Protein Kinase C Isoforms Are Translocated to Microtubules in Neurons*

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Activation of protein kinase C (PKC) increases microtubule (MT) growth lifetimes, resulting in extension of a nocodazole-sensitive population of MTs in Aplysia growth cones. We examined whether the two phorbol ester-activated PKCs in Aplysia, the Ca\(^{2+}\)-activated PKC Apl I and the Ca\(^{2+}\)-independent PKC Apl II, are associated with these MTs. Phorbol esters translocated PKC to the Triton X-100-insoluble fraction, and a significant portion of this translocated pool was sensitive to low concentrations of nocodazole. Low doses of nocodazole had no effect on the amount of PKC in the Triton X-100-insoluble fraction in the absence of phorbol esters, whereas, higher doses of nocodazole reduced basal levels of PKC Apl II. The F-actin cytoskeletal disrupter, latrunculin A, removed both PKCs from the Triton X-100-insoluble fraction in both control and phorbol ester-treated nervous systems. PKC Apl II also directly interacted with purified MTs. In detergent-extracted cells, both PKCs immunolocalized predominantly with MTs. PKCs were associated with newly formed MTs invading the actin-rich peripheral growth cone domain after PKC activation. Our results are consistent with a central role for PKCs in regulating MT extension.

A large amount of research has been directed at how protein kinase C (PKC) regulates cytoskeletal plasticity (1). Most of this research has focused on the actin cytoskeleton because PKCs bind directly to actin filaments (2–4) and a number of important actin regulatory proteins have been shown to be controlled by PKC phosphorylation (5–7). In contrast, roles for PKC in regulating MT extension, guidance, and/or delivery of important molecules to the plasma membrane (11–13).

Examination of PKC action is simplified in the Aplysia nervous system, as there are only two phorbol ester-activated PKCs, the Ca\(^{2+}\)-activated PKC Apl I (homologous to PKC\(\alpha\), PKC\(\beta\), and PKC\(\gamma\) in vertebrates) and the Ca\(^{2+}\)-independent PKC Apl II (homologous to PKC\(\epsilon\) and PKC\(\gamma\) in vertebrates) (14, 15). Both isoforms of PKC co-assemble with actin in vitro, and this is enhanced by phorbol esters and by inhibitors of PKC phosphorylation (3). Using immunocytochemistry, PKC Apl II was localized to actin cables in the growth cone (3). Both PKCs also translocated to a Triton X-100-insoluble fraction by phorbol esters, and this was initially assumed to represent the actin cytoskeleton (3). However, given our recent report of PKC regulation of MT dynamics, we have further investigated the cytoskeletal pools with which PKC associates in neurons. We have also examined the distribution of PKC on MTs before and after PKC activation, using different cell extraction protocols that retain native associations of PKC with the cytoskeleton. We show that, upon activation by phorbol esters, both isoforms of PKC rapidly translocate onto newly assembled MTs, suggesting a direct role for PKC in regulating MT extension in neuronal growth cones.

**EXPERIMENTAL PROCEDURES**

*Aplysia californica*—*A. californica* (50–200 g) were purchased from Marine Specimens Unlimited (Pacific Palisades, CA) or the Aplysia resource facility at the University of Miami and kept in an aquarium for at least 3 days before experimentation. The animals were first placed in a bath of isotonic MgCl\(_2\)/artificial seawater (1:1, v/v) and then anesthetized by injection of isotonic MgCl\(_2\). The ganglia were dissected from the animals and trimmed of connective tissue in ice-cold dissecting medium (230 mM NaCl, 220 mM MgCl\(_2\), 1 mM CaCl\(_2\), 10 mM KCl, 10 mM HEPES, 0.2× low methionine amino acid mixture, 0.2× minimal essential medium nonessential amino acids (Invitrogen), 0.5× minimal essential medium vitamin solution, 0.1 mM glutamine, and 0.1% glucose, final pH 7.8).

