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PHOSPHORYLATION AT THE HYDROPHOBIC SITE OF PROTEIN KINASE C Apl II IS INCREASED DURING INTERMEDIATE TERM FACILITATION

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Abstract—In *Aplysia*, persistent increases in synaptic strength are paralleled by the persistent activation of the novel protein kinase C Apl II. We raised a phosphospecific antibody against serine 725, the hydrophobic motif in protein kinase C Apl II. Phosphorylation of serine 725 increased in parallel to the persistent activation of the kinase. We expressed protein kinase C where this site was mutated to an alanine to prevent phosphorylation. The mutated protein kinase C showed decreased specific activity consistent with a model where the kinase is less stable in the absence of phosphorylation of this site. Endogenous phosphorylation of protein kinase C Apl II at serine 725 was unaffected by either activation of protein kinase C by phorbol esters, or inhibition of protein kinase C using two distinct inhibitors, suggesting the site is not autophosphorylated. Consistent with this, overexpressed kinase-dead protein kinase C Apl II still was phosphorylated at serine 725, although to a lesser extent than wild-type protein kinase C Apl II. While PDK appears to interact with the serine 725 site, it is not responsible for its phosphorylation. Finally inhibition of phosphoinositide-3 kinase or the target of rapamycin by pharmacological agents did not block basal phosphorylation of serine 725 in *Aplysia* ganglia. Our results suggest trans-phosphorylation of protein kinase C Apl II as Ser 725 occurs during persistent activation of the kinase, but this does not appear to be downstream of phosphoinositide-3 kinase.

Key words: *Aplysia*, learning and memory, phosphoinositide dependent kinase I, persistent protein kinase, rapamycin, autophosphorylation.

The *Aplysia* sensory-motor neuron synapse is a leading model for understanding the cellular basis of memory (Kandel, 2001). A shock to the animal leads to increases in defensive reflexes, a process known as sensitization. At the cellular level, sensitization is partly mediated by increases in the strength of the synapse between the sensory and motor neuron, a process known as facilitation (Kandel, 2001) Facilitation can be mimicked in isolated ganglia or in sensory-motor neuron cultures by the addition of the facilitating neurotransmitter serotonin (5-HT) (Byrne and Kandel, 1996). Facilitation can be short term, lasting under 30 min, intermediate term, lasting 2 h or more, and long term lasting more than 24 h (Ghirardi et al., 1995; Mauelshagen et al., 1996). The maintenance of intermediate-term facilitation is mediated by persistent activation of protein kinase A or C depending on the stimulation paradigm (Yanow et al., 1998; Sutton and Carew, 2000). We have been interested in the regulation of protein kinase C (PKC) in this system and how it could be persistently activated (Sossin et al., 1994; Sossin, 1997).

The PKC family consists of three broad groups: conventional, novel and atypical. Conventional PKCs have regulatory domains regulated by the conjunction of calcium and diacylglycerol, while novel PKCs are activated by diacylglycerol, but not calcium, and atypical PKCs are insensitive to both (Newton, 2003). In mammals there are 10 unique identified PKCs, while in *Aplysia* there are three: PKC Apl I, PKC Apl II and a recently identified atypical PKC Apl III (unpublished observations), one from each family of conventional, novel and atypical PKCs, respectively (Sossin et al., 1993). This allows for easier dissection of the distinct roles of these isotypes.

PKCs are also regulated by phosphorylation (Newton, 2003). The enzyme phosphoinositide-dependent kinase I (PDK-1) phosphorylates PKCs and many other related kinases at a conserved site in the catalytic domain (Chou et al., 1998; Dutil et al., 1998; Le Good et al., 1998; Toker and Newton, 2000). This site is required for initial formation of the catalytic domain and PKCs with a mutation at this site are inactive (Keranen et al., 1995; Newton, 1997). Once this site is phosphorylated, PKCs are then phosphorylated at a minimum of two additional sites (Keranen et al., 1995; Newton, 1997). One of these, the turn site, is an autophosphorylation site (Newton, 2003) and the other, termed the hydrophobic site, is either autophosphorylated or phosphorylated by a heterologous kinase whose identity is controversial (Behn-Krappa and Newton, 1999; Parekh et al., 1999; Ziegler et al., 1999; Cenni et al., 2002; Rybin et al., 2003; Dong and Liu, 2005). Once these two sites have been phosphorylated, phosphorylation of the PDK-1 site no longer is required, at least for some isoforms of PKC (Newton, 2003).

