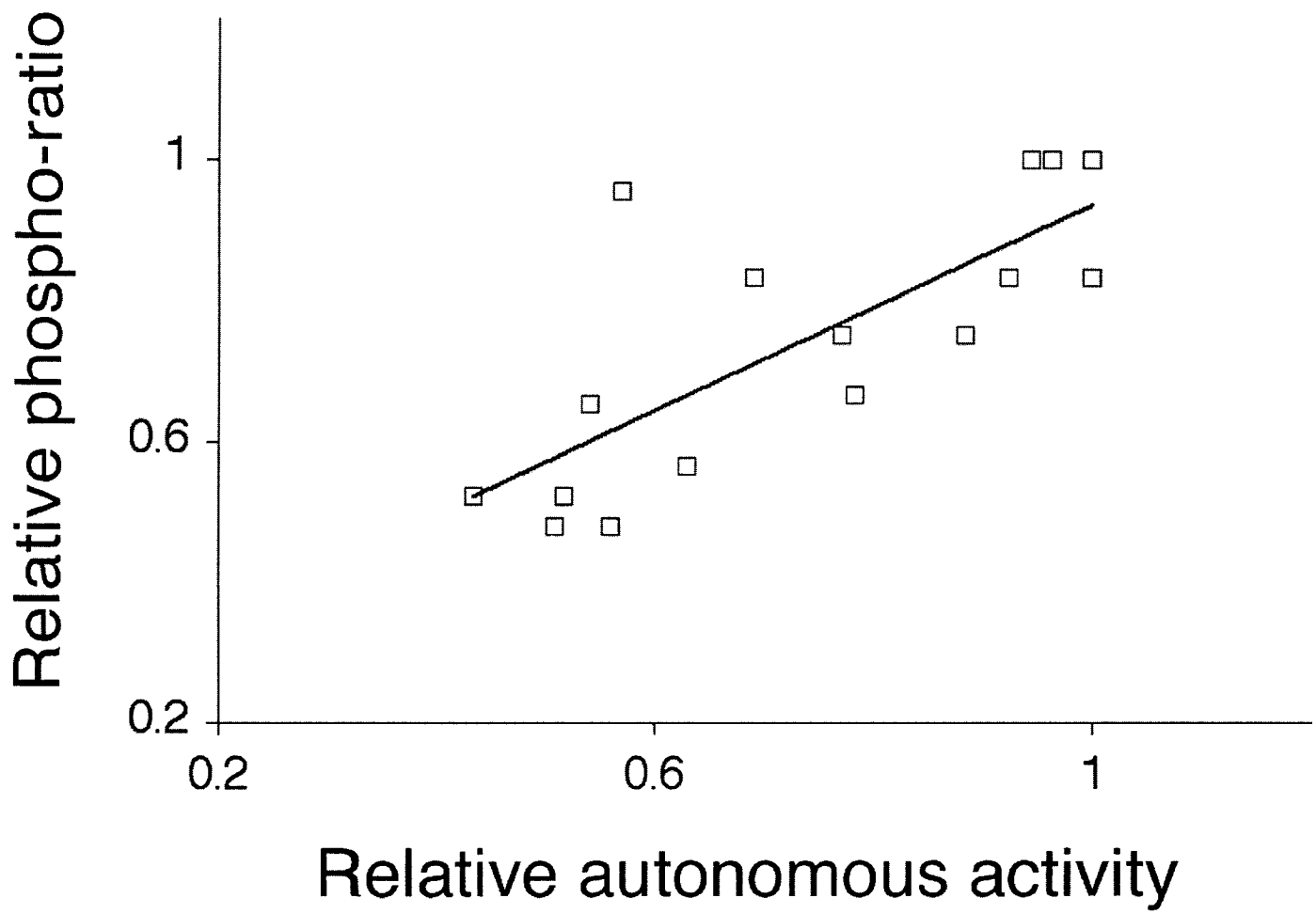

Relative phospho-ratio
1
0.6
0.2
0.2
0.6
1
Relative autonomous activity

