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Mechanism of protein kinase C activation during the induction and maintenance of long-term potentiation probed using a selective peptide substrate

(hippocampus/synaptic plasticity/phosphorylation)

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ABSTRACT **Previous reports using various protein kinase inhibitors have suggested that protein kinase activity is necessary for both the induction and maintenance of hippocampal long-term potentiation (LTP), a cellular phenomenon likely to contribute to mammalian memory formation. We designed and characterized a selective peptide substrate for protein kinase C (PKC), corresponding to amino acids 28 to 43 of the neuronal protein neurogranin, and used the substrate to obtain direct biochemical evidence for activation of PKC in both the induction and maintenance phases of LTP. As the effect cannot be accounted for by either of two well-known mechanisms for persistent PKC activation, membrane insertion, or proteolysis, the persistent activation of PKC in the maintenance phase of LTP appears to occur via another mechanism. The maintenance phase of LTP is associated with decreased immunoreactivity of PKC, an effect that can be reversed with phosphatase treatment. Thus, PKC appears to be both phosphorylated and persistently activated in the maintenance phase of LTP.**

Long-term potentiation (LTP) is a use-dependent increase in synaptic strength widely studied as a cellular mechanism contributing to memory formation in mammals. The biochemical mechanisms responsible for this change in synaptic strength are unclear, but recent evidence indicates that inhibitors of a number of protein kinases can block both the induction and maintenance of LTP (1–7). Also, genetically engineered mice missing either the gene for the α subunit of Ca^{2+}/calmodulin-dependent protein kinase (CaMKII) or missing the gene for the tyrosine kinase Fyn have been shown to have a deficiency in LTP induction (8, 9). In addition to these studies indicating a necessity for intact protein kinase activity in LTP, we have shown (10) that the maintenance phase of LTP is associated with an increase in protein kinase activity.

We sought to identify the particular protein kinase(s) activated in the induction and maintenance of LTP. Identification of specific protein kinases activated *in vivo* has in general been limited by the lack of potent and selective protein kinase substrates. We have developed a peptide substrate selective for protein kinase C (PKC) that corresponds to the phosphorylation site (aa 28–43) of neurogranin/RC3 [NG-(28–43)], a neuronal protein that is an endogenous selective PKC substrate (11–13). In the present studies, we have characterized the properties of NG-(28–43) phosphorylation in hippocampal homogenates and used the peptide to test the hypothesis that PKC is activated in the induction and maintenance phases of LTP.

MATERIALS AND METHODS

Protein Kinase Assays. Rat hippocampi were homogenized in buffer containing 50 mM Tris·HCl (pH 7.4), 10 mM benzamidine, aprotinin (100 ng/ml), leupeptin (100 ng/ml), 1 mM EDTA, and 1 mM EGTA. The amount of protein used for assays was 2–10 μg in Fig. 1 and 0.5–2 μg for LTP experiments. Reaction mixtures (final volume, 50 μl) contained 5 μl of homogenate, 20 mM Tris·HCl (pH 7.4), 10 μM NG-(28–43), 10 mM $MgCl_2$, 2 mM sodium pyrophosphate, 0.1 mM phenylmethylsulfonyl fluoride, leupeptin (25 μg/ml), and 100 μM [γ-^{32}P]ATP. Assays for PKC activity were carried out with EGTA for basal activity (final concentration, 2.5 mM) or in the presence of Ca^{2+} (100 μM in the presence of 500 μM EGTA) and lipid cofactors for total activity [final concentrations: phosphatidylserine (PS), 320 μg/ml; *sn*-1,2-dioctanoylglycerol (DAG), 30 μg/ml]. Reactions were started by addition of the homogenate to the reaction mixture, incubated for 2 min at 37°C, and terminated by adding 25 μl of ice-cold stop solution containing 100 mM ATP and 100 mM EDTA. Duplicate 25-μl aliquots were spotted onto P-81 phosphocellulose filter papers. The papers were washed with 150 mM H_3PO_4, dried, and immersed in 2.5 ml of Aquasol-2, and radioactivity was measured by scintillation counting. For each experimental condition, values for control reactions lacking the substrate peptide were subtracted as blanks. NG-(28–43) phosphorylation was linear with respect to time and protein added under these conditions. Data are expressed as either specific activity (pmol per min per μg of protein) or as percent of control for LTP experiments. CaMKII activity and cAMP-dependent protein kinase (PKA) activity were stimulated with either 500 μM Ca^{2+} and calmodulin (CaM) at 10 μg/ml or 100 μM cAMP and 1 mM 3-isobutyl-1-methylxanthine. Under these conditions, substantial phosphorylation of the CaMKII substrate autocamtide (50 μM, ref. 14) and the PKA substrate kemptide (25 μM, ref. 15) could be detected. The small amount of NG-(28–43) phosphorylation observed with addition of Ca^{2+}/CaM is likely due to Ca^{2+} stimulation of PKC, as pure CaMKII is unable to phosphorylate NG-(28–43) (16).

