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Protected-Site Phosphorylation of Protein Kinase C in Hippocampal Long-Term Potentiation

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Abstract: One important aspect of synaptic plasticity is that transient stimulation of neuronal cell surface receptors can lead to long-lasting biochemical and physiological effects in neurons. In long-term potentiation (LTP), generation of autonomously active protein kinase C (PKC) is one biochemical effect persisting beyond the NMDA receptor activation that triggers plasticity. We previously observed that the expression of early LTP is associated with a phosphatase-reversible alteration in PKC immunoreactivity, suggesting that autophosphorylation of PKC might be elevated in LTP. In the present studies we tested the hypothesis that PKC phosphorylation is persistently increased in the early maintenance of LTP. We generated an antiserum that selectively recognizes the α and βII isoforms of PKC autophosphorylated in the C-terminal domain. Using western blotting with this antiserum we observed an NMDA receptor-mediated increase in phosphorylation of PKC 1 h after LTP was induced. How is the increased phosphorylation maintained in the cell in the face of ongoing phosphatase activity? We observed that dephosphorylation of PKC in vitro requires the presence of cofactors normally serving to activate PKC, i.e., Ca^{2+}, phosphatidylserine, and diacylglycerol. Based on these observations and computer modeling of the three-dimensional structure of the PKC catalytic core, we propose a "protected site" model of PKC autophosphorylation, whereby the conformation of PKC regulates accessibility of the phosphates to phosphatase. Although we have proposed the protected site model based on our studies of PKC phosphorylation in LTP, phosphorylation of protected sites might be a general biochemical mechanism for the generation of stable, long-lasting physiologic changes. **Key Words:** Protein kinase C—Autophosphorylation—Dephosphorylation—Hippocampus.
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One intriguing problem in contemporary neuroscience is understanding the mechanisms by which a transient stimulation of cell surface receptors can lead to a long-lasting effect in neurons. One phenomenon for which this question is particularly relevant is long-term potentiation (LTP) of synaptic transmission in the hippocampus. In the most widely studied form of LTP, a transient activation of NMDA receptors leads to a robust, long-lasting increase in synaptic transmission. Understanding the mechanisms of the persistence of LTP therefore requires an understanding of the means of generating lasting sequelae downstream of the NMDA receptor.

In LTP, there is good evidence that generation of autonomously active protein kinase C (PKC) is one biochemical effect persisting beyond the period of NMDA receptor activation and that autonomously active PKC plays a role in the expression of the early phase of synaptic potentiation, i.e., potentiation lasting 30 min–2 h into LTP, referred to as early LTP (or E-LTP). For example, inhibitors of PKC can reverse expression of LTP (Lovinger et al., 1987; Malinow et al., 1988; Colley et al., 1990; Wang and Feng, 1992). Moreover, it is known that activation of PKC is sufficient to elicit increased synaptic transmission in the hippocampus (Malenka et al., 1986, 1987; Hu et al., 1987; Hvalby et al., 1988; Muller et al., 1988), and autonomously active PKC has been shown to be present during the expression of both early and late LTP (Lovinger et al., 1985; Klann et al., 1991, 1993; Gianotti et al., 1992; Leahy et al., 1993; Sacktor et al., 1993; Schwartz, 1993; Osten et al., 1996). Therefore, in LTP, one mechanism by which transient signals are converted to a long-lasting physiologic effect is through the generation of autonomously active PKC.

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Abbreviations used: APV, 2-amino-5-phosphonovalerate; CaMKII, calcium/calmodulin-dependent protein kinase II; DAG, 1,2-dioctanoyl-*sn*-glycerol; EPSP, excitatory postsynaptic potential; HFS, high-frequency stimulation; LTP, long-term potentiation; PAGE, polyacrylamide gel electrophoresis; PKA, protein kinase A; PKC, protein kinase C; PP, protein phosphatase; PS, L-α-phosphatidylserine; SDS, sodium dodecyl sulfate.

As part of earlier studies on the mechanisms and consequences of persistent PKC activation in LTP, Klann et al. (1993) observed that early LTP is associated with a phosphatase-reversible alteration in PKC immunoreactivity, suggesting that increased phosphorylation of PKC might occur in LTP. In the present studies we tested the hypothesis that PKC phosphorylation is increased during early LTP expression, using an antibody we generated that is selective for autophosphorylated PKC.

MATERIALS AND METHODS

Production of antisera

A peptide was synthesized at the Baylor College of Medicine Protein Core Facility corresponding to residues 629–644 of PKC βII that had phosphothreonine at amino acids 634 and 641. The peptide was coupled to keyhole limpet hemocyanin, and the conjugate was injected into rabbits. Antisera were generated in albino New Zealand rabbits, using intradermal injection of antigen in Titermax adjuvant (total volume, 0.1 ml). Approximately every 2 months sera were tested for appropriate immunoreactivity, and the rabbits were boosted with antigen. Antisera were screened using western blotting procedures as described below.

PKC autophosphorylation

Rat brain PKC was purified according to the technique of Huang et al. (1986). For the experiments presented in Fig. 3, PKC was autophosphorylated by incubating in the presence of [^{32}P]ATP, with and without activators [1.5 m*M* $CaCl_2$, 320 μg/ml L-α-phosphatidylserine (PS), and 30 μg/ml 1,2-dioctanoyl-*sn*-glycerol (DAG)]. Reaction conditions were as follows: 20 m*M* Tris-HCl (pH 7.5), 0.5 m*M* EGTA, 0.5 m*M* EDTA, 20 μ*M* ATP (10 μCi per reaction), 10 m*M* $MgCl_2$, 1 μg/ml aprotinin, 50 μg/ml leupeptin, and 2 m*M* $Na_4P_2O_7$. To obtain a time course of PKC autophosphorylation, reaction times ranged from 2 to 60 min. Reactions were stopped with addition of sample buffer [75 m*M* Tris-HCl (pH 6.8), 10% glycerol, 2% sodium dodecyl sulfate (SDS), 0.01 mg/ml bromophenol blue, and 50 m*M* dithiothreitol]. The protein was electrophoresed on a 10% polyacrylamide gel, lightly Coomassie Blue-stained, and exposed to Kodak X-Omat AR film. Using the autoradiograph as a guide, the ^{32}P-labeled PKC band was dissected from the gel, chopped finely, and immersed in 3 ml of Aquasol. Radioactivity was measured by scintillation counting. An adjacent blank piece of gel was excised and used to subtract out background ^{32}P binding.

For the experiments described in Figs. 4, 5, and 8, preparation of detergent-soluble extracts of Sf-21 cells overexpressing various forms of PKC (PKC γ, PKC βII, and a T641A/T634A PKC βII mutant) was as described (Orr et al., 1992; Keranen et al., 1995). PKC activity toward a synthetic peptide was assayed, and dephosphorylation of PKC by protein phosphatase (PP) 1 or 2a was performed as described (Dutil et al., 1994). Pure PKC βII (100 ng per lane) or T634A/T641A PKC extract (~100 ng of PKC per lane) was autophosphorylated as described (Keranen et al., 1995) in the presence of PS/DAG membranes (140 and 4 μ*M*, respectively) and 500 μ*M* Ca^{2+} for 1 h at 30°C, unless otherwise indicated. Autophosphorylated PKC was analyzed by western blotting, or phosphorylation was quantified directly by phosphoimaging (Molecular Dynamics), either before or after further manipulation by proteolysis or dephosphorylation. For proteolysis experiments, Lys-C (0.25 unit/ml) was added to autophosphorylated PKC, and the reaction mixture was incubated at 30°C. At the indicated time, aliquots were removed into SDS-polyacrylamide gel electrophoresis (PAGE) sample buffer. Samples were electrophoresed using 9% SDS-PAGE, transferred to nitrocellulose, and analyzed as described (Keranen et al., 1995).