Figure 4

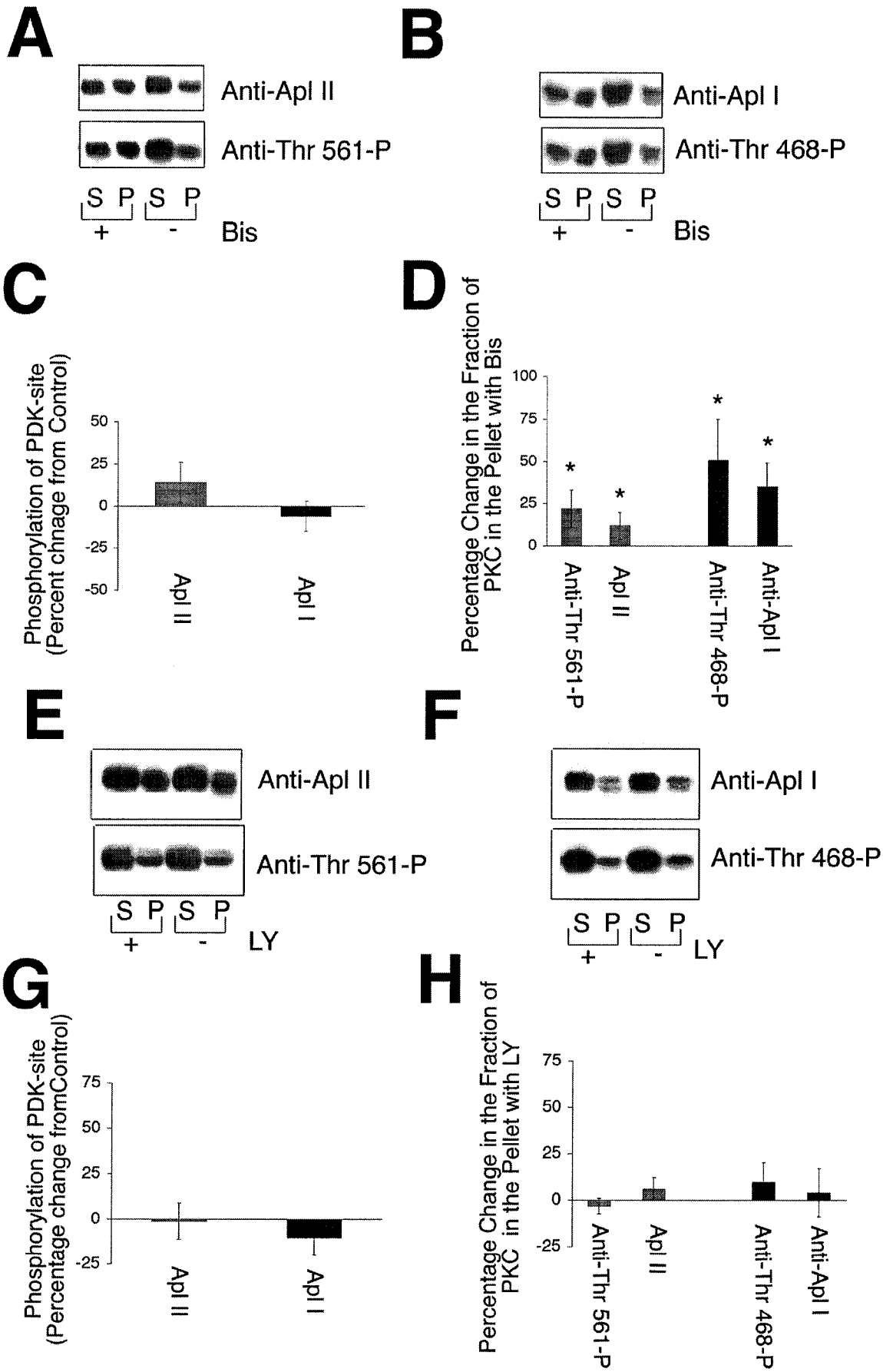
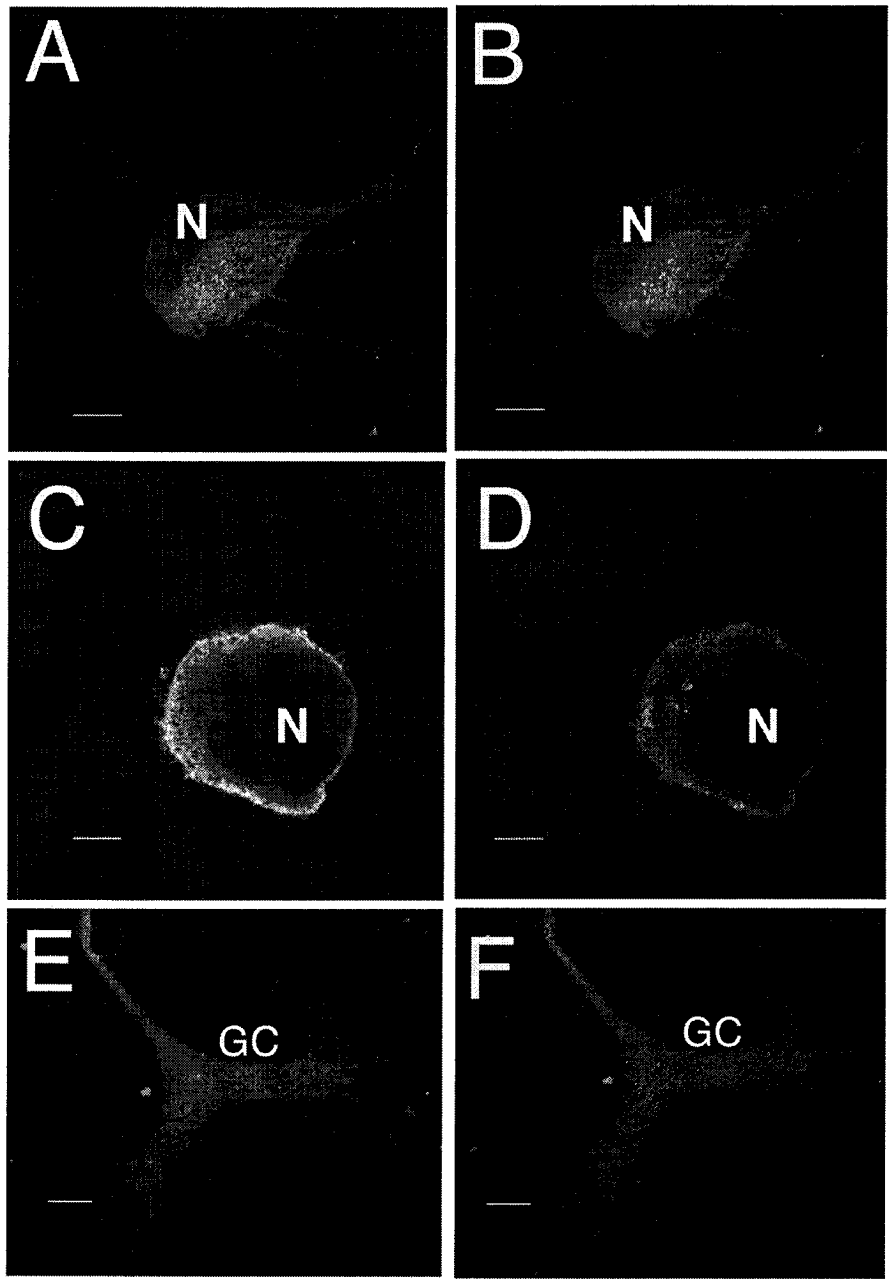