During the maintenance of intermediate-term facilitation induced by a continuous pulse of 5-HT, PKC Apl II is persistently and autonomously active (Sossin et al., 1994; Sossin, 1997). This persistent kinase activity is not due to cleavage of the regulatory subunit to form a protein kinase M (PKM), since the enzyme can still be inhibited by the regulatory domain specific inhibitor calphostin C, albeit at higher concentrations than for the non-autonomous enzyme (Sossin, 1997). The phosphorylation of PKC Apl II at

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Abbreviations: PDBu, phorbol dibutyrate; PDK-1, phosphoinositide-dependent kinase I; PI-3 kinase, phosphoinositide-3 kinase; PKC, protein kinase C; PS, phosphatidylserine; SF9, *Spodoptera frugiperda*; TOR, target of rapamycin; 5-HT, 5-hydroxytryptamine (serotonin).

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the PDK site is also increased in concert with the autonomous activity of the kinase (Pepio et al., 2002). Whether this is a cause or a consequence of autonomous activation is not clear. To further examine the state of PKC Apl II phosphorylation during intermediate-term facilitation, we have raised a phosphopeptide antibody to the hydrophobic site in PKC Apl II. We find that phosphorylation at this site is also increased during persistent activation of PKC Apl II. We provide evidence that this is a trans-phosphorylation event, but one that does not appear to be regulated similarly to trans-phosphorylation of novel PKCs in vertebrates.

EXPERIMENTAL PROCEDURES

Experimental animal system

Aplysia californica were purchased from either Marinus Scientific (Garden Grove, CA, USA) or the University of Miami *Aplysia* Resource Facility (Miami, FL, USA) and maintained in an aquarium for at least 3 days prior to experimentation. All experiments were approved by the McGill Animal Care Committee. Care was taken to minimize the number of animals used. The animals were first placed in a bath of isotonic $MgCl_2$/artificial seawater (1:1, vol:vol) and then anesthetized by injection of isotonic $MgCl_2$. Pleural–pedal paired ganglia (for experiments involving intermediate term facilitation) or abdominal, cerebral and buccal ganglia (for pharmacological experiments) were dissected out as described (Sossin et al., 1994; Pepio et al., 2002). Each experimental trial control vs. treatment was paired left vs. right side (randomly) from within the same animal.

Cytosolic vs. membrane fractions

After treatments, nervous system tissue samples were homogenized in a low salt homogenization buffer (10 mM $MgCl_2$, 50 mM Tris pH 7.5, 10% v/v glycerol) containing protease inhibitors 100 μM leupeptin, 5 mM benzamidine 2 mM and 2 μM aprotinin and phosphatase inhibitors 50 μM NaF and 5 mM sodium pyrophosphate. Homogenates were then spun at 51,000 r.p.m. (100,000$\times g$) for 30 min to separate the homogenate into a supernatant and pellet fraction. The pellet was resuspended in homogenization buffer and after a fraction was removed for measuring levels of protein using a modified Bradford reaction (Sossin et al., 1994), both supernatant and pellet were denatured with Laemmli buffer, heated to 95 °C for 5 min, separated on 9% SDS-PAGE, transferred to nitrocellulose and immunoblotted as described (Pepio et al., 2002).