Western blotting of purified PKC

For Figs. 4, 5, and 8, samples were analyzed by SDS-PAGE using 7 or 9% acrylamide gels. Proteins were electrophoretically transferred to nitrocellulose using an LKB Multiphor II transfer unit at 0.8 mA/cm^2 area of filter. Molecular weight markers were visualized after transfer by Ponceau S staining of the filter. Blots were blocked in phosphate-buffered saline [50 m*M* sodium phosphate (pH 7.5), 150 m*M* NaCl, 0.05% Tween 20, and 5% milk powder], probed sequentially with antiserum 96160 (1:1,000) followed by incubation with a horseradish peroxidase-linked goat anti-rabbit IgG (1:20,000), and developed using enhanced chemiluminescence (Pierce, Rockford, IL, U.S.A.). Western blotting using the catalytic domain antibody and silver staining were performed as previously described (Poehling and Neuhoff, 1981; Keranen et al., 1995).

Immunoprecipitation of autophosphorylated PKC

Purified rat brain PKC was stimulated to undergo autophosphorylation by incubation (15 min, 37°C) with Ca^{2+} (1.5 m*M*) and lipid activators (320 μg/ml PS and 30 μg/ml DAG), using [^{32}P]ATP [100 μ*M* ATP (2 μCi/nmol) plus 10 m*M* Mg^{2+} in 20 m*M* Tris-HCl (pH 7.5), 0.5 m*M* EGTA, and 0.5 m*M* EDTA] in the incubation mixture to radiolabel the phosphorylated enzyme. Labeled autophosphorylated PKC was then incubated at 4°C for 12 h with either immunoprecipitation buffer alone [50 m*M* Tris-HCl (pH 7.5), 50 m*M* NaCl, 10 m*M* EGTA, 5 m*M* EDTA, and 1% Triton X-100] or a 1:500 dilution of antiserum 96160 in immunoprecipitation buffer. Then, protein A-Sepharose beads were added, and the incubations were continued for 1.5 h. The beads with attendant material were separated by centrifugation and washed three times using immunoprecipitation buffer. Beads were then incubated overnight at 4°C with or without phosphopeptide antigen.

Electrophysiology and LTP

Rat hippocampal slices were prepared, and electrophysiological responses in the CA1 subfield were monitored as described by Klann et al. (1993), as modified by English and Sweatt (1996). In brief, slices were maintained at 32°C, and responses to Schaffer collateral stimulation were monitored for at least 15 min preceding the delivery of tetanic stimulation to assure stable baseline responses. Responses were measured as the initial slope of field excitatory postsynaptic potentials (EPSPs) of an average of six individual traces. Tetanic stimulation consisted of three sets of stimuli, with each set delivered 10 min apart. Each set comprised two 1-s trains of stimuli (100 Hz), given 20 s apart. This protocol produced LTP with an average 82 ± 15% (n = 7) increase in the initial slope of the population EPSP 45 min after the final tetanus. Experimental and control slices were removed from the slice chamber 45–60 min after the last period of tetanic stimulation and frozen on dry ice, and the CA1 region between the position of the stimulating and recording electrodes was dissected. The microdissected subre-

gions were homogenized in 100 μl of electrophoresis sample buffer before subsequent western blotting.

Western blotting of LTP samples

Western blot procedures were performed essentially as described for Figs. 2, 3, and 6 (Klann et al., 1993). Proteins from control and LTP homogenates (5–10 μg) were separated on 8.5% polyacrylamide gels. All molecular weights were estimated by comparison with Rainbow colored molecular weight markers (Amersham, Arlington Heights, IL, U.S.A.). Proteins were transferred onto Immobilon-P polyvinylidine difluoride transfer membranes using a Transphor Power Lid (Hoefer) set at maximal voltage for 1–1.5 h at 4°C. Nonspecific protein binding to Immobilon-P was blocked using 3% dried milk in Tris-buffered saline. Blots were incubated with antiserum 96160 and developed as described above. In some cases blots were stripped with stripping buffer [62.5 m*M* Tris-HCl (pH 6.7), 2% SDS, and 0.1 *M* β-mercaptoethanol] and subsequently reprobed with a different antiserum. Blots were exposed for an interval appropriate to be in the linear range of the film and were quantified by digital densitometry using NIH Image. Values for immunoreactivity of protein bands on western blots were normalized to protein content in the individual sample and then to immunoreactivity observed in a within-animal control slice.

Computer modeling of PKC

Modeling of the carboxy-terminal phosphorylation domain of PKC was performed essentially as described by Orr and Newton (1994). A three-dimensional model of the catalytic core of PKC (amino acids 340–628), based on the reported structure of cyclic AMP-dependent protein kinase A (PKA) and reported by Orr and Newton (1994), was kindly provided by J. Orr. In the present studies, the model was extended through amino acid 644. This was accomplished using the PKA crystal structure (Zheng et al., 1993) of the homologous region as a starting point and substituting where appropriate the amino acid side chain to correspond to the phosphorylated PKC βII sequence using Hyperchem (AutoDesk, Sausalito, CA, U.S.A.). The resulting construction was then subjected to 30 cycles of energy minimization using the AMBER force field in Hyperchem and the Polak–Ribiere conjugation gradient optimizer. Molecular graphics were rendered using RASMOL (Roger A. Sayle, Glaxo, Middlesex, U.K.).

Dephosphorylation of autophosphorylated PKC

To the autophosphorylated PKC reaction described above for Figs. 4, 5, and 8, PS/DAG membranes and Ca^{2+} were added to final concentrations of 210 μ*M* (97 mol%)/6 μ*M* (3 mol%) and 400 μ*M*, respectively (activating conditions), or activators were diluted with Triton X-100 (2 m*M*) to form mixed micelles containing 2 mol% PS and 0.05 mol% DAG in a solution with 100 μ*M* Ca^{2+} (nonactivating conditions). PP1 (2 units/ml) was added with 200 μ*M* $MnCl_2$, and the reaction mixture was incubated at 23°C. At the indicated times, aliquots were removed into SDS-PAGE sample buffer. Samples were electrophoresed using 7% SDS-PAGE and transferred to nitrocellulose, and ^{32}P was quantified by phosphoimaging or western blotting with antiserum 96160 performed as described above.

Materials

Animal experimental protocols were approved by the Baylor College of Medicine Animal Care and Use Institutional Review Committee and meet the guidelines of the National Institutes of Health. PKC βII (rat) overexpressed using the baculovirus system was purified to homogeneity from a Triton X-100-soluble fraction as described (Orr et al., 1992). This PKC is fully phosphorylated at positions T641 and S660 and ~50% phosphorylated at T500 (Keranen et al., 1995). PKC βI (rat) or α (bovine brain) was overexpressed and purified using the same protocol. PP1 and PP2a were provided by A. DePaoli-Roach (Indiana University, Indianapolis, IN, U.S.A.). Brain PS was from Avanti Polar Lipids (Alabaster, AL, U.S.A.). DAG was from Sigma (St. Louis, MO, U.S.A.) or Calbiochem (La Jolla, CA, U.S.A.). Endoproteinase Lys-C was from Boehringer Mannheim (Indianapolis). A polyclonal antibody raised against the catalytic domain of PKC βII and a recombinant baculovirus encoding a T641A/T634A PKC βII mutant were gifts of Andrew Flint and Daniel E. Koshland, Jr. (University of California, Berkley, CA, U.S.A.).

RESULTS

Design of antigen

Klann et al. (1993) previously reported that LTP is associated with a phosphatase-reversible decrease in PKC immunoreactivity and that autophosphorylation of purified PKC led to a similar decrease in its immunoreactivity using the same antibody. As Klann et al. (1993) reported, the most parsimonious interpretation of these observations is the hypothesis that PKC autophosphorylation increases in LTP.

To test this hypothesis we sought to generate an antiserum selective for autophosphorylated PKC. PKC was originally found to autophosphorylate by an intramolecular reaction in three domains in vitro: an amino-terminal pair of sites near the autoinhibitory domain (S15 and T16), a pair of sites in the hinge region (T314 and T324), and a carboxy-terminal pair of sites (T634 and T641) (Newton and Koshland, 1987; Flint et al., 1990). Recent studies have revealed that the enzyme is phosphorylated at two additional sites: a transphosphorylation on the activation loop (T500 in PKC βII) and an autophosphorylation at an additional C-terminal site (S660 in PKC βII) (Keranen et al., 1995; Tsutakawa et al., 1995). Three of the known phosphorylation sites (T500, T641, and S660) are likely to be phosphorylations occurring concomitant with maturation of the kinase, as PKC overexpressed in unstimulated Sf-21 insect cells is stoichiometrically phosphorylated at these three positions (Keranen et al., 1995; Tsutakawa et al., 1995).