A
Anti-Apl II
Anti-Thr 561-P
S P S P
+ -
Bis
B
Anti-Apl I
Anti-Thr 468-P
S P S P
+ -
Bis
C
Phosphorylation of PDK-site
(Percent chnage from Control)
50
25
0
-25
-50
Apl II
Apl I
D
Percentage Change in the Fraction of
PKC in the Pellet with Bis
100
75
50
25
0
*
Anti-Thr 561-P
Apl II
Anti-Thr 468-P
Anti-Apl I
E
Anti-Apl II
Anti-Thr 561-P
S P S P
+ -
LY
F
Anti-Apl I
Anti-Thr 468-P
S P S P
+ -
LY
G
Phosphorylation of PDK-site
(Percentage change fromControl)
75
50
25
0
-25
Apl II
Apl I
H
Percentage Change in the Fraction of
PKC in the Pellet with LY
75
50
25
0
-25
Anti-Thr 561-P
Apl II
Anti-Thr 468-P
Anti-Apl I

Figure 5



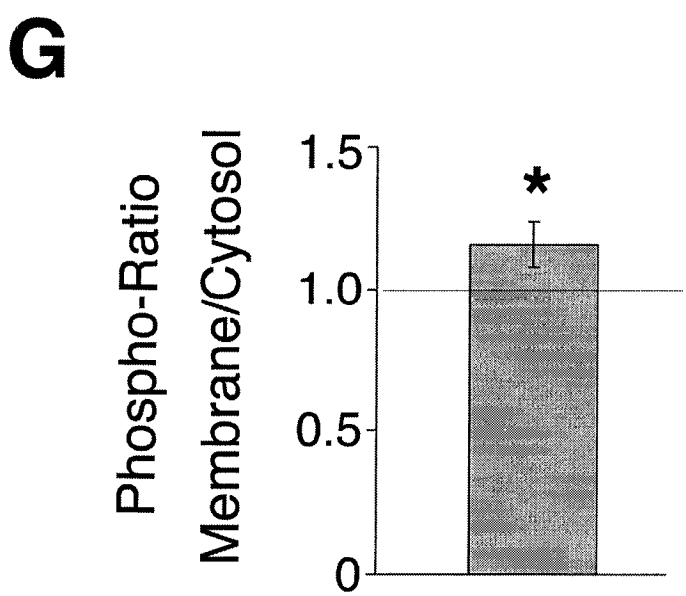


Figure 6

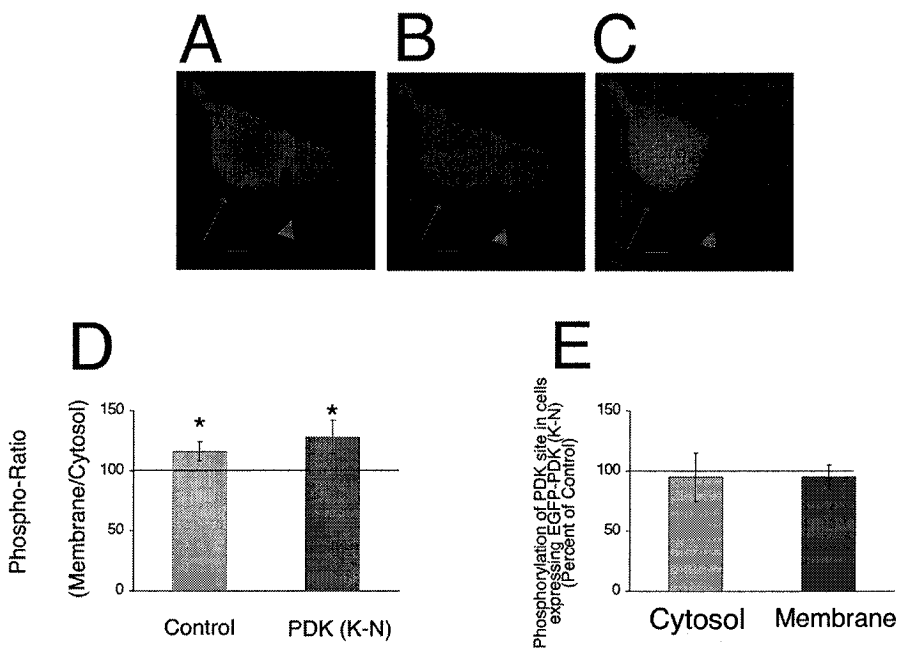

A
B
C
D
Phospho-Ratio
(Membrane/Cytosol)
150
100
50
0
*
*
Control
PDK (K-N)
E
Phosphorylation of PDK site in cells expressing EGFP-PDK (K-N)
(Percent of Control)
150
100
50
0
Cytosol
Membrane

Figure 7

Chapter 3 – Translocation of PKC can be monitored using a green fluorescent protein tag

Introduction

Binding to the plasma membrane plays two important roles in PKC regulation. The first is removal of the pseudosubstrate from the kinase domain, resulting in PKC activation (Dutil, 2000). The second is bringing PKC nearer to its substrates, hence increasing its efficiency (Jaken, 2000). *In vivo,* this translocation is a result of activation of G-protein linked receptors coupled to phospholipase C. The resulting increase in DAG and calcium levels induces translocation of PKC to the membrane via its C1 and C2 domains. This translocation can be observed in real-time by using a green fluorescent protein (GFP) tag to monitor the movement of PKC.

