Phosphorylation of calcium-activated protein kinase Cs (PKCs) at threonine 634 and/or threonine 641 increases during long term potentiation or associative learning in rodents. In the marine mollusk Aplysia, persistent activation of the calcium-activated PKC Apl I occurs during long term facilitation. We have facilitated an antibody to a peptide from PKC Apl I phosphorylated at threonines 613 and 620 (sites homologous to threonines 634 and 641). This antibody recognizes PKC Apl I only when it is phosphorylated at threonine 613. Both phorbol esters and serotonin increase the percentage of kinase phosphorylated at threonine 613 in Aplysia neurons. Furthermore, the pool of PKC that is phosphorylated at threonine 613 in neurons is resistant to both membrane translocation and down-regulation. Replacement of threonine 613 with alanine increased the affinity of PKC Apl I for calcium, suggesting that phosphorylation of this site may reduce the ability of PKC Apl I to translocate to membranes in the presence of calcium. We propose that phosphorylation of this site is important for removal of PKC from the membrane and may be a mechanism for negative feedback of PKC activation.

Protein kinase Cs (PKCs) represent a large family of proteins that undergo complex regulation. Classical isoforms of PKC are activated by a combination of two second messengers, calcium and diacylglycerol. Calcium increases the affinity of the enzyme for lipids through its interaction with the C2 domain, and diacylglycerol induces a high affinity interaction with the membrane that leads to enzyme activation (1, 2). In order to be activated by second messengers, PKC is first activated through phosphorylation by both phosphoinositide-dependent kinase 1 (3–5) and by autophosphorylation (6). Two major autophosphorylation sites have been identified in classical PKCs. Autophosphorylation at threonine 641 (PKC βII) is required for formation of active PKC (6–10), and autophosphorylation at serine 660 (PKC βII) is important for stabilization of enzyme conformation (11, 12). Interestingly, mutations at serine 660 also affect the affinity of the enzyme for calcium, suggesting that in the intact kinase interactions occur between the C2 domain and the carboxyl terminus (12). A number of other autophosphorylation sites have been identified after activation of PKCs by phorbol esters (13), but these sites are not important for PKC activity (8, 14). However, one of these sites, threonine 634 in PKC βII, is conserved over evolution. Furthermore, phosphorylation at threonine 634 and threonine 641 increases after long term potentiation or after associative learning in rodents (15, 16), suggesting a role for phosphorylation at these sites in regulation of PKC in the nervous system.

In the marine mollusk Aplysia californica, only two phorbol ester-activated isoforms of PKC exist, PKC Apl I and PKC Apl II (17). PKC Apl I is a classical PKC that contains a C2 domain that is regulated by calcium, while PKC Apl II contains an amino-terminal C2 domain that is homologous to those of PKCs and PKCζ and not regulated by calcium (18). The major autophosphorylation sites in vertebrate PKCs are conserved in the Aplysia isoforms, as is the site at threonine 634 (Fig. 1).

PKC is persistently activated during the formation of long term synaptic changes in both vertebrates and invertebrates (19–23). In both model systems, an autonomous form of PKC is formed that may be important for the maintenance of synaptic changes at intermediate times (19, 20, 24). The autonomous form of PKC in vertebrates has been suggested to be due to phosphorylation of a classical isofrom or the formation of protein kinase M from the atypical ζ form (25, 26). In contrast, the autonomous PKC in Aplysia is generated by a modification in the regulatory domain of the novel PKC Apl II (24). Translocation of PKC Apl I to the membrane and the amount of activable PKC Apl I in the membrane also increase at this time, demonstrating that PKC Apl I is persistently activated during this period although not constitutively active (21).

To determine if phosphorylation of PKC Apl I at the equivalent positions of threonine 634 and threonine 641 (threonine 613 and threonine 620) is involved in the persistent activation of PKC Apl I, we raised a phosphopeptide antibody to this site. This antibody only recognized PKC Apl I when phosphorylated at threonine 613. We found that this site is phosphorylated in neurons and that the percentage of kinase phosphorylated at this site increased after phorbol ester and serotonin treatment. Interestingly, PKC Apl I phosphorylated at this site was found in the cytoplasmic fraction to a greater extent, and was more resistant to down-regulation, than the bulk of PKC. These results suggest that phosphorylation of this site is involved in removing PKC from the membrane.