We chose the sequence containing the carboxy-terminal pair of autophosphorylation sites (T634 and T641) as the phosphospecific epitope for several reasons. First, there is a high degree of sequence conservation among the classical PKC isoforms surrounding T634 and T641 in this domain (see Fig. 1). Second, site-directed mutagenesis studies suggested that autophosphorylation in this domain has important functional consequences (Zhang et al., 1993, 1994). Finally, Klann et al. (1993) made indirect observations suggesting carboxy-terminal phosphorylation of PKC

ISOZYME		
βII	629	F D R F F T* R H P P V L T* P P D 644
βI		F D K E F T R Q P V E L T P T D
α		F D K F F T R G Q P V L T P P D
γ		F D K F F T R A A P A L T P P D

FIG. 1. Structure of the antigen used for production of the phospho-PKC-selective antiserum used in these studies. A synthetic peptide corresponding to amino acids 629–644 of the PKC βII sequence was synthesized, using phosphothreonine at the positions corresponding to amino acids 634 and 641 (marked with asterisks). The sequences from the homologous regions of the other classical isoforms of PKC are shown for comparison, to illustrate the sequence conservation in this domain.

in LTP. Therefore, we generated antisera against a peptide corresponding to amino acids 629–644 of the βII isoform of PKC that had phosphothreonine substituted at both T634 and T641 (Fig. 1).

Characterization of antiserum 96160

One antiserum, 96160, showed a high degree of selectivity for recognizing phosphorylated (vs. unphosphorylated) peptide antigen (Fig. 2). The unphosphorylated PKC βII 629–644 peptide or dually phosphorylated PKC βII 629–644 peptide was coupled to ovalbumin and then probed with antiserum 96160 by western blotting. As shown in Fig. 2, antiserum 96160 recognized the phosphopeptide but not the unphosphorylated peptide or the ovalbumin carrier protein.

We next determined whether stimulation of PKC to undergo autophosphorylation elicited an increase in immunoreactivity to antiserum 96160 (Fig. 3). To assess the ability of PKC to undergo autophosphorylation, we measured ^{32}P incorporation into purified PKC by incubating the enzyme for various intervals with

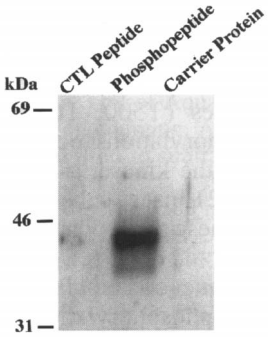

FIG. 2. Antiserum 96160 selectively recognizes the phosphopeptide antigen. Nonphosphorylated or dually phosphorylated PKC βII 629-644 peptide was coupled to ovalbumin, resolved on 8.5% polyacrylamide gels, and probed with antiserum 96160. Antiserum 96160 detected dually phosphorylated peptide (Phosphopeptide) but not the nonphosphorylated peptide (CTL Peptide) or ovalbumin alone (Carrier Protein). The other antiserum, 97349, that was generated recognized both the dephosphorylated and phosphorylated antigens coupled to ovalbumin but did not recognize ovalbumin alone (data not shown).

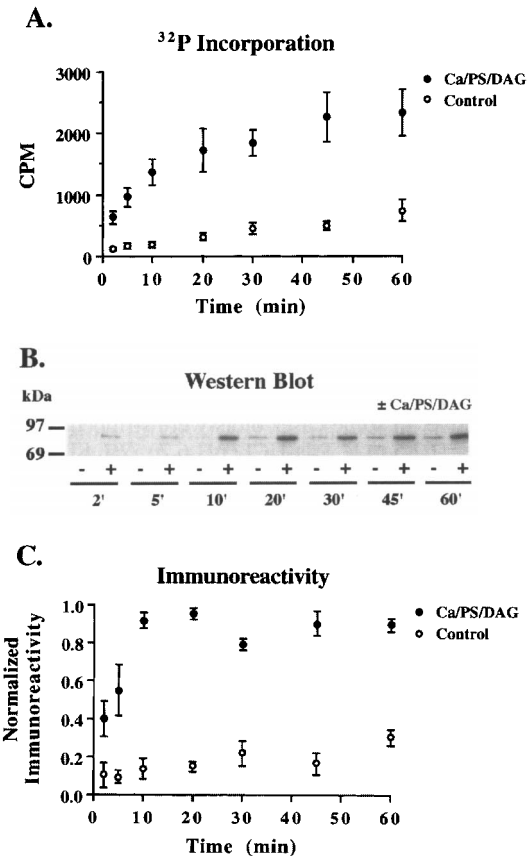

FIG. 3. Time course of PKC autophosphorylation and increased immunoreactivity to antiserum 96160. **A:** PKC was incubated for various times (2–60 min) with [^{32}P]ATP in the absence or presence of PKC activators (Ca/PS/DAG). Data are mean ± SEM (bars) values (n = 6). **B:** Representative western blot of the time course of PKC autophosphorylation using antiserum 96160. PKC was incubated as described in A but without [^{32}P]ATP. **C:** Normalized immunoreactivity using antiserum 96160. Data are mean ± SEM (bars) values (n = 5).

[^{32}P]ATP and Mg^{2+}, with or without activating cofactors (Ca^{2+}/PS/DAG). As shown in Fig. 3A, stimulation of PKC with Ca^{2+}/PS/DAG increased the incorporation of ^{32}P into PKC. We next determined whether stimulation of PKC to autophosphorylate led to increased immunoreactivity with antiserum 96160. As shown in Fig. 3B and C, PKC autophosphorylation led to a dramatic increase in immunoreactivity with antiserum 96160.

However, it is possible, although unlikely, that a contaminating kinase in the preparation was increasing PKC immunoreactivity via transphosphorylation. Thus, we assessed the effect of the selective PKC inhibitor peptide PKC_{19-36} on the Ca^{2+}/PS/DAG-stimulated increase in PKC immunoreactivity. In these experiments, we observed that PKC_{19-36} (50 μM) blocked the effects of Ca^{2+}/PS/DAG on PKC immunoreactivity (percentage of basal immunoreactivity: control, 100%; Ca^{2+}/PS/DAG, 856%; Ca^{2+}/PS/DAG plus

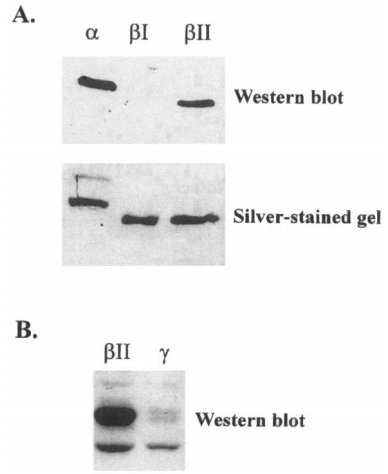

FIG. 4. Antiserum specificity for PKC isoforms. **A:** Western blot of pure PKC α, βI, and βII (200 ng) probed with antiserum 96160 (**top**) and the silver-stained gel of PKC α, βI, and βII (**bottom**). **B:** Western blot of extracts containing PKC βII or PKC γ (0.3 activity unit, in nmol of phosphate/min) probed with antiserum 96160. Equal amounts of PKC βII and γ were probed as assessed by phosphotransferase activity (data not shown).

PKC_{19-36}, 133%; and PKC_{19-36}, 58%; n = 2). These results indicate that the increased immunoreactivity was a consequence of the phosphotransferase activity of PKC.

To assess whether antiserum 96160 recognizes PKC in its native conformation, we immunoprecipitated purified PKC that had been stimulated to undergo autophosphorylation. We observed that antiserum 96160 immunoprecipitated purified PKC autophosphorylated in vitro and that antibody binding to native PKC could be competed with antigen (data not shown).