**MT Binding Assay**—PKC was obtained for these experiments from the cytosol of S9 cells infected with baculovirus encoding PKC Apl I or Apl II as described (3). Purified bovine brain tubulin (10 mg/ml; ICN Biomedicals, Inc., Aurora, OH) was stored in 80 mM PIPES (sesquioctammonium salt), 1 mM EGTA, 1 mM MgCl\(_2\), 1 mM GTP, and 10% glycerol, pH 6.8, at –70 °C. Varying amounts of tubulin from the above stock solution were preincubated for 30 min at 37 °C with an equal volume of

The abbreviations used are: PKC, protein kinase C; MT, microtubule; P domain, peripheral domain; PIPES, piperazine-N,N'-bis(2-ethanesulfonic acid); MES, 2-(N-morpholino)ethanesulfonic acid; PDBu, phorbol 12,13-dibutyrate; CSB, cytoskeletal stabilization buffer.
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Feasibly buffer (0.1 M MES, 1 mM EGTA, and 0.5 mM MgCl₂) containing 1 mM GTP and 10% glycerol (RGG buffer) plus 10 μg/ml Taxol (Sigma) for some experiments. These samples were then further diluted in RGG buffer to obtain the desired concentration of microtubules. Finally, the samples were incubated in 1 μM 4μ-phorbol 12,13-dibutyrate (PDBu; the PKC activator) or 1 μM 4α-PDBu (the inactive analog of the phorbol ester) in RGG buffer with 10 μg of S9 cell cytosolic fraction expressing PKC Apl I or Apl II for 30 min at room temperature. The samples were centrifuged at 94,000 × g for 30 min at 25 °C. Supernatants were removed and added to 20 μl of sample buffer (2% SDS, 10% glycerol, 100 mM dithiothreitol, 60 mM Tris, pH 6.8, and 0.001% bromphenol blue). Following resuspension of the pellets, samples were loaded onto a 9% SDS-polyacrylamide gel.

**Trichoplax Aplasia**—Most ganglia are symmetrically paired on either side of the animal. In all experiments, the ganglion on one side of the animal was used as a control for the corresponding one on the other side. The ganglia were desheathed to facilitate penetration of phorbol ester and transferred to resting medium (the same as dissecting medium, but with 460 mM NaCl, 11 mM CaCl₂, and 55 mM MgCl₂). The ganglia were incubated in either drug solution (300 nM nocodazole (Molecular Probes, Inc., Eugene, OR) and/or 5 μM latrunculin A (Molecular Probes, Inc.) or vehicle in resting medium containing 10 mM glutamine and 0.1% glucose. Subsequently, phorbol esters were added to both samples to a final concentration of 1 μM 4α-PDBu to examine PKC translocation or 1 μM 4α-PDBu (inactive isomer) to examine basal PKC levels. Ganglia were incubated for 1 h at 15 °C; this leads to maximum translocation of PKC (16). The ganglia were then washed twice and homogenized in 120 μl of cold homogenization buffer (50 mM Tris-HCl (pH 7.5), 10 mM MgCl₂, 1 mM EGTA, 5 mM mercaptoethanol, 20 μg/ml aprotinin, 5 mM benzamidine, and 0.1 mM leupeptin). The samples were centrifuged at 4 °C for 3 min at 800 × g to remove any unhomogenized debris. A sample (10 μl) from each condition was removed for analysis of total protein concentration. The remaining homogenates (100 μl) were centrifuged at 100,000 × g for 30 min. Supernatants (S1) were extracted; a small sample was put aside for analysis of the protein concentration; and the remaining 80 μl was added to 20 μl of sample buffer. Pellets were resuspended in 80 μl of 1% Triton X-100 in homogenization buffer. The tubes were incubated at 4 °C for 15 min and then centrifuged at 4 °C for 30 min at 100,000 × g. Supernatants (S2, Triton X-100-soluble) were removed and added to 20 μl of sample buffer. The pellets (P, Triton X-100-insoluble) were resuspended in 100 μl of sample buffer. Equal protein amounts (S1 lanes containing 10 μg of protein and S2 and P lanes containing the maximum amount to allow equal loading) from control and experimental samples were loaded onto 9% SDS-polyacrylamide gels and analyzed by immunoblotting.