**EXPERIMENTAL PROCEDURES**

Antibody Production and Immunoblotting—A 17-amino acid carboxyl-terminal peptide, containing residues 606–623 of Aplysia PKC Apl I with threonine 613 and threonine 620 converted to phosphothreonine...
was formed by the mutagenesis and was used to confirm the cloning. The clone was sequenced over the entire amplified region to confirm no additional changes were made.

Recombination of the transfer vectors into baculovirus and generation of hig h titer baculovirus stocks were performed as described (29).

In Vitro Phosphorylation—Phosphorylation was initiated by the addition of purified kinases to the phosphorylation mix (50 mM TPA, 5 μg/ml PS, 500 mM CaCl₂, 50 μM ATP (10 μM when incorporation of γ-32P)ATP (1–3 μCi, NEN Life Science Products) was investigated), 45 mM MgCl₂, 2.6 mM 2-mercaptoethanol, 20 μg/ml aprotinin, 5 mM benzamidine, and 0.1 mM leupeptin. All further steps were performed at 4 °C. Control experiments demonstrated that after this calibration, values were linear with respect to the amount of protein over a wide range of values (28).

Generation of PKC T613A and PKC T620E, S639E—Single-amino acid mutations were generated with a two-step mutagenic procedure using the polymerase chain reaction (PCR). For the T613A mutation, first round PCR used the ApI I DNA in BlueScriptSK (Invitrogen) (29) as a template and either the outside 5′ primer 5′-CCTATGAGGATTGTT-GCTGTACG and the inside 3′ primer 5′-TTCACTCGCGAACTCTCGG-TAGGACGC or the inside 5′ primer 5′-GCTGTACGACTCACTATAGGGC (T7 primer) as the outside 3′ primer. The products from the first round synthesis were combined and used as the template for second round synthesis using the two outside primers. The resultant product was cut with AvrII and NdeI and inserted into the ApI I cDNA in the baculovirus transfer vector Bluebac4 (Invitrogen) (29) cut with the same enzymes. An NruI site was formed by the mutagenesis and was used to confirm the cloning. For the S639E mutation, an internal EcoRI site in PKC ApI I was used for a single PCR procedure using the 5′ primer 5′-GGAAA-TGGAGCAGTTCTCAGATG-3′ and the T7 outside primer. The PCR product was cut with EcoRI and NdeI and inserted into the ApI I cDNA in the baculovirus transfer vector Bluebac4 (29) cut with the same enzymes. A BsrII site was formed by the mutagenesis and was used to confirm the cloning. For the T620E mutation, the S639E mutation, and an internal EcoRI site in PKC ApI I was used for a single PCR procedure using the 5′ primer 5′-GGAAATTCGAGGCTCCTCACATGTC-3′ and the T7 outside primer. The PCR product was cut with EcoRI and NdeI and inserted into the ApI I cDNA in the baculovirus transfer vector Bluebac4 (29) cut with the same enzymes. A NruI site was formed by the mutagenesis and was used to confirm the cloning.

Treatment of Ganglia with PDBu or 5-HT—Aplysia (50–250 g, Marine Specimens, Pacific Palisades, CA) were kept in an aquarium for at least 3 days before experimentation. The animals were first placed in a bath of isotonic MgCl₂/artificial sea water (1:1, v/v) and then anesthetized by injection of isotonic MgCl₂. Ganglia were isolated from the animal and pinned to silicone plastic in ice-cold dissecting medium containing high magnesium and low calcium (21). The two pleural and pedal ganglia from each animal were dissected, desheathed, and rested for 1–3 h in resting media at 15 °C (21) with 10 mM glutamine and 0.1% glucose added just before use. One ganglion from each animal was treated with vehicle (control ganglion) while the other ganglion received either 2 μM 4-p-PDBu (experimental ganglion) for 1 h or 20 μM 5-HT for 30 min at 15 °C. In experiments using 5-HT, both ganglia were then gently washed in resting medium three times and incubated in resting media for 30 min at 15 °C. At the end of the incubations, the resting medium was replaced with ice-cold homogenization media, and the ganglia were desheathed and transferred to homogenizing microtubes ( Kontes) containing 125–150 μl of supplemented homogenization buffer (same as above in addition to 50 mM NaF, 5 mM sodium pyrophosphate (pH 8.5), and 1 μM microcystin). The ganglia were homogenized on ice in the microtubes and centrifuged at 2500 × g at 4 °C for 3 min. Subsequently, these homogenates were centrifuged at 100,000 × g for 30 min at 4 °C. The supernatant was taken, and the pellets were resuspended in 100 μl of supplemented homogenization buffer. A sample from each condition was removed for analysis of total protein, the remaining sample was boiled in Laemmli buffer, and equal amounts of protein from control and experimental homogenates were loaded on 9% SDS-polyacrylamide gels. After transfer to nitrocel lulose, membranes were immunoblotted with anti-Apl I-P followed by stripping of the membrane and Western blotting with anti-Apl I.
this report use antibody from rabbit 2018 (anti-Apl I-P). To ensure specificity to phosphorylated PKC, the purified antibody was also incubated with nonphosphorylated peptide prior to immunoblotting. Under these conditions, anti-Apl I-P did not recognize PKC Apl I expressed in SF9 cells using baculovirus (Fig. 2A). Stimulation of purified PKC Apl I with PKC activators and ATP led to the phosphorylation of PKC Apl I and the appearance of immunoreactivity with anti-Apl I-P (Fig. 2A). These results suggest that immunoreactivity depends on the phosphorylation of threonine 613, since PKCs isolated from SF9 cells are likely to be phosphorylated at threonine 620 before activation (6, 31), but the isolated PKC is only recognized by anti-Apl I-P after stimulation with PKC activators and ATP in vitro.