GFP-PKC is thus a useful tool for monitoring the effect of various agonists on PKC localization. The effects of various mutations on the response of PKC to these agonists can also be monitored, providing clues as to the roles of particular phosphorylation sites or particular domains.

Although PKC activation *in vivo* depends on the phorbol ester DAG, experimentally DAG is not a convenient tool for the induction of translocation, as its turnover is high and the activation of PKC it induces is short-lived. The phorbol ester PDBu is more potent than DAG, and induces a more persistent translocation to the membrane (Feng, 1998).

We have begun developing microinjection of GFP-PKC as a tool for the study of PKC translocation. We have found that PDBu produces a rapid and persistent translocation to the plasma membrane. PDBu also induces PKC translocation to discrete

regions within the cell which may include the cytoskeleton as well as other PKC binding sites.

Materials and Methods

GFP-PKC Apl II construct

A full-length clone of PKC Apl II was present in pBluescript SK (Invitrogen, Carlsbad, CA) and baculo-virus vectors (Sossin, 1996). Initially, hemagglutinin (HA) tags were attached to the 5' end. PKC Apl II was excised from pBluescript SK with *Eco*RI, filled in with Klenow, and inserted into the *Stu*I site of the HA vector. This constructs were then excised from the HA vector with *Kpn*I and *Sac*I and inserted into pNEX-3 (gift from B. Kaang, Seoul National University, Seoul, South Korea) cut with the same enzymes. HA-PKC Apl II was excised using *Xho*I /*Eco*RI and inserted into BB4 cut with the same enzymes. EGFP-NI (Clontech, Palo Alto, CA) was amplified by PCR and inserted into the 5' end of PKC Apl II using *Xho*I. The *Xho*I site was cut and filled in to create the correct reading frame. The EGFP-PKC was then excised with *Sac*I and inserted into BB4 cut with *Sac*I. All clonings were confirmed by sequencing over insertion sites.

Sensory neuron culture

Aplysia californica (50-250 g) were obtained from Marine Specimens Unlimited at Pacific Palisades, California, and maintained in an aquarium for at least 3 days before experimentation. The animals were first placed in a bath of isotonic $MgCl_2$ /artificial sea water (1:1, vol/vol) and then anesthetized by injection of isotonic $MgCl_2$. Pleural and pedal ganglia were isolated from the animal. The sensory cluster was removed from the pleural ganglion and digested for 90 minutes in 1% protease–artificial seawater. The neurons were mechanically dissociated and transferred to dishes (Falcon #1008; Becton

Dickinson Canada Inc., Mississauga, Canada) containing a mixture of L-15 (modified for *Aplysia*) (Schacher and Proshansky, 1983), hemolymph (10 %), and bovine serum albumin (0.01%) (Klein, 1995). Glass-bottom dishes (no. 1.5, Mattek Corp, Ashland, MA, USA) were treated with 0.1 % poly L-lysine in sodium borate for 24-72 hours. These dishes were then rinsed thoroughly with distilled water, and on day 1 after isolation, sensory neurons (usually round and devoid of any processes) were plated on the poly-lysine coated dishes in L15 and allowed to adhere. Once cells were attached, hemolymph was added to the dish to a final concentration of 20%. Microinjections of plasmid solutions (2% fast green and 200 ng/μl DNA in distilled water) were done with back-filled glass pipettes (~ 5 MΩ) using a pico-injector (PLI-100; Medical Systems, Greenvale, NY). After impalement, sensory neurons were rapidly filled by delivering short air puffs (50 –150 psi) until the cell soma became uniformly green. Cells were left for two to three days following injection to allow for adequate expression.

GFP-PKC translocation

Cultures were viewed at room temperature at 63X magnification on a Zeiss LSM 510 confocal microscope with Axiovert 200M inverted microscope. A z-stack of the soma (~50 μm thick) was obtained at two five-minute intervals to monitor basal changes in PKC distribution. In some cells, a varicosity was examined; these stacks were ~ 4 μm thick. PDBu was added to the dish to a final concentration of 1 μM. Starting immediately with the addition of PDBu, a series of image z-stacks were obtained, 3 to 15 minutes apart. Once the stacks were collected, the cultures were rinsed with artificial seawater, and fixed for 30 minutes in 4% paraformaldehyde in phosphate-buffered saline

(PBS) with 30% sucrose. Cells were then permeabilized in 0.1% Triton X-100 in PBS with 30% sucrose for 10 minutes, and washed three times with PBS. Free aldehydes were quenched with a 15 minute incubation in 50 mM ammonium chloride in PBS. Non-specific antibody binding was inhibited by incubating with a blocking solution (10% Normal Goat Serum, 0.5% Triton X-100, in PBS) for 1 hour. Cultures were incubated overnight with rabbit anti-PKC 1:1000 in blocking solution. The cultures were washed 4 x 10 minutes with PBS, and were then incubated with secondary antibody in blocking solution (1:400 CY3 donkey anti-rabbit) for 1 to 2 hours. Finally, the cells were washed 4 x 10 minutes with PBS, and the culture dish was covered with a cover slip, using Mowiol as the mounting medium. Fixed cultures were viewed on a Zeiss LSM 510 with Axiovert 200M inverted microscope. Cells were imaged in multi-tracking mode using a 488 nm laser line for the excitation of GFP and the 543 nm laserline for CY3.