Overall, these data characterizing antiserum 96160 indicate that the antiserum selectively recognizes autophosphorylated PKC.

Selectivity of antiserum 96160 for isoforms of PKC

To identify the isoforms recognized by antibody 96160, we tested its reactivity with equal amounts of PKC α, βI, βII, or γ. Figure 4A shows the result of probing equal amounts of pure PKC α, βI, and βII with antiserum 96160. The antiserum recognized PKC α and βII equally well but did not recognize PKC βI, nor did the antibody recognize PKC γ (Fig. 4B). Thus, antiserum 96160 selectively recognizes PKC α and βII but not βI or γ.

Selectivity of antiserum 96160 for sites of autophosphorylation of PKC

We sought to determine which phosphorylated sites in PKC βII are recognized by antiserum 96160. T500, T641, and S660 are stoichiometrically phosphorylated in mature, unstimulated PKC from resting Sf-21 insect cells (Keranen et al., 1995; Tsutakawa et al., 1995). Using a long-duration western blot exposure, we observed that unstimulated PKC exhibited basal immunoreactivity, suggesting that antiserum 96160 recognizes T641 (Fig. 5A, control lane). To test this hypothesis, we examined the effect of removing this phosphate on immunoreactivity. Figure 5A shows that treatment of PKC βII with PP1, which dephosphorylates all three PKC phosphorylation sites normally phosphorylated in resting cells [T500, T641, S660 (Keranen et al., 1995)], abolished this immunoreactivity. In contrast, treatment with PP2a, which dephosphorylates positions T500 and S660 but not T641 (Keranen et al., 1995), had no significant effect on immunoreactivity. Thus, of the three amino acids that are phosphorylated stoichiometrically in mature, unstimulated PKC βII, antiserum 96160 recognizes phospho-T641 but does not recognize phospho-T500 or phospho-S660.

We next examined the specificity of the antiserum for the T634 autophosphorylation site identified from prior in vitro studies (Flint et al., 1990). This amino acid is not usually phosphorylated in unstimulated cells, but stimulation of PKC to undergo autophosphorylation in vitro greatly increases phosphorylation at this position (Flint et al., 1990). Figure 5B shows that autophosphorylation of PKC in vitro results in a dramatic increase in immunoreactivity with antiserum 96160. These results indicate that phosphorylation at T634 contributes to the immunoreactivity observed with antiserum 96160; in fact, given that purified PKC is likely already phosphorylated at T641, the data in Figs. 3 and 5B strongly suggest that the majority of immunoreactivity with antiserum 96160 is due to recognition of T634.

As an additional confirmation that phosphorylation at T634 and T641 contributes to the immunoreactivity of antiserum 96160, we determined if mutating T634 and T641 to alanines affected antiserum 96160 reactivity. With this mutant PKC (T634A/T641A), only a modest increase in immunoreactivity was seen when PKC was autophosphorylated (Fig. 5B). This observation indicates that the vast majority of immunoreactivity observed with antiserum 96160 is due to recognition of these two C-terminal autophosphorylation sites, T634 and T641.

To confirm that the increase in immunoreactivity of in vitro autophosphorylated PKC resulted from autophosphorylation at the C terminus, i.e., T634, in vitro autophosphorylated PKC was cleaved into the regulatory and catalytic fragments, and the distribution of ^{32}P and immunoreactivity was examined. Both domains were phosphorylated to a similar extent based on incorporation of ^{32}P (data not shown). However, antiserum 96160 labeled the catalytic domain >10 times more that the regulatory fragment (Fig. 5C). Thus, although antiserum 96160 can weakly recognize autophosphorylation sites in the regulatory half, >90% of the observed immunoreactivity results from phosphorylation of the C terminus.

Overall, our data indicate that antiserum 96160 is selective for autophosphorylated PKC, specifically the

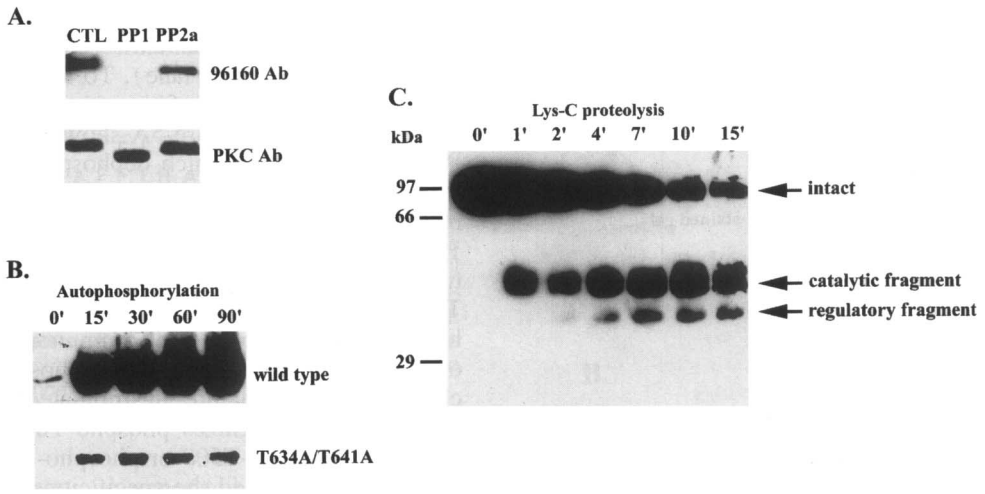

FIG. 5. Antiserum 96160 recognizes phospho-T641 and phospho-T634. **A. Upper panel:** Extensively developed western blot of native (CTL) PKC βII (150 ng), PP1-treated PKC βII, or PP2a-treated PKC βII, probed with antiserum 96160. Note that these samples were not stimulated to undergo autophosphorylation in vitro; therefore, the PKC is predominantly phosphorylated at the "processing" sites, T500, T641, and S660. **Lower panel:** The same blot shown in the upper panel was reprobed with a polyclonal antibody raised against the catalytic domain of PKC βII. **B. Upper panel:** Antiserum 96160 western blot of purified PKC βII (100 ng per lane) that has been autophosphorylated for the indicated time. **Lower panel:** Antiserum 96160 western blot of an extract containing T634A/T641A PKC βII (~100 ng per lane) that has been autophosphorylated for the indicated time. **C:** Antiserum 96160 labeling of the catalytic and regulatory fragments of autophosphorylated PKC βII (~100 ng PKC per lane). PKC βII was maximally autophosphorylated, then digested with endoprotease Lys-C for the indicated time.

α and βII isoforms. The vast majority of the immunoreactivity is accounted for by recognition of the C-terminal autophosphorylation domain from both phosphorylated residues, T634 and T641.

PKC phosphorylation in LTP

We next determined if the phosphorylation of PKC was increased in an NMDA receptor-dependent manner during the expression phase of early LTP, as assessed using antiserum 96160 in western blots of LTP samples. We chose as our LTP induction protocol three pairs of 1-s, 100-Hz stimulations, with each pair separated by 10 min, which is similar to the protocol we have used previously to demonstrate autonomous PKC activation in LTP (Klann et al., 1993) (Fig. 6A). This protocol elicits robust LTP that is NMDA receptor-dependent (Fig. 6A) (Klann et al., 1993). For each individual experiment one control slice, which was tested electrophysiologically for healthy responses, and one experimental slice, which received either tetanic stimulation or low-frequency stimulation, were used. At 45 min to 1 h after LTP induction, slices were quickly frozen on dry ice, and the CA1 areas were dissected out and subsequently solubilized in sample buffer for gel electrophoresis and western blotting. After western blotting with antiserum 96160 and densitometry to quantify PKC phosphorylation, blots were stripped with stripping buffer, reprobed with an antiserum (erk1-CT) against p44 mitogen-activated protein kinase, and quantitated densitometrically to normalize for protein loading of individual samples. We have observed previously that p44 mitogen-activated protein kinase levels do not change in LTP (English and Sweatt, 1996).