**Immunoblotting**—Blotting and probing with antibodies to PKCs Apl I and Apl II were as described (3). The blots were then stripped and reprobed with antibody to actin (ICN Biomedicals, Inc.) or tubulin (Sigma Immunotechnology) and scanned. The analysis was performed using the NIH Image program. We calibrated our data with the uncalibrated OD feature of NIH Image, which transforms the data using the formula $y = \log(10)\{255/255 - x\}$, where $x$ is the pixel value (0–254). Control experiments demonstrated that, after this calibration, values were linear with respect to the amount of protein over a wide range of values (3).

First, the percentage of protein in each of the compartments (supernatant, Triton X-100-soluble, and Triton X-100-insoluble) was calculated. The percentage change in this value with nocodazole and/or latrunculin A was then calculated between the paired ganglia. Statistical tests were paired $t$ tests between control and experimental ganglia.

**Immunocytochemistry**—Bag cell neurons were cultured and treated with drugs as previously described (8). Cells were chemically fixed as described previously (8) with 3.7% formaldehyde in CSB. After fixation, both groups were triple-labeled with F-actin, MTs, and PKCs. Alexa 594-phalloidin (Polysciences, Inc.) was used for F-actin, and Alexa 550-labeled goat anti-mouse and Alexa 488-labeled goat anti-rabbit secondary antibodies were used for MTs and PKCs, respectively (Alexa-labeled reagents were from Molecular Probes, Inc.). Triple-labeled samples were imaged with a Coolsnap HQ cooled CCD camera (Roper Scientific, Trenton, NJ) and MetaMorph control software (Universal Imaging Corp., Downingtown, PA). Controls demonstrated no bleed-through between channels.

**RESULTS**

**PKC Translocation to the Triton X-100-insoluble Fraction Is Sensitive to Nocodazole**—Phorbol esters translocate PKC to the Triton X-100-insoluble fraction of the nervous system (3). Phorbol esters also increase the amount of tubulin that sediments after ganglia are extracted in buffers that stabilize MTs (8). Furthermore, both the extended MTs seen in growth cones and the increased tubulin sedimented in the presence of phorbol esters are sensitive to low concentrations (10–100 nM) of nocodazole (8). These low concentrations of nocodazole primarily damp MT dynamics, having insignificant effects on cellular MT mass (17, 18) or the amount of tubulin sedimented in the absence of phorbol esters (8). We thus examined whether low concentrations of nocodazole could reverse phorbol ester-mediated translocation of PKCs to the Triton X-100-insoluble fraction. Paired ganglia were treated with either the active or inactive phorbol ester in the presence or absence of 300 nM nocodazole. Nocodazole had no effect on the amount of either PKC in the Triton X-100-insoluble fraction when the inactive phorbol ester was used (Fig. 1A; quantitated in Fig. 1B). In contrast, when added in the presence of the active phorbol ester, 300 nM nocodazole significantly decreased the amount of both PKCs Apl I and Apl II in the Triton X-100-insoluble fraction and increased the amount of both PKCs in the supernatant fraction (Fig. 1A; quantitated in Fig. 1B). In the presence of phorbol ester, nocodazole also decreased the amount of tubulin found in the Triton X-100-insoluble fraction, but nocodazole had no effect on the amount of tubulin in the Triton X-100-insoluble fraction in the absence of phorbol esters (Fig. 1A; quantitated in Fig. 1B). These results suggest that PKCs are translocated to a dynamic population of MTs by phorbol ester treatment.

Nocodazole did not affect the amount of PKC found in the Triton X-100-soluble pellet either before or after phorbol ester treatment (Fig. 1A; quantitated in Fig. 1B). The phorbol ester-mediated translocation of PKCs to the Triton X-100-soluble pellet (3, 16) is presumably to either plasma membrane or internal membranes and would not be expected to be sensitive to disruption of the cytoskeleton.