Quantitation of GFP-PKC translocation

Quantitative analysis of fluorescence was performed using the 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/). Line intensity profiles were determined, and the first ten pixels were included in measurements of membrane staining. This membrane staining was then normalized against the average staining throughout the cell as a control for bleaching.

Results

Following the addition of PDBu, there was a clear increase in the amount of GFP-PKC on the plasma membrane of the cell body. This translocation began immediately after the addition of PDBu and continued to rise until a plateau was achieved within 15 minutes. The plateau was a 60% increase in the intensity of membrane staining relative to total cell body staining. In addition to this membrane translocation, PKC translocated to particular regions within the cell. Distinct lines of PKC were formed within the cell body. Immunocytochemistry (ICC) using an antibody against PKC revealed a staining that paralleled that of GFP-PKC, with the exception of the nucleus: although GFP staining was seen in the nucleus, the antibody did not detect any PKC in the nucleus.

Imaging of the varicosities revealed the formation of hot spots subsequent to the addition of PDBu. In addition, the shape of GFP-PKC staining in the growth cone was altered: staining in the growth cone tip narrowed, while staining mid-way through the process (~ 20 μm thick) was broadened.

Discussion

Through fluorescent microscopy, we were able to observe the effects of the DAG analog PDBu on the translocation of GFP-tagged PKC. DAG induced significant and persistent translocation of PKC to the membrane. A plateau in the amount of translocation was reached within 15 minutes. Although this response is consistent with that observed in other studies with phorbol esters (Wang, 1999), it differs from the translocation of PKC induced by physiological stimuli, which results in a much more transient translocation. Physiological signals such as stimulation of angiotensin II type 1A receptor and endothelin A receptor induce a rapid, transient translocation to the membrane. In spite of the continued presence of the agonists of these receptors, PKC undergoes only a single, rapid cycle of translocation to the membrane. This is thought to be due to desensitization of the receptors, since subsequent treatment of these cells with phorbol esters results in persistent translocation (Feng, 1998).

PKC also translocated to linear regions within the cell body. One possibility is that these linear regions are actin and/or tubulin. Both Apl I and Apl II have been shown to bind to actin in a phorbol ester-dependent manner. Vertebrate PKC isoforms have also been found to bind actin. PKC binding of actin is associated with actin reorganization, as well as increased cell motility. Since the actin cytoskeleton often retains vesicles from reaching the readily releasable pool, actin disassembly may promote neurotransmitter release (Nakhost, 1998). Activation of PKC by phorbol esters also induces advance of the microtubules to distal growth cones, and this advance is blocked by PKC inhibitors (Kabir, 2001). The vertebrate PKC βII binds to proteins associated with microtubules, and PKC βI is part of a tubulin-enriched cytoskeletal complex (Kiley, 1995; Volkov,

2001). PKC phosphorylates microtubule associated protein-2 (MAP-2), stimulating tubulin polymerization (Ainsztein, 1994); PKC also phosphorylates the microtubule-associated protein tau (Hoshi, 1987).

To determine whether the staining we observed truly was a function of binding to actin or tubulin, ICC using antibodies to actin and/or tubulin could be done. In addition, co-injection of cyan fluorescent protein (CFP)-tagged PKC with yellow fluorescent protein (YFP)-tubulin or actin could be used to follow the translocation in real time.

The formation of hot spots in varicosities may be a function of PKC binding proteins within the varicosity. Binding proteins can play an important role in bringing PKC within proximity to its substrates, as well as to points of convergence of secondary messenger pathways. Syndecan, a proteoglycan involved in cell adhesion, is thought to play a role in the translocation of PKC to points of contact following treatment with phorbol esters. Substrates that interact with C-kinase (STICKs) are proteins that localize to the interface between the cytoskeleton and plasma membrane. They are phosphorylated by PKC, and are thought to be a link between PKC activation and changes in cell morphology and cross-talk. STICKs bind PKC with a high affinity (K_d<100 nM). Both STICKs and receptors for activated C-kinases (RACKs) require PS binding in order to bind PKC. RACKs bring PKC within proximity to its substrates (Jaken, 2000).

The change in the morphology of the growth cone might be due to either a change in the distribution of PKC, or to a change in the morphology of the growth cone itself. Shifts in PKC distribution within the cell body, as well as the formation of hot spots, might suggest that this shift is due to PKC's movement to an area of the cell from which

it was previously excluded. However, this is not what we observed in the cell body, where changes simply reflected a shift in intensity in particular regions. Phorbol esters have been known to induce growth cone collapse, and this may be what we have observed. One way to determine which of the above scenarios is occurring would be to co-inject GFP-PKC and CFP. Fluorescent proteins distribute evenly throughout the cell when not fused to other proteins, and so CFP could give the outline of the cell.