As shown in Fig. 6B and C, there is increased antiserum 96160 immunoreactivity in LTP samples, indicating increased phosphorylation of PKC in LTP (LTP = 305 ± 52% of control samples, n = 9). In control experiments we used an anti-PKC antiserum that recognizes both phosphorylated and unphosphorylated PKC equally well and observed no change in PKC protein levels in these same samples (LTP = 114 ± 29% of control samples, n = 9). This observation is consistent with the findings of Osten et al. (1996), who also observed no LTP-associated change in total PKC when using a similar LTP-inducing protocol. As is also shown in Fig. 6B and C, there was no increase in 96160 immunoreactivity in samples receiving a comparable duration of test stimulation in the absence of LTP-inducing tetanic stimulation (test stimulation = 85 ± 34% of control samples, n = 3). It should be emphasized that all LTP samples were taken at 45 min–1 h after the last pair of tetani were delivered, i.e., these samples are from the early maintenance phase of LTP. Overall these data indicate a lasting increase in the phosphorylation of the C-terminal domain of PKC during LTP.

We next sought to determine if the increased PKC phosphorylation was dependent on NMDA receptor activation. The increase in PKC immunoreactivity was blocked when slices were incubated with the NMDA receptor antagonist 2-amino-5-phosphonovalerate (APV) for the duration of the experiment [Fig. 6B and C; high-frequency stimulation (HFS) + APV = 94 ± 26% of

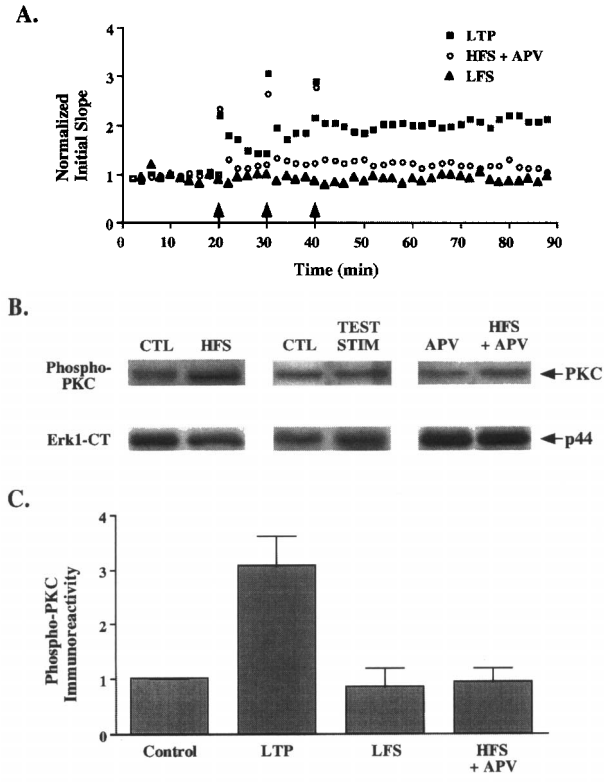

FIG. 6. Increased PKC phosphorylation in LTP. **A:** Representative physiologic recording data for the three conditions used to investigate PKC phosphorylation in LTP. The *y*-axis is the normalized initial slope of the field EPSP. Note that the HFS and HFS + APV (50 μM D, L-APV) samples each received three pairs of tetanic stimulation trains (marked with arrows). All HFS and HFS + APV samples were taken 45 min–1 h after delivery of the third period of tetanic stimulation. LFS, low-frequency stimulation. **B:** Representative western blots of individual area CA1 subregions for each of the three conditions used in these studies. Each is paired with an appropriate control slice (CTL) from the same animal. **Upper panels:** Blotting with antiserum 96160 (phospho-PKC). **Lower panels:** Anti-p44 MAP kinase blot to control for protein loading (erk1-CT). **C:** Normalized phospho-PKC immunoreactivity. Data are mean ± SEM (bars) values [LTP, n = 9; test stimulation (LFS), n = 3; HFS + APV, n = 4]. HFS is significantly different from control, LFS, and HFS + APV ($p < 0.05$ by one-way ANOVA).

control samples, n = 4]. This observation indicates a necessity for the activation of NMDA receptors for the phosphorylation of PKC to increase in response to HFS. As APV blocks both the induction of LTP and autonomous activation of PKC in LTP, this observation also strengthens the correlation between PKC phosphorylation and these phenomena.

Modeling the C-terminal autophosphorylation sites on PKC

In the preceding experiments it was observed that the increased phosphorylation of PKC persisted for 45 min–1 h after tetanic stimulation. We sought to gain insight into how the increased phosphorylation is maintained. Previous reports have indicated that dephosphorylation of PKC, either in vitro or in LTP samples, requires the presence of cofactors normally serving to activate PKC, i.e., Ca^{2+}/PS/DAG (Klann et al., 1993; Dutil et al., 1994). These observations lead to the hypothesis that phosphatase accessibility of phosphorylation sites on PKC is conformation-dependent. How are the sites of phosphorylation on PKC rendered inaccessible to phosphatases? We propose a "protected site" model, whereby the conformation of PKC regulates accessibility of the phosphates to phosphatase. In this model, in the unstimulated conformation, sites of PKC phosphorylation are protected through steric hindrance by the surrounding areas of the protein. On binding of activating ligands, a conformational change occurs that makes the phosphorylation sites accessible at the surface of the molecule. This model has the interesting implication that the dephosphorylation of PKC is regulatable. Only when PKC is in the appropriate conformation, e.g., in the presence of activating factors, will the protein be susceptible to dephosphorylation.

To gain preliminary insights into the structural correlates of protected sites of PKC phosphorylation, we undertook modeling studies of the catalytic core and carboxy-terminal autophosphorylation domain of PKC, based on the known crystal structure of the catalytic subunit of PKA (see Materials and Methods). Figure 7 shows several renderings of our model of the PKC catalytic core and C-terminal phosphorylation sites.

Our modeling suggested two potential types of protected phosphorylation sites on PKC. The first type is exemplified by the T641 phosphorylation site. The phosphate at T641 is immersed in a cleft in the upper lobe of the catalytic core, pointed inward toward the center of the molecule (Fig. 7). In addition, T641 sits on the interior of a pronounced angle in the peptide backbone of the adjacent residues, limiting accessibility of the phosphate from the exterior of the molecule. This conformation suggests that the phosphate at T641 is normally well protected by the catalytic core on one face and also by the adjacent peptide backbone. Consistent with the hypothesis, this residue is stoichiometrically phosphorylated in the PKC present in detergent-soluble fractions of unstimulated cells (Borner et al., 1989; Dutil et al., 1994; Zhang et al., 1994; Keranen et al., 1995). A corresponding residue, S338, in PKA is also stably phosphorylated (Shoji et al., 1979).

It is interesting that T634 presents another type of configuration. In our model of the catalytic core of PKC, the phosphate at T634 appears to point outward and be at the surface of the catalytic core. In addition, Orr and Newton (1994) have previously reported that the amino-terminal pair of autophosphorylated residues may be in a similar conformation. Thus, protection of this type of site would necessitate that other regions of PKC beyond the catalytic core extend over or near the site to limit phosphatase accessibility. Although at present no direct structural information is