**Latrunculin A Removes PKC from the Triton X-100-insoluble Fraction Independently of Phorbol Ester Treatment**—We have previously shown that both Aplysia PKCs co-sediment with actin filaments in a phorbol ester-dependent manner (3). To further characterize possible PKC-actin filament interactions, we examined the effect of latrunculin A, a sponge toxin that effectively and specifically disrupts actin filaments (19), on the amount of PKC translocated to the Triton X-100-insoluble fraction. Paired ganglia were treated with either the active or inactive phorbol ester in the presence or absence of 5 μM latrunculin A. Latrunculin A decreased the level of PKCs Apl I and Apl II in the Triton X-100-insoluble fraction both in the presence and absence of phorbol esters (Fig. 2A; quantitated in Fig. 2B). There was a concomitant decrease in the amount of actin in the Triton X-100-insoluble fraction (Fig. 2A; quantitated in Fig. 2B). This decrease is comparable to the amount of actin released from the Triton X-100-insoluble fraction in other preparations (20, 21). Latrunculin A did not cause a significant change in the amount of PKC found in the Triton X-100-soluble

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membrane fraction either in the presence or absence of phorbol esters (Fig. 2A; quantitated in Fig. 2B). These results demonstrate that a significant fraction of the PKC that is Triton X-100-insoluble is sensitive to disruption of the actin cytoskeleton.

To demonstrate that the effects of nocodazole and latrunculin A were on different pools of PKC, we tested whether nocodazole or latrunculin A effects persisted in the presence of the other drug. Paired ganglia were treated with phorbol esters plus 300 nM nocodazole in the presence or absence of 5 μM latrunculin A or, alternatively, with phorbol esters plus 5 μM nocodazole. We found that the actions of the two cytoskeletal disrupters were independent of one another. Both decreased the percentage of PKCs found in the Triton X-100-insoluble fraction even in the presence of the other inhibitor (Fig. 3, A and B; quantitated in Fig. 3C). Indeed, the changes appear to be a bit larger as expected if the effects were independent (i.e. nocodazole removed a higher percentage of the Triton X-100-insoluble PKC, as the actin-associated PKC was no longer present because of the addition of latrunculin A to both control and experimental samples). Thus, there appear to be two separate pools of cytoskeleton-associated PKCs, one associated with actin filaments and one associated with microtubules.

These experiments allowed us to approximate the percentage of PKC that is Triton X-100-insoluble and sensitive to cytoskeletal disrupters. Under basal conditions, 18 ± 5% of PKC Apl II (n = 8) and 17 ± 3% of PKC Apl I (n = 8) were associated with the Triton X-100-insoluble fraction. Some of this kinase was sensitive to disruption of the cytoskeleton because, in the presence of both nocodazole and latrunculin,
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DISCUSSION

The Cytoskeleton Is a Major Anchoring Site for PKC in Aplysia Neurons—One important result from this study is that actin, tubulin, and cytoskeleton-associated proteins are major anchoring proteins for PKC in Aplysia neurons. Although our immunocytochemical experiments were restricted to growth cones, these structures make up only a small fraction of the material used for biochemistry; and thus, the cytoskeleton is presumably an important target for PKC throughout neurons. PKCs bind directly to actin and to many components of the cytoskeleton. In addition, some PKC-dependent MT advance and some perturbation of F-actin are observed at the distal tip of the MTs in the presence of PKC (22, 23). The constitutive binding of PKC to MTs is dependent on actin, tubulin, and cytoskeleton-associated proteins (24). The constitutive binding of PKC to MTs is dependent on actin, tubulin, and cytoskeleton-associated proteins. PKC-Apl II interacts with purified MTs in a 1 μM PDBu or 10 μM Gö 6976-sensitive manner. The constitutive binding of PKC to MTs is dependent on actin, tubulin, and cytoskeleton-associated proteins. PKC-Apl II interacts with purified MTs in a 1 μM PDBu or 10 μM Gö 6976-sensitive manner.
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In particular, PKCβ was shown to associate with microtubules both in T-cells (28) and in U937 cells (29). This association is important for cell motility in T-cells (30). Similar to our results with the Ca²⁺-activated PKC Apl I, interactions of the Ca²⁺-activated PKCβII with tubulin in U937 cells were shown to be indirect (31).