To confirm that the increase in immunoreactivity was not due to in vitro phosphorylation at threonine 620, we replaced both this threonine and the constitutively phosphorylated serine 639 with glutamic acid (PKC Apl I T620E, S639E). The kinase was purified from SF9 cells infected with a baculovirus expressing the construct. Similar to wild type PKC Apl I, this kinase was only detected by anti-Apl I-P after stimulation with PKC activators and ATP (Fig. 2B). Thus, a negative charge at threonine 620 was not sufficient for immunoreactivity by itself but permitted immunoreactivity after phosphorylation of threonine 613 in vitro. Multiple bands were recognized by anti-Apl I-P in PKC Apl I T620E, S639E (Fig. 2B). These bands may still represent differentially phosphorylated forms of PKC Apl I, since preliminary phosphopeptide mapping of PKC Apl I suggests that additional autophosphorylation sites are present in PKC Apl I apart from threonine 613, threonine 620, and serine 639 (data not shown).

To demonstrate conclusively that phosphorylation at threonine 613 was required for immunoreactivity, we examined phosphorylation of PKC Apl I after replacing threonine 613 with alanine (PKC Apl I T613A). This construct was not immunoreactive with anti-Apl I-P before stimulation in vitro (Figs. 2B and 3B), despite the high specific activity of this protein (Fig. 2C) and its ability to autophosphorylate at other positions (Fig. 3B).

These results demonstrate that anti-Apl I-P recognizes PKC when it is phosphorylated at threonine 613. Evidence from vertebrate PKCs suggests that the enzyme is inactive unless phosphorylated at threonine 620 (8, 9) and that this site is quantitatively phosphorylated in cells (6, 31). Thus, we expect that most kinase phosphorylated at threonine 613 will also be phosphorylated at threonine 620. However, our results do not demonstrate whether anti-Apl I-P can recognize PKC phosphorylated at threonine 613 without a negative charge at threonine 620 but permitted immunoreactivity after phosphorylation at threonine 613 in vitro.

Phosphorylation of purified PKC Apl I at threonine 613 is slow, increasing throughout a 60-min period of activation (Fig. 3A). This is similar to the bulk of PKC Apl I autophosphorylation measured by incorporation of ATP (Fig. 3A). Threonine 613 is not the only site phosphorylated under these conditions, since incorporation of ATP into PKC Apl I T613A was comparable with that of PKC Apl I (Fig. 3B), despite the lack of threonine 613 phosphorylation in this kinase.
Phosphorylation of PKC Apl I at Threonine 613

Characterization of Phosphorylation of Threonine 613 in Neurons—To determine if threonine 613 is phosphorylated in neurons, we examined Aplysia nervous systems that were treated for 1 h with 2 µM R-5652 or 1 h with 2 µM of vehicle (0.02% dimethyl sulfoxide). As previously reported, R-5652 treatment led to the translocation of PKC Apl I, followed by a 30-min wash in resting medium. 40 µg of the supernatant fraction from each ganglion were separated on a 9% SDS-PAGE gel, transferred to nitrocellulose, and immunoblotted with anti-Apl I-P. The blot was stripped and reprobed with the anti-Apl I. Different preparations showed a specific decrease in cytosolic PKC Apl I that is not phosphorylated at threonine 613, due to down-regulation. Consistent with this interpretation, the increase in the percentage of kinase phosphorylated at threonine 613 correlated with the amount of PKC Apl I down-regulated (Fig. 4C). Thus, phosphorylation at threonine 613 may protect PKC Apl I from down-regulation, this protection could be due solely to the lack of translocation, since down-regulation may be stimulated by membrane binding (33, 34).