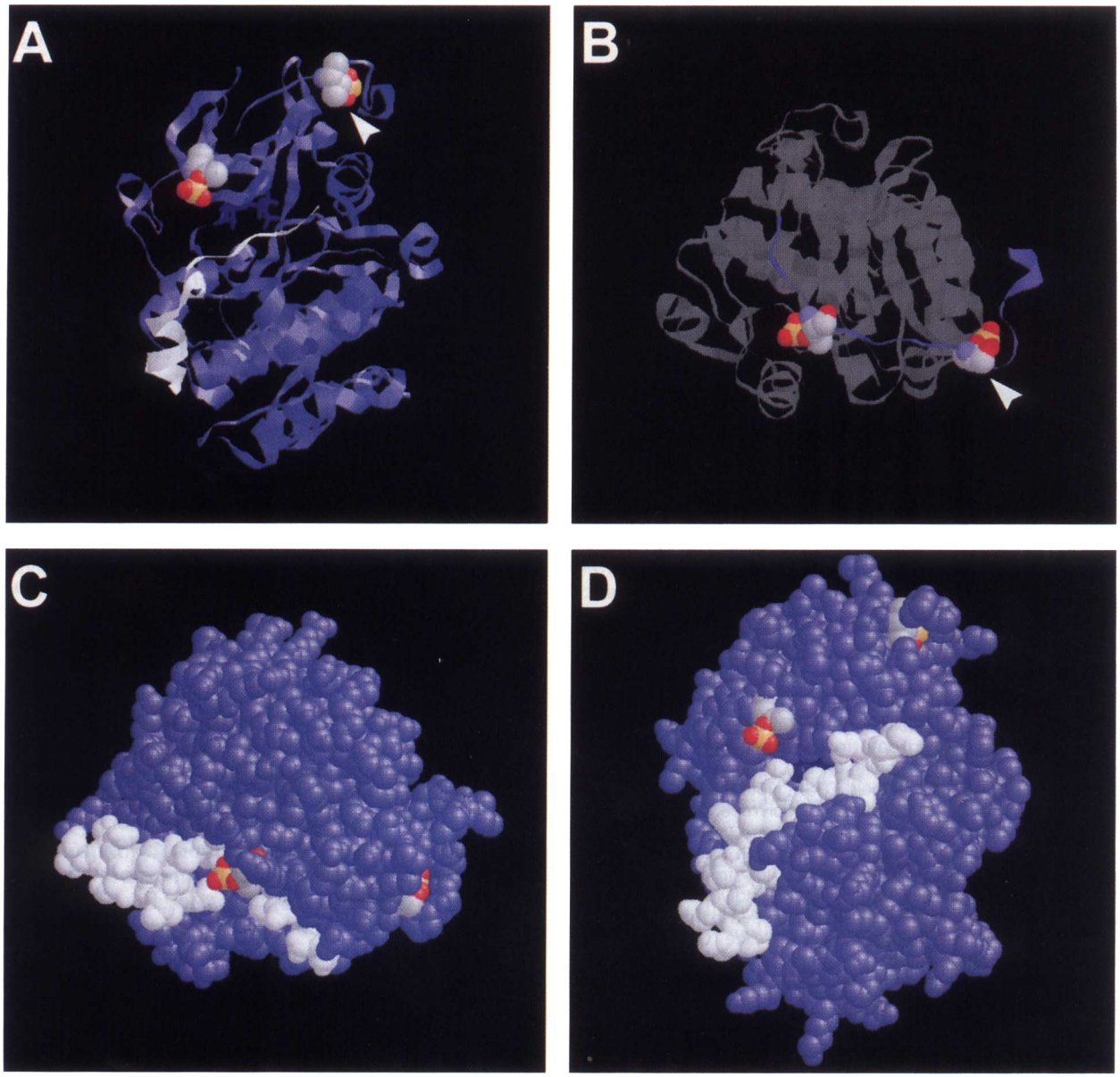

FIG. 7. Proposed structure of the carboxy-terminal autophosphorylation domain in PKC. The structure of the catalytic core of PKC was modeled based on the known structure of the PKA catalytic subunit as described in Materials and Methods and by Orr and Newton (1994). **A:** Ribbon diagram of the proposed structure of PKC βII. The autoinhibitory domain is shown in white, ATP is dark blue, and the C-terminal catalytic core shown in light blue. Phospho-T634 and phospho-T641 (T641 indicated with the white arrow) are rendered in space-fill using the CPK color scheme. **B:** Proposed structure of amino acids 629–642 of PKC βII demonstrates that T641 (white arrowhead) resides on the interior of a sharp angle in the peptide backbone in this domain, whereas T634 is in a more exposed position. Phosphorylated T641 and T634 are shown in space-fill. This view is looking downward onto the upper lobe of the catalytic core. **C:** Space-filling model demonstrates the positions of T641 and T634. This view is the same orientation as B, and the color scheme is identical to A. Note that the side chain of T634 is exposed, whereas the side chain of T641 points inward toward the core and is on the interior side of the peptide backbone. **D:** Space-fill lateral view of the same rendering as in C. This is the same orientation as A.

available, domains potentially involved include both the Ca^{2+}- and lipid-binding domains of PKC (Newton, 1995). Overall our finding of persistent PKC autophosphorylation in LTP and our previous findings of conformation-dependent PKC dephosphorylation (Klann et al., 1993; Dutil et al., 1994) led us to hypothesize a "protected site" model of PKC autophosphorylation. Our modeling of the catalytic core led us to hypothesize that whereas protection of T641 from dephosphorylation may be achieved by local structural determinants, protection of T634 may require a direct contribution from the regulatory domain of the molecule.

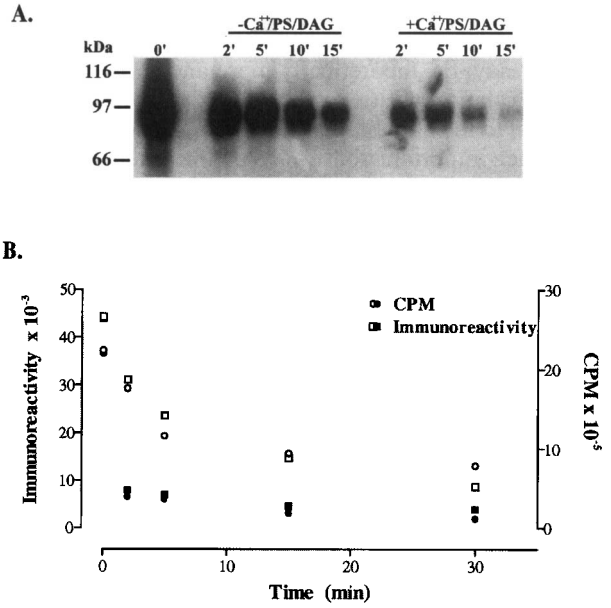

FIG. 8. Dephosphorylation of autophosphorylated PKC by PP1 is accelerated in the presence of activators. **A:** Western blot using antiserum 96160 of purified PKC βII (100 ng per lane) that was autophosphorylated as described in Materials and Methods and then dephosphorylated with PP1 for the indicated time in the presence of either 210 μ*M* PS/6 μ*M* DAG (97 and 3 mol%, respectively) and 400 μ*M* Ca^{2+} (activating conditions) or Triton X-100 (2 m*M*) mixed micelles containing 35 μ*M* PS/1 μ*M* DAG (2 and 0.05 mol%, respectively) with 100 μ*M* Ca^{2+} (nonactivating conditions). **B:** Quantitation of the western blot shown above. PKC was dephosphorylated in the presence (solid symbols) or absence (open symbols) of activators Ca^{2+}/PS/DAG. Dephosphorylation of PKC was monitored using antiserum 96160 (left *y*-axis; squares) or ^{32}P release (right *y*-axis; circles).

Phosphatase susceptibility of phosphorylated PKC

To confirm and extend our previous observations of allosteric regulation of PKC autophosphorylation, we examined the phosphatase sensitivity of PKC in the presence and absence of its activators, Ca^{2+}/PS/DAG. PKC was stimulated to undergo autophosphorylation with [^{32}P]ATP in vitro and then dephosphorylated with PP1, which is not regulated by Ca^{2+}/PS/DAG (Cohen, 1982). As shown in Fig. 8, PKC was 82% dephosphorylated after incubation with PP1 in the presence of activators for 2 min, whereas without activators, PKC was only 30% dephosphorylated after 2 min of phosphatase treatment, as assessed by western blotting with antiserum 96160. Likewise, after a 30-min incubation with phosphatase, PKC incubated in the presence of activators was completely dephosphorylated, whereas control incubations exhibited notable phosphatase resistance (Fig. 8B). Analysis using ^{32}P release as a measure of PKC dephosphorylation paralleled the rate at which immunoreactivity with antiserum 96160 decreased (Fig. 8B). A similar result was obtained when Ca^{2+} was present in both incubation mixtures, and the only difference was the presence or absence of lipids (data not shown). Thus, dephosphorylation of PKC autophosphorylated in vitro was markedly stimulated in the membrane-bound conformation of PKC. These observations are supported by studies by Klann et al. (1993) and Dutil et al. (1994), who found that the dephosphorylation of PKC in LTP samples or of PKC overexpressed in Sf-21 insect cells required the presence of Ca^{2+}/PS/DAG. These results and our own support the model that T634 and T641 are protected from phosphatases unless the enzyme is in an activated conformation. Furthermore, as [^{32}P]phosphate incorporates into sites in the N terminus and hinge regions of PKC, our ^{32}P-labeling data may imply that additional sites outside the C terminus are also protected from dephosphorylation.