Induction of LTP. Rat hippocampal slices were prepared and physiologic responses were recorded at 32°C (10). Briefly, responses to Schaffer collateral stimulation in the CA1 region were monitored for at least 20 min before tetanic stimulation to assure a stable base line. Responses were measured as an average of at least four individual traces. Tetanic stimulation consisted of three sets of stimuli, each set delivered 5 min apart. Each set included two 1-s trains of stimuli (110 Hz), given 20 s apart. This produced LTP with an average 99 ± 9% (n = 55) increase in the initial slope of the population excitatory postsynaptic potential (EPSP) 45 min after the final tetanus. Bath application of 50 μM DL-2-amino-

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Abbreviations: PKC, protein kinase C; LTP, long-term potentiation; NG-(28–43), neurogranin-(28–43); CaM, calmodulin; CaMKII, Ca^{2+}/CaM-dependent protein kinase; PKA, cAMP-dependent protein kinase; PS, phosphatidylserine; DAG, *sn*-1,2-dioctanoylglycerol; AP5, DL-2-amino-5-phosphonovaleric acid; NMDA, *N*-methyl-D-aspartate; EPSP, excitatory postsynaptic potential.

5-phosphonovaleric acid (AP5) inhibited the potentiation of the EPSP slope of the EPSP to an increase of 9 ± 4% (n = 8). Experimental and control slices were removed at various times after the final period of tetanic stimulation, frozen on dry ice, and dissected. The microdissected subregion (area CA1) was homogenized in 100 μl of buffer and assayed for PKC activity.

Fractionation of Hippocampal Homogenates. Control and LTP homogenates (100 μl) were centrifuged for 45 min at 133,000 × g and separated into pellet and soluble fractions. Pellet fractions were resuspended in 100 μl of homogenization buffer. The fractions were subsequently assayed for basal and total (cofactor dependent) activity as described above. The soluble PKC activity was on average 50% of the total PKC activity.

Western Blots for PKC. Western blots were performed essentially as described (17). Proteins from control and LTP hippocampal homogenates (5 μg) were separated by SDS/PAGE on 10% gels. Proteins were transferred onto Immobilon-P transfer membranes. Blots were incubated with a polyclonal PKC antibody diluted 1:2000 (generously provided by Freesia Huang, National Institutes of Health), exposed to a secondary antibody diluted 1:2000, exposed to 20 μCi of ^{125}I-labeled protein A (1 Ci = 37 GBq), and then placed on film at −70°C. This antibody has been reported to recognize the catalytic and regulatory subunits of the enzyme (18, 19). We have also observed antibody binding to pure PKM, the catalytic domain of PKC (data not shown). Moreover, proteolytic activation of PKC in hippocampal homogenates induced by a brief incubation with Ca^{2+} results in the generation of a proteolytic fragment of PKC that corresponds to PKM and can be recognized by this antibody (20). Additionally, we observed no change in PKC immunoreactivity when the enzyme was persistently activated by mild oxidation in hippocampal homogenates (21).