Using antibodies raised in rabbits against PKC, we compared PKC ICC staining to GFP-PKC staining. Comparing the two, the staining was identical with the exception of the nucleus. Staining was detected in the nucleus, whereas antibody staining excluded the nucleus. This is likely due to GFP alone as a degradation product of GFP-PKC. That staining seen elsewhere in the cell is GFP fused to PKC is supported by two lines of evidence: first, it parallels that seen with the PKC antibody; second, it translocated in response to PDBu. In addition, these constructs have been shown to retain their kinase activity when expressed in Sf9 cells (Manseau, 2001).

Phosphorylation plays an important role in the regulation of PKC by influencing the response of PKC to its allosteric activators. This technique would allow examination of the effects of individual phosphorylation sites on PKC translocation, through the use of mutants. The particular role of the C2 domain in translocation may be elucidated using mutants in which this domain is deleted. Effects of different physiological activators such as 5-HT on GFP-PKC localization could be observed. In addition, may provide the opportunity to look beyond translocation to the plasma membrane, to observe in real-time the translocation of PKC to other subcellular structures.

References for Chapter 3

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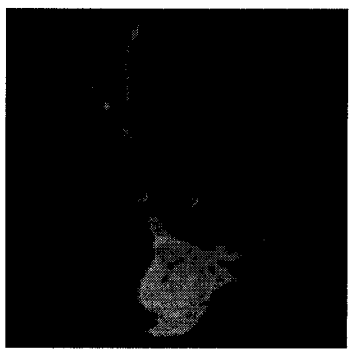
Figure legends

Figure 8. PDBu induces translocation of GFP-PKC to the membrane, as well as to structures within the cell. (A) Cell body of a neuron expressing GFP-PKC prior to treatment with PDBu. (B) Translocation of GFP-PKC to the membrane is visible immediately following PDBu treatment. (C) 10 minutes following PDBu. A semi-circular GFP-PKC staining is observed. (D) Translocation persists 30 minutes following PDBu treatment. (E) ICC using an antibody against PKC reveals staining which is indistinguishable from that of GFP-PKC with the exception of the nucleus, where antibody staining is absent.

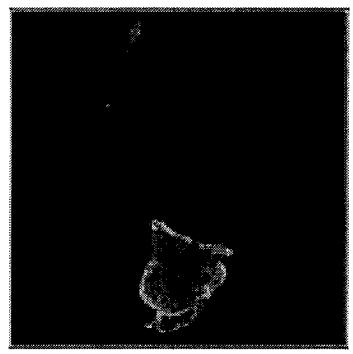
Figure 9. PDBu induces the formation of GFP-PKC hot-spots in neuronal varicosities. (A) A varicosity prior to PDBu treatemnt. (B) Immediately after PDBu treatment, a bright spot of GFP-PKC forms in the varicosity. This spots persists over (B) 10 minutes and (C) 30 minutes following the treatment, as well as after fixation and ICC with an antibody against PKC (E).

Figure 10. A change in GFP-PKC morphology was observed following PDBu treatment. (A) Three-dimensional reconstruction of two neurites, seen from the horizontal plane, slightly askew., prior to PDBu treatment (B) 10 minutes after the addition of PDBu, staining at the tip of the neurites narrow, and a bulbous structure forms at approximately 20 μm from the tip. (C) The change in morphology is more pronounced at 30 minutes following PDBu treatment.