DISCUSSION

In these studies we have tested the hypothesis promulgated by Klann et al. (1993) of increased PKC phosphorylation in the early maintenance phase of LTP, using an antiserum we developed that is selective for phosphorylated PKC. Consistent with this hypothesis, we observed increased antiserum 96160 immunoreactivity in area CA1 of hippocampal slices 45 min–1 h after LTP-inducing stimulation. In addition, induction of the increased PKC immunoreactivity was dependent on activation of NMDA receptors. Overall, our data indicate increased phosphorylation of PKC in LTP. These findings complement a substantial body of data indicating that in LTP, NMDA receptor activation leads to long-lasting posttranslational alterations in PKC (Klann et al., 1993; Sacktor et al., 1993; Powell et al., 1994; Osten et al., 1996).

Our studies have provided an insight into the likely mechanism of increased phosphorylation of PKC in LTP. Our data demonstrating that PKC autophosphorylation is sufficient to account for the altered PKC immunoreactivity we have observed in LTP indicate that the most parsimonious explanation for our data is autophosphorylation of PKC in LTP, although transphosphorylation of PKC by another kinase in LTP is a possibility. Although we cannot rule out a contribution from increased phosphorylation in the amino-terminal domain, these results indicate that autophosphorylation in the carboxy-terminal domain at positions T634 and T641 is sufficient to cause the LTP-associated increased immunoreactivity we have observed in the present studies.

The functional consequences of PKC autophosphorylation are being actively investigated. Site-directed mutagenesis has suggested that the phosphorylation of T641 is essential for PKC to be active (Zhang et al., 1993, 1994). Similarly, dephosphorylation of phospho-T641 results in an inactive kinase (Dutil et al., 1994; Keranen et al., 1995). Autophosphorylation of PKC in vitro lowers its K_m for Ca^{2+}, analogous to the effects of DAG, and a subtle increase in basal activity, i.e., activity in the absence of Ca^{2+}, occurs (Huang et al., 1986). Phosphorylation may also alter the subcellular distribution of the enzyme and its protease sensi-

tivity (Mitchell et al., 1989; Ohno et al., 1990; Zhang et al., 1993; Blobe et al., 1996).

Our observations provide direct evidence for a long-lasting posttranslational modification of PKC in LTP. How is the phosphorylation of PKC preserved in the presence of ongoing phosphatase activity in the neuron? One possibility is that autonomously active PKC continually rephosphorylates itself after phosphatase removes phosphate from the C-terminal autophosphorylation domain. However, the data in Fig. 8 and from Klann et al. (1993) and Dutil et al. (1994) indicate that phosphatase is unable to dephosphorylate effectively PKC, even the autonomously active PKC present in LTP samples (Klann et al., 1993), unless the enzyme is "reactivated" with second messengers. Therefore, even if an autonomously active PKC is able to rephosphorylate, this mechanism is not a complete explanation for the persistence of increased PKC autophosphorylation in LTP; even in this model the necessary first step is dephosphorylation of the enzyme.

Therefore, to explain more completely our observations we have developed the "protected site" model for PKC phosphorylation in LTP. In this model, PKC phosphorylation occurs at sites protected sterically by adjacent portions of the PKC molecule, limiting accessibility of phosphatase and helping preserve the enzyme in a phosphorylated state. As described above, our data suggest that the protection is conformation-dependent; in the presence of its activators PKC is readily dephosphorylated (Fig. 8) (Klann et al., 1993; Dutil et al., 1994). This observation suggests that PKC phosphorylation is reversible under specific conditions, such as what might occur when the cell receives a depotentiating signal. Specifically, we propose that on entry of low levels of Ca^{2+}, PP2b is activated, leading to dephosphorylation of PP inhibitor 1, thereby causing activation of PP1. The Ca^{2+} signal (in conjunction with other activators, for example, transient DAG production) changes the conformation of PKC, exposing the phosphate at the "protected" site. PP1 can then dephosphorylate PKC, returning the enzyme to its original state. Alternatively, PP2b could directly dephosphorylate PKC. In this regard, we have observed that PP2b also selectively dephosphorylates the membrane-bound conformation of PKC (E. M. Dutil and A. Newton, unpublished data).

This model is inspired by the model of calcium/calmodulin-dependent protein kinase II (CaMKII) activation in LTP by Lisman (1985) but has several distinct features. Our focus on PKC as opposed to CaMKII has an interesting mechanistic implication. CaMKII autophosphorylation occurs via an intersubunit reaction. Lisman (1985) capitalized on this feature to propose that the autonomous kinase activation is self-perpetuating, via a positive feedback loop of one CaMKII subunit phosphorylating another. PKC autophosphorylation, however, is intramolecular and therefore cannot be self-perpetuating. This feature accounts for the necessity of the "protected" autophosphorylation site in our model, for the phosphorylation to be persistent. Therefore, although CaMKII autophosphorylation may be theoretically permanent, PKC autophosphorylation will at a minimum decay with a half-life equal to the half-life of the protein; these considerations place important constraints on the potential physiologic roles of autophosphorylated PKC and CaMKII.

Our model also has two interesting implications concerning mechanisms operating in depotentiation. First, the model predicts that depotentiation will involve phosphatase activation. This is consistent with data already available in the literature (Mulkey et al., 1993; O'Dell and Kandel, 1994). Second, for PKC autophosphorylation to be reversed, the PKC molecule must receive a second messenger signal to be in the right conformation for the phosphatase to have access to the "protected" site. This may explain one aspect of an apparent enigma that has arisen in prior studies of depotentiation: Like LTP, depotentiation requires the activation of NMDA receptors. Our model incorporates this finding by postulating that NMDA receptors are the source of the signal necessary to put the PKC molecule in the proper conformation for dephosphorylation.

Although we have proposed the protected site model based on our studies of PKC phosphorylation in LTP, phosphorylation of protected sites might be a general biochemical mechanism for the generation of stable, long-lasting physiologic changes. Such a mechanism confers three attributes on a change in an enzymatic system: stability, constancy, and regulated reversibility. The change is stable in the sense that it is long-lasting in the cell. The magnitude of the change is constant because a constant fraction of the enzyme stays phosphorylated, unless additional stimulation occurs. Finally, although the change can be long-lasting, on receiving a specific signal the change can be readily reversed, restoring the system to its original state.

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REFERENCES

Blobe G. C., Stribling D. S., Fabbro D., Stabel S., and Hannun Y. A. (1996) Protein kinase C betaII specifically binds to and is activated by F-actin. *J. Biol. Chem.* **271,** 15823–15830.

Borner C., Filipuzzi I., Wartman M., Eppenberger U., and Fabbro D. (1989) Biosynthesis and posttranslational modifications of protein kinase C in human breast cancer cells. *J. Biol. Chem.* **264,** 13902–13909.

Cohen P. (1982) The role of protein phosphorylation in neural and hormonal control of cellular activity. *Nature* **296,** 613–620.

Colley P. A., Sheu F.-S., and Routtenberg A. (1990) Inhibition of protein kinase C blocks two components of LTP persistence, leaving initial potentiation intact. *J. Neurosci.* **10,** 3353–3360.

Dutil E. M., Keranen L. M., DePaoli-Roach A. A., and Newton A. C. (1994) In vivo regulation of protein kinase C by transphosphorylation followed by autophosphorylation. *J. Biol. Chem.* **269,** 29359–29362.