Antibodies

The phospho-peptide EFRGF[pS]FANPD and the corresponding non-phosphorylated peptide EFRFGSFANPD corresponding to the hydrophobic site in PKC Apl II were purchased from Biosource International (Hopkington, MA, USA). The phospho-peptide was coupled to bovine serum albumin using EDC (Pierce, Rockford, IL, USA) and used to immunize rabbits with the adjuvant Titer-Max. The resultant serum was affinity purified over a peptide column generated using the EDC/Diaminodiipropylaminde kit (Pierce) and then concentrated before use. Phospho antibodies were preblocked with non-phosphopeptide at a concentration of 25:1 molar ratio for 10 min, centrifuged, and the supernatant diluted to a final concentration of antibody of 1 μg/μl for blotting. The antibody to total PKC Apl II has been previously characterized (Sossin et al., 1994; Pepio et al., 2002).

Quantitation of immunoblotting

Western blot films were scanned and the bands were quantified using Image J software with uncalibrated OD. We have found this to give a linear result over a large range of input protein (Nakhost et al., 1998, 1999). We define phosphoratio as immunoreactivity revealed by the phosphospecific antibody/immunoreactivity with the total antibody. Effects of 5-HT and pharmacological agents were calculated as the fold change in phosphoratio between control and drug-treated samples. PKC translocation was measured by comparing immunoreactivity between cytoplasm and membrane fractions, corrected by the percentage of the total protein loaded. Statistics were done using Student's paired *t*-tests between phosphoratios in control and drug-treated samples.

Expression in *Spodoptera frugiperda* (SF9) cells

Plasmids encoding PKC Apl II, PKC Apl II S725-A and PKC Apl II S725-E were made in BbacHis2c (Invitrogen, Burlington, ON, Canada) and high titer baculoviruses generated (Invitrogen). SF9 cells were infected at a multiplicity of infection (MOI) of 5 and extracts were generated after three days of infection for both immunoblotting and purification experiments.

Enzyme purification

Baculovirus expressed PKCs were purified using Invitrogen's Probond® His-affinity resin columns using a modified purification buffer (20 mM HEPES pH 7.5, 10 mM $MgCl_2$, 1 mM DTT, 100 mM KCl, 10% glycerol, aprotinin (2 μM), benzamidine (5 mM), leupeptin (100 μM). The enzymes were then eluted into a storage buffer (50 mM Tris pH 7.5, 1 mM $MgCl_2$, 100 μM $ZnSO_4$, 10% v/v glycerol) and concentrated.

Kinase assays

Enzymatic activity of our kinases was assayed in an *in vitro* reaction, with all parameters as previously described (Sossin and Schwartz, 1992; Sossin et al., 1996). Briefly, PKC Apl II was incubated with 10 μM substrate A-ε peptide (Sossin and Schwartz, 1992; Sossin et al., 1996), radioactive ^{32}P-ATP, phosphatidylserine (PS) (Avanti, Alabaster, AL, USA)/TPA or water, and PKC inhibitors or water. Reactions were performed at 20 °C for 30 min. Each reaction was blotted onto P81 Whatman® filter paper, stopped in a 2% w/v cold ATP solution, washed 4$\times$ with 0.425% v/v phosphoric acid, and then counted in a scintillation counter to determine levels of radioactivity incorporated into the substrate. Specific activity was determined as activity divided by total protein as determined by Bradford assays of purified proteins and where purity was confirmed by Coomassie staining.

RESULTS

The hydrophobic site is conserved in PKC Apl II (Fig. 1A), however due to interspecies differences, we found that commercial phosphopeptide specific antibodies generated against the homologous sites in PKC epsilon or delta were not effective in *Aplysia* (data not shown). Thus we raised a phospho-specific antibody to the sequence in PKC Apl II. The affinity-purified antibody recognized a single band at 100 kDa, that co-migrated with the band immunoreactive for PKC Apl II in both the *Aplysia* nervous system and when PKC Apl II was expressed in SF9 cells (Fig. 1B). The antibody is phospho-specific as it did not recognize PKC Apl II when serine 725 was converted to an alanine (Fig. 1B) or when a catalytic fragment of PKC Apl II was expressed in bacteria (data not shown).

During intermediate term facilitation induced by 90 min of 5-HT treatment, there is an increase in the activity of an autonomous kinase derived from PKC Apl II (Pepio et al.,