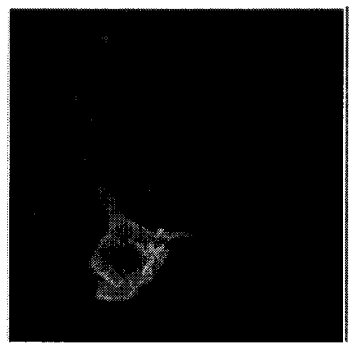
before PDBu

immediately after PDBu



10 minutes
after PDBu

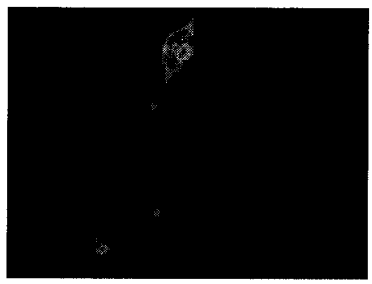
30 minutes
after PDBu



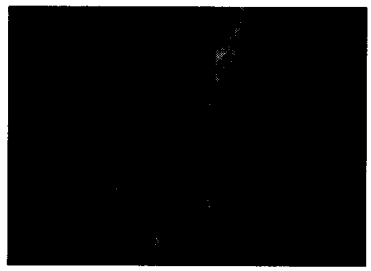
fixed

Figure 8

before PDBu

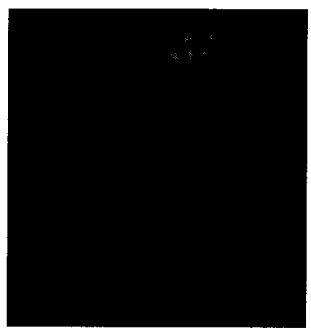


immediately
after PDBu



10 minutes
after PDBu

30 minutes after PDBu



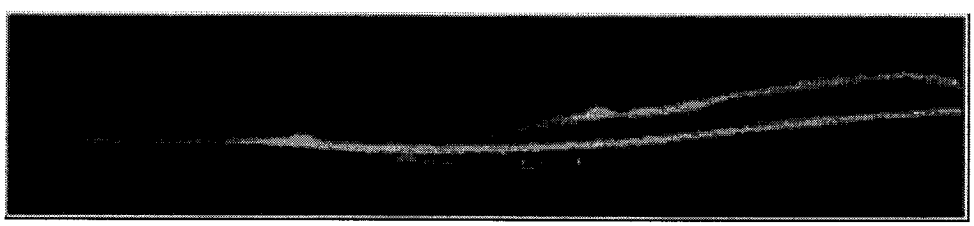
fixed

Figure 9

A

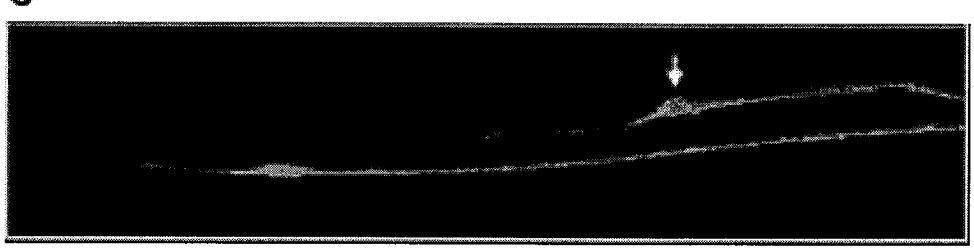
before PDBu

B



10 minutes
after PDBu

C



30 minutes
after PDBu

Figure 10

COMPLIANCE ATTESTATION FORM
POSTDOCTORAL/GRADUATE STUDENT
STIPEND AND RESEARCH ALLOWANCE AWARDS

RESEARCH PROJECT INFORMATION: (Must be completed by Award Holder, and include signatures)		
Name of Postdoc/Graduate Student:	Miss Ginette Thibault	
Research Director/Supervisor:	Dr. Wayne Sossin	
Project Title of Postdoc/Graduate Student:	Characterization of the phosphorylation of protein kinase C by phosphoinositide-dependent kinase-1	
Project Title: (as appears on certificate(s))	PKC, transmitter release and re-arrangement of the cytoskelton	
If your project involves any of the areas specified below, this completed Attestation of Compliance form must accompany the Fund Opening Approval Form. **IF YOUR FUNDING INCLUDES A RESEARCH ALLOWANCE, YOU MUST ALSO ATTACH RELEVANT CERTIFICATES.**	**Yes**	**Certificate Expiry Date:**
HUMAN SUBJECTS		
ANIMAL SUBJECTS	Yes	March 31, 2003
MICROORGANISMS, LIVING CELLS, OTHER BIOHAZARDS		
RADIOACTIVE MATERIALS	Yes	April 30, 2003
For research allowances: if your research is covered by the certificate of your supervisor, please attach your supervisor's certificate along with this completed "Attestation of Compliance".		

Investigators and members of their research teams must adhere to policies and guidelines as stipulated by NSERC, CIHR and SSHRC in their *Tri-Council Policy Statement on Ethical Conduct for Research Involving Humans*, as well as standards stipulated by CCAC, Laboratory Biosafety Guidelines, CNSC, and all requirements concerning Licences for Research in the Yukon, the Northwest Territories and Nunavut. For further information concerning compliance, please contact the appropriate staff member of the Research Grants Office. Consult the RGO website at: http://www.mcgill.ca/rgo

I ATTEST THAT THE RESEARCH PROJECT OF THE POSTDOC/GRADUATE STUDENT, AS INDICATED ABOVE, IS COVERED BY MY CERTIFICATE. IF AWARD INCLUDES A RESEARCH ALLOWANCE, I HAVE INCLUDED A COPY OF CERTIFICATE(S) WITH THIS "ATTESTATION OF COMPLIANCE". ANY FUNDS AWARDED UNDER MY SUPERVISION WILL BE USED IN ACCORDANCE WITH ALL REQUIRED RESEARCH REGULATIONS AND POLICIES.

SIGNATURES:

1) THE INFORMATION ABOVE IS TRUE AND MY CERTIFICATE COMPLIES WITH THE SPONSOR AND UNIVERSITY REGULATIONS AND POLICIES

RESEARCH DIRECTOR/SUPERVISOR _____ DATE Oct. 17, 2002

DEPARTMENTAL CHAIR/DELEGATE _____ DATE 17 OCT. 2002

Note: OFTEN, THE PERIOD FOR WHICH A COMPLIANCE CERTIFICATE IS ISSUED WILL NOT COINCIDE EXACTLY WITH THE AWARD START AND END DATES. BEARING IN MIND THAT THESE CERTIFICATES HAVE AN EXPIRY DATE, PLEASE BE ADVISED THAT IT IS THE POSTDOC/ STUDENT'S RESPONSIBILITY TO ENSURE THEIR RESEARCH MEETS WITH REQUIRED RESEARCH REGULATIONS AND POLICIES FOR THE DURATION OF THE PROJECT. IN THE CASE OF RENEWABLE AWARDS, PLEASE FORWARD UPDATED COMPLIANCE CERTIFICATE(S) APPROXIMATELY 4-6 WEEKS BEFORE EACH ANNIVERSARY DATE OF AWARD, TO AVOID INTERRUPTIONS IN PAYMENT.