We also examined whether treatment with the physiological activator 5-HT led to an increase in PKC Apl I phosphorylation. A significant increase in the percentage of cytosolic PKC Apl I phosphorylated at threonine 613 was observed 30 min after a 5-HT application of 20 µM 5-HT (Fig. 5). In this case, we did not observe down-regulation of cytosolic PKC Apl I (Fig. 5). Thus, increased phosphorylation at threonine 613 occurs during the persistent activation of PKC in Aplysia ganglia, similar to results seen during persistent activation of PKC in vertebrate hippocampus (16), although in the vertebrate study increased phosphorylation at other sites or an increase in PKC expression may also have contributed to the signal (16).

PKC Apl I T613A Has a Higher Affinity for Calcium than Wild Type PKC—To determine the importance of threonine 613 for activation and translocation of PKC, we examined the requirements for kinase activity of the PKC Apl I T613A mutant (Fig. 6). PKC Apl I T613A had similar phospholipid requirements as wild type PKC in the absence of calcium. In contrast, PKC Apl I T613A required significantly less calcium at limiting PS levels for activation (Fig. 6). These results suggest that this site may regulate the binding of calcium to the C2 domain of PKC Apl I. Previously, it has been reported that phosphorylation of serine 660 is involved in the binding of calcium (12), but this is the first evidence that phosphorylation at threonine 613 (threonine 634 in PKC βII) is also important for the interaction of calcium with the C2 domain. This observation suggests the
phosphorylation only occurred with activation by phorbol esters and not by calcium ions. However, we observed a similar phosphorylation of threonine 613 in PKC Apl I after stimulation by either PS and calcium or PS and TPA in vitro (data not shown).

Our study is also in agreement with the recent work of Feng and Hannun (36), which demonstrated that PKC βII requires autophosphorylation to be removed from membranes. In this study, conversion of threonine 614 and serine 660 to alanine substantially reduced the ability of PKC to be removed from the membrane. However, this double alanine mutant was still significantly removed from the membrane compared with catalytically inactive PKC. These results are consistent with phosphorylation at threonine 634 (threonine 634 in Aplysia) being critical for removal from the membrane, since phosphorylation at threonine 614 and serine 660 may be important for the ability of PKC to autophosphorylate at this position (6).

We observed an increase in phosphorylation of threonine 613 after 5-HT addition, corresponding to a time there is a persistent increase in PKC Apl I activity and autonomous PKC activity (21). We do not believe that phosphorylation at threonine 613 is important for persistent activation of PKC Apl I or formation of the autonomous kinase, since all threonine 613-phosphorylated PKC is in the cytosol, and there is no increase in the amount of cytosolic activable PKC Apl I or in cytosolic autonomous activity at this time point (21). Furthermore, no autonomous activity from the cytosol can be immunoprecipitated with the antibody to PKC Apl I (21, 24). We favor a model whereby increased phosphorylation at threonine 613 is a consequence of persistent activation of PKC but not a cause of persistent activation of PKC.

**Regulation of PKC by Phosphorylation**—A number of aspects of PKC regulation have been linked to the phosphorylation state of the enzyme, and these phosphorylation events all lead to an increase in PKC activity (6). In contrast, we suggest that phosphorylation at threonine 613 is not important for kinase activity and instead may lead to less PKC activity by preventing PKC from associating with the membrane. This lack of PKC translocation could be explained in multiple ways. The change could be due to a decrease in the affinity for lipids, although we have not been able to demonstrate a decrease in lipid binding in vitro (data not shown). There may be a decrease in the affinity of the enzyme for calcium, and translocation of PKC Apl I by phorbol esters depends on calcium in vitro (37). Since we cannot demonstrate the lack of translocation with in vitro phosphorylated PKC, additional proteins may be required to prevent PKC Apl I translocation in neurons. In particular, a cytosolic protein might specifically bind to PKC phosphorylated at threonine 613 and retain it in the cytoplasm. Whatever the mechanism for prevention of PKC translocation, the lack of translocation may lead to a decrease in activable PKC. We suggest therefore that phosphorylation of this residue is a mechanism for negative feedback regulation of PKC.

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