Phosphatase Treatment of Control and LTP Homogenates. To reverse the loss in PKC immunoreactivity, PS (320 μg/ml), DAG (30 μg/ml), and 5 units of alkaline phosphatase (Boehringer Mannheim) were added to 5 μg of homogenate from control and LTP slices and incubated for 45 or 90 min. Subsequent SDS/PAGE and Western blots were performed as described above. Control experiments with purified PKC revealed that, when PKC was autophosphorylated (22), PKC immunoreactivity was decreased. To ensure that PKC was autophosphorylated, [γ-^{32}P]ATP was added to reaction mixtures and autophosphorylation was confirmed either by SDS/PAGE or phosphocellulose binding. Also, incubation of 5 units of alkaline phosphatase with 5 μg of hippocampal homogenate for 30 min at 37°C resulted in an increase in PKC immunoreactivity.

RESULTS

NG-(28–43) is a selective substrate for PKC in rat hippocampal homogenates (Fig. 1). The phosphorylation of NG-(28–43) is increased by addition of the PKC activators PS, DAG, and Ca^{2+} to homogenates. Phosphorylation of NG-(28–43) is not appreciably increased by activators of PKA or CaMKII (Fig. 1*A*). Ninety-five percent of NG-(28–43) phosphorylation is blocked by the addition of a selective inhibitor peptide of PKC [10 μM PKC-(19–36); ref. 2; Fig. 1*B*]. These data indicate that NG-(28–43) is a very selective and potent substrate for PKC vs. CaMKII or PKA in hippocampal homogenates. Thus, utilization of NG-(28–43) permits a selective assay of changes in PKC activity in physiological preparations (see also ref. 16).

We proceeded to use NG-(28–43) to test the hypothesis that persistent PKC activation is associated with LTP. Our previous studies suggested that PKC activity was elevated 45–60 min after the final tetanic stimulation (10). To confirm

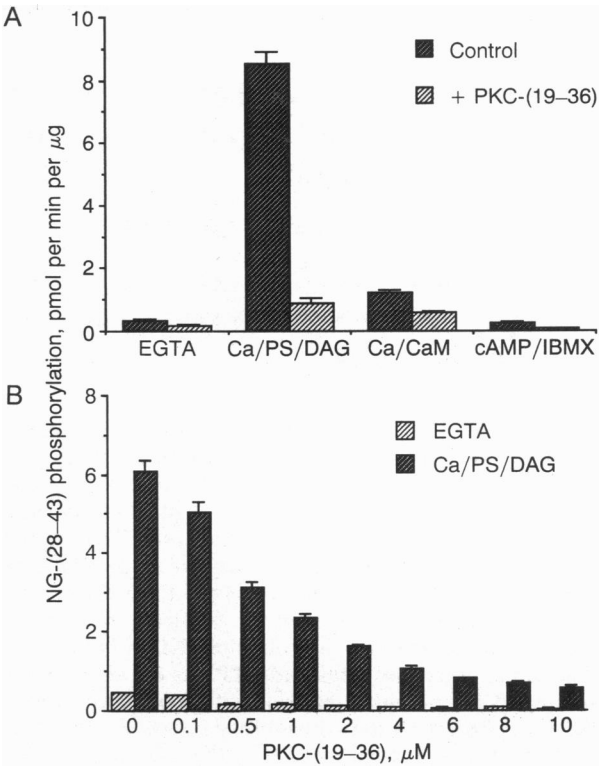

FIG. 1. NG-(28–43) is a selective substrate for PKC in hippocampal homogenate. (*A*) Phosphorylation of NG-(28–43). NG-(28–43) (10 μM) was phosphorylated in hippocampal homogenate in the presence of EGTA, Ca^{2+}/PS/DAG, Ca^{2+}/CaM, or cAMP/3-isobutyl-1-methylxanthine (IBMX). The homogenate was preincubated with H_2O (no inhibitor) or with 5 μM PKC-(19–36) at 4°C for 10 min before the addition of substrate. Error bars show the SE for four determinations. Each determination was assayed in duplicate. (*B*) Effect of PKC-(19–36) on NG-(28–43) phosphorylation. Error bars are the SE for four determinations. Each determination was assayed in duplicate.