English J. D. and Sweatt J. D. (1996) Activation of p42 mitogen-activated protein kinase in hippocampal long term potentiation. *J. Biol. Chem.* **271,** 24329–24332.

Flint A. J., Paladini R. D., and Koshland D. E. Jr. (1990) Autophosphorylation of protein kinase C at three separated regions of its primary sequence. *Science* **249,** 408–411.

Gianotti C., Nunzi M. G., Gispen W. H., and Corradetti R. (1992) Phosphorylation of the presynaptic protein B-50 (GAP-43) is increased during electrically induced long-term potentiation. *Neuron* **8,** 843–848.

Hu G. Y., Hvalby O., Walaas S. I., Albert K. A., Skjeflo P., Andersen P., and Greengard P. (1987) Protein kinase C injection into hippocampal pyramidal cells elicits features of long term potentiation. *Nature* **328,** 426–429.

Huang K. P., Chan K. J., Singh T. J., Nakabayashi H., and Huang F. L. (1986) Autophosphorylation of rat brain Ca^{2+}-activated and phospholipid-dependent protein kinase. *J. Biol. Chem.* **261,** 12134–12140.

Hvalby O., Reymann K., and Andersen P. (1988) Intracellular analysis of potentiation of CA1 hippocampal synaptic transmission by phorbol ester application. *Exp. Brain Res.* **71,** 588–596.

Keranen L. M., Dutil E. M., and Newton A. C. (1995) Protein kinase C is regulated in vivo by three functionally distinct phosphorylations. *Curr. Biol.* **5,** 1394–1403.

Klann E., Chen S.-J., and Sweatt J. D. (1991) Persistent protein kinase activation in the maintenance phase of long-term potentiation. *J. Biol. Chem.* **266,** 24253–24256.

Klann E., Chen S.-J., and Sweatt J. D. (1993) Mechanism of protein kinase C activation during the induction and maintenance of long-term potentiation probed using a selective peptide substrate. *Proc. Natl. Acad. Sci. USA* **90,** 8337–8341.

Leahy J. C., Luo Y., Kent C. S., Meiri K. F., and Vallano M. L. (1993) Demonstration of presynaptic protein kinase C activation following long-term potentiation in rat hippocampal slices. *Neuroscience* **52,** 563–574.

Lisman J. E. (1985) A mechanism for memory storage insensitive to molecular turnover: a bistable autophosphorylating kinase. *Proc. Natl. Acad. Sci. USA* **82,** 3055–3057.

Lovinger D. M., Akers R. F., Nelson R. B., Barnes C. A., McNaughton B. L., and Routtenberg A. (1985) A selective increase in phosphorylation of protein F1, a protein kinase C substrate, directly related to three day growth of long term synaptic enhancement. *Brain Res.* **343,** 137–143.

Lovinger D. M., Wong K. L., Murakami K., and Routtenberg A. (1987) Protein kinase C inhibitors eliminate hippocampal long-term potentiation. *Brain Res.* **436,** 177–183.

Malenka R. C., Madison D. V., and Nicoll R. A. (1986) Potentiation of synaptic transmission in the hippocampus by phorbol esters. *Nature* **321,** 175–177.

Malenka R. C., Ayoub G. S., and Nicoll R. A. (1987) Phorbol esters enhance transmitter release in rat hippocampal slices. *Brain Res.* **403,** 198–203.

Malinow R., Madison D. V., and Tsien R. W. (1988) Persistent protein kinase activity underlying long-term potentiation. *Nature* **335,** 820–824.

Mitchell F. E., Marais R. M., and Parker P. J. (1989) The phosphorylation of protein kinase C as a potential measure of activation. *Biochem. J.* **261,** 131–136.

Mochly-Rosen D. and Koshland D. E. Jr. (1987) Domain structure and phosphorylation of protein kinase C. *J. Biol. Chem.* **262,** 2291–2297.

Mulkey R. M., Herron C. E., and Malenka R. C. (1993) An essential role for protein phosphatases in hippocampal long-term depression. *Science* **261,** 1051–1055.

Muller D., Turnbull J., Baudry M., and Lynch G. (1988) Phorbol ester-induced synaptic facilitation is different than long-term potentiation. *Proc. Natl. Acad. Sci. USA* **85,** 6997–7000.

Newton A. C. (1995) Protein kinase C: structure, function, and regulation. *J. Biol. Chem.* **270,** 28495–28498.

Newton A. C. and Koshland D. E. Jr. (1987) Protein kinase C autophosphorylates by an intrapeptide reaction. *J. Biol. Chem.* **262,** 10185–10188.

O'Dell T. and Kandel E. (1994) Low-frequency stimulation erases LTP through an NMDA receptor-mediated activation of protein phosphatases. *Learn. Mem.* **1,** 129–139.

Ohno S., Konno Y., Akita Y., Yano A., and Suzuki K. (1990) A point mutation at the putative ATP-binding site of protein kinase C alpha abolishes the kinase activity and renders it down-regulation-insensitive. A molecular link between autophosphorylation and down-regulation. *J. Biol. Chem.* **265,** 6296–6300.

Orr J. W. and Newton A. C. (1994) Intrapeptide regulation of protein kinase C. *J. Biol. Chem.* **269,** 8383–8387.

Orr J. W., Keranen L. M., and Newton A. C. (1992) Reversible exposure of the pseudosubstrate domain of PKC by phosphatidylserine and diacylglycerol. *J. Biol. Chem.* **267,** 15263–15266.

Osten P., Valsamis L., Harris A., and Sacktor T. C. (1996) Protein synthesis-dependent formation of protein kinase Mζ in long-term potentiation. *J. Neurosci.* **16,** 2444–2451.

Poehling H. M. and Neuhoff V. (1981) Visualization of proteins with a silver "stain": a critical analysis. *Electrophoresis* **2,** 141–147.

Powell C., Johnston D., and Sweatt J. D. (1994) Proteolytic activation of PKC in the maintenance phase of NMDA receptor independent LTP. *J. Biol. Chem.* **269,** 27958–27963.

Sacktor T. C., Osten P., Valsamis H., Jiang X., Naik M. U., and Sublette E. (1993) Persistent activation of the zeta isoform of protein kinase C in the maintenance of long-term potentiation. *Proc. Natl. Acad. Sci. USA* **90,** 8342–8346.

Schwartz J. H. (1993) Cognitive kinases. *Proc. Natl. Acad. Sci. USA* **90,** 8310–8313.

Shoji S., Titani K., Demaille J. G., and Fischer E. H. (1979) Sequence of two phosphorylated sites in the catalytic subunit of bovine cardiac muscle adenosine 3′:5′-monophosphate-dependent protein kinase. *J. Biol. Chem.* **254,** 6211–6214.

Tsutakawa S. E., Medzihradszky K. F., Flint A. J., Burlingame A. L., and Koshland D. E. Jr. (1995) Determination of in vivo phosphorylation sites in protein kinase C. *J. Biol. Chem.* **270,** 26807–26812.

Wang J. H. and Feng D. P. (1992) Postsynaptic protein kinase-C essential to induction and maintenance of long-term potentiation in the hippocampal CA1 region. *Proc. Natl. Acad. Sci. USA* **89,** 2576–2580.

Zhang J., Wang L., Petrin J., Bishop W. R., and Bond R. W. (1993) Characterization of site-specific mutants altered at protein kinase C beta 1 isozyme autophosphorylation sites. *Proc. Natl. Acad. Sci. USA* **90,** 6130–6134.

Zhang J., Wang L., Schwartz J., Bond R. W., and Bishop W. R. (1994) Phosphorylation of Thr642 is an early event in the processing of newly synthesized protein kinase C beta 1 and is essential for its activation. *J. Biol. Chem.* **269,** 19578–19584.

Zheng J., Knighton D. R., ten Eyck L. F., Karlsson R., Xuong N., Taylor S. S., and Sowadski J. M. (1993) Crystal structure of the catalytic subunit of cAMP-dependent protein kinase complexed with MgATP and peptide inhibitor. *Biochemistry* **32,** 2154–2161.