Translocation of PKC to Nocodazole-sensitive MTs—We have shown that phorbol esters translocated both PKCs Apl I and Apl II to the Triton X-100-insoluble pool and that this was partly reversed by low concentrations of nocodazole. One explanation for this result is that PKC constitutively associates with MTs. Phorbol ester activation of PKC present on MTs may result in phosphorylation of a protein important in promoting MT (+)-end assembly (8). Increased MT mass after phorbol ester treatment would lead to an increase in the number of sites for PKC binding and thus increased levels of MT-bound PKC. Nocodazole blocks new MT assembly and thus prevents generation of substrate for PKC translocation. This is consistent with the immunocytological results in extracted neurons showing that both PKCs are associated constitutively with MTs along their entire observable lengths. Note that there does not appear to be preferential PKC binding near MT (+)-ends that extend distally into the P domain (Fig. 7), suggesting that PKC does not bind preferentially to newly synthesized (nocodazole-
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PKC interactions with MTs are not apparent in growth cones fixed before membrane extraction. Bag cell neurons were first fixed with 3.7% formaldehyde in artificial seawater containing 400 mM sucrose. After fixation, the cells were permeabilized with 1% Triton X-100 and then stained for F-actin (A and D), MTs (B and E), and PKC Apl II (ApII; C and F). After activating PKC with 1 μM PDBu for 30 min (A–C), no obvious co-localization with either F-actin or MTs was observed. Inactivation of PKC with 10 μM Go 6976 showed some PKC Apl II co-localization with F-actin in a manner similar to controls. Scale bar = 20 μm.

PKC Localization to Actin Filaments—Our results with latrunculin A show that a sizable fraction of each PKC isoform was associated with the actin cytoskeleton both before and after phorbol ester treatment (Fig. 2). PKCs bind to purified actin in a phorbol ester-dependent manner (3). However, inhibitors of PKC reduce interactions of PKC with actin (3), suggesting that the translocation to actin may be transient and thus difficult to see in steady-state experiments.

PKC Localization in Growth Cones—When growth cones were fixed and then extracted and labeled, PKC Apl II appeared to be mainly cytosolic, with some concentration in puncta. This punctate labeling in the F-actin-rich P domain tended to increase after PKC inhibition (Fig. 6F), consistent with our previous findings (3). However, under these conditions, localization to MTs was not readily apparent. In contrast, we found that live cell extraction in CSB revealed clear PKC Apl I and Apl II interactions with MTs, both after PKC activation (Fig. 7) and in the presence of PKC inhibitors (Fig. 8). These results suggest that PKCs Apl I and Apl II associate constitutively with MTs and that this localization does not depend critically on the enzymatic activity of PKC. PKC-MT interactions in the P domain were not apparent under normal fixation conditions probably because of relatively high levels of cytosolic and membrane-associated PKC labeling. We cannot rule out the possibility that the observed PKC-MT interactions result from removal of cytosolic factors that normally inhibit PKC-MT interactions. These issues may be resolved by direct visualization of green fluorescent protein-PKC dynamics in living cells in future studies. With that said, it is important to emphasize that the relocalization of PKC and MTs in Figs. 7 and 8 is not MT signal bleed-through, as it was observed in preparations labeled for PKC alone (data not shown). MT labeling in the live cell-extracted growth cones also appears to be specific, as there was essentially no PKC localization to the robust F-actin structures present in Figs. 7 and 8. These findings suggest that much of the PKC normally associated with F-actin structures in growth cones fixed before extraction (Fig. 6) is labile and associated with the Triton X-100-sensitive fraction, localization to MTs was not readily apparent. In contrast, latrunculin-sensitive PKC-actin interactions in the biochemical experiments involved Triton X-100-insoluble actin. This pool of PKC bound to actin is either not enriched in growth cones or sensitive in some other manner to the conditions used for extraction and fixation.

Summary—PKC activation by phorbol esters stimulates distal MT advance in the growth cone. It is likely that the association of PKC with MTs is important for this ability. In addition, PKCs are associated with more proximal and stable MTs in the central domain. Kinases are often anchored near their substrates, and this anchoring is often critical for efficient signal transduction (33–35). The large percentage of PKCs associated with microtubules and actin filaments in neurons suggests that the cytoskeleton is one of the major targets of PKC action in these cells. In the future, it will be important to...
understand how the activity of PKC on microtubules is regulated under physiological conditions to regulate neuronal outgrowth, delivery of materials, and growth cone guidance.

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