C-level

McGill University
Animal Use Protocol – Research

Guidelines for completing the form are available at www.mcgill.ca/rgo/animal

Protocol #: 3401
Investigator #: 763
Approval End Date: MARCH 31, 2003
Facility Committee: MUC

☐ Pilot ☐ New Application ☒ Renewal of Protocol # 3401

Title PKC, transmitter release and re-arrangement of the cytoskeleton
(must match the title of the funding source application)

1. Investigator Data:

Principal Investigator: Wayne SOSSIN Office #:
Department: Neurology & Neurosurgery Fax#:
Address: Montreal Neurological Institute Email:

2. Emergency Contacts: Two people must be designated to handle emergencies.

Name: Wayne Sossin Work #: Emergency #:
Name: Xiaotang Fan Work #: Emergency #:

ACTION	✓	DATE
P.I.	✓	Apr 3 '02
FACC	✓	"
RGO	✓	"
VET	✓	"
DB		
Renewed		

3. Funding Source:

External ☒
Source (s): NSERC
Peer Reviewed: ☒ YES ☐ NO**
Status: ☒ Awarded ☐ Pending
Funding period: Apr 1, 2002-Mar 31, 2004

Internal ☐
Source (s): ____
Peer Reviewed: ☐ YES ☐ NO**
Status: ☐ Awarded ☐ Pending
Funding period: ____

** All projects that have not been peer reviewed for scientific merit by the funding source require 2 Peer Review Forms to be completed. e.g. Projects funded from industrial sources. Peer Review Forms are available at www.mcgill.ca/fgsr/rgo/animal/

Proposed Start Date of Animal Use (d/m/y): ____ or ongoing ☒

Expected Date of Completion of Animal Use (d/m/y): ____ or ongoing ☒

Investigator's Statement: The information in this application is exact and complete. I assure that all care and use of animals in this proposal will be in accordance with the guidelines and policies of the Canadian Council on Animal Care and those of McGill University. I shall request the Animal Care Committee's approval prior to any deviations from this protocol as approved. I understand that this approval is valid for one year and must be approved on an annual basis.

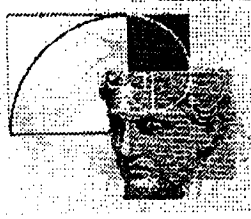
Principal Investigator's signature: Date: March 6/02

Approval Signatures:

Chair, Facility Animal Care Committee:		Date: 22/3/02
University Veterinarian:		Date: 4/2/02
Chair, Ethics Subcommittee(as per UACC policy):		Date:
Approved Period for Animal Use	Beginning: April 1, 2002	Ending: March 31, 2003

☐ This protocol has been approved with the modifications noted in Section 13.

APR 0 2 2002



Montreal Neurological Institute and Hospital

A Teaching and Research Institute at McGill University

Internal Radioisotope User Permit

Issued by:

The Radiation Safety Committee of the Montreal Neurological Institute and Hospital

Authorized by the Canadian Nuclear Safet Commission

CNSC Radioisotope Licence Number: 01187-2-03.0

1. Radioisotope User Permit Number : MNI_021
   Classification : BASIC
   Date of Issue : May 1, 2001
   Expiry Date : April 30, 2003

2. Name of Principal Investigator : SOSSIN, WAYNE

3. Department : CBET

4. Locations approved by this permit : 776

5. Radioisotopes approved by this permit :

^{3}H	^{14}C	^{35}S	^{32}P	[redacted]

6. Personal Dosimeters Required : YES (if >10 mCi ^{32}P used)

7. Method of Disposal : **Quantities higher than these limits must be disposed of as radioactive waste**

	^{3}H	^{14}C	^{35}S	^{32}P	[redacted]
Liquid/sewer (µCi/l)	10	1	0.1	0.1	[redacted]
Solid/ garbage (µCi/Kg)	1000	100	10	10	[redacted]
Gas/Atmosphere (µCi/ CubicMeter)	1	0.1	0.01	0.01	[redacted]

8. Special Conditions:

Gloves and labs coats mandatory.

Weekly wipe tests required in areas where radioisotopes are used.

[redacted]

The Radiation Safety Officer, MNH/I: (E. Meyer, RSO)

The Journal of Biological Chemistry
Published by
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September 11, 2002

Dear Dr. Ginette L. Thibault

Please note your recent request for copyright permission has been granted for the following:

Vol	Page	Year
277	37116-37123	2002

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Sincerely yours,

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AND MOLECULAR BIOLOGY

7475 Lusk Boulevard
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September 24, 2002

Ms. Ginette Thibault
Montreal Neurological Institute
3801 University Street Rm #776
Montreal, PQ H3A 2B4
CANADA

RE: "ATTN: Ginette Thibault - MSc Thesis Waiver for use of JBC article: Vol. 277, pp. 37116-37123"

To Whom It May Concern:

This letter acknowledges my understanding and authorization for Ginette L. Thibault to use the JBC Manuscript vol. 277, pp 37116-37123 as part of her masters thesis entitled: "Characterization of the phosphorylation of protein kinase C by phosphoinositide-dependent kinase-1".

Sincerely,

Anthony M. Pepio, Ph.D.
Clinical Scientist, Clinical Development
Elan Pharmaceuticals

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INSTITUT ET HÔPITAL NEUROLOGIQUES DE MONTRÉAL

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Wayne Sossin, PhD
Research Group on Cell Biology of Excitable Tissues
Associate Professor
Department of Neurology and Neurosurgery
McGill University

Oct 25, 2002

To whom it may concern,

This letter acknowledges my understanding and authorization for Ginette L. Thibault to use the JBC manuscript vol 277: pp37116-37123 as part of her Masters thesis entitled, " Characterization of the phosphorylation of protein kinase C by phosphoinositide-dependent kinase 1.

Sincerely,

3801, rue University
Montréal, Québec

Wayne Sossin