this, we assayed for PKC activity in control and LTP homogenates at the same time point employing NG-(28–43) as the substrate. The results are shown in Fig. 2*A*. PKC activity was increased in homogenates of area CA1 prepared 45 min after LTP-inducing tetanic stimulation (195 ± 26% of control, n = 25). EGTA (2 mM) was included in these assays; therefore, the increased PKC activity was expressed independent of Ca^{2+}. This Ca^{2+}-independent activity will be referred to as basal PKC activity. As the homogenates are diluted ≈1:1000 relative to their state *in situ*, it is unlikely that the increased activity is due to any residual increase in second messenger levels. Expression *in vitro* of the LTP-associated increase in basal PKC activity could be blocked by addition of the selective PKC inhibitor peptide PKC-(19–36) to the assay (5 μM; Fig. 2*B*). These results are strong evidence that PKC is persistently activated in the maintenance phase of LTP. While NG-(28–43) appears to be a potent and selective substrate for PKC and PKC-(19–36) was used at a concentration that should selectively inhibit PKC, we cannot completely rule out the possibility that another protein kinase might contribute to part of the increased NG-(28–43) phosphorylation we observed.

We next determined whether the persistent increase in basal PKC activity was associated specifically with LTP-inducing stimuli. To ensure that the increase in basal PKC activity was not dependent on the number of stimuli given to the slice, we stimulated slices with the same number of stimuli as given during high-frequency stimulation, but at low frequency (0.1 Hz). These slices exhibited no increase in

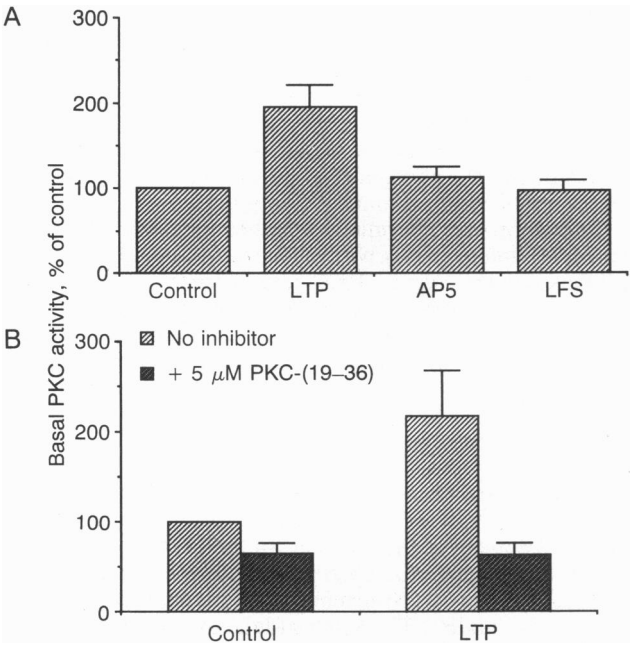

FIG. 2. LTP-associated persistent increase in basal PKC activity. (*A*) Measurement of basal PKC with NG-(28–43) as an exogenous substrate. NG-(28–43) phosphorylation in CA1 hippocampal homogenates of control and LTP ($n = 25$) slices. Error bars are the SE for the indicated number of determinations. Each determination was assayed in duplicate. For these and all subsequent experiments, PKC activity in experimental slices was directly compared to a control slice with similar electrophysiological responses from the same hippocampus. NG-(28–43) phosphorylation is expressed as percent control for control slices, LTP slices, slices given tetanic stimulation in the presence of bath-applied AP5 ($n = 4$, 50 μM), and slices stimulated at low frequency [LFS ($n = 4$)] with the same number of stimuli given to the LTP slices. Data expressed as specific activity: control = 0.94 ± 0.14 pmol per μg per min; LTP = 1.46 ± 0.20 pmol per μg per min; $P < 0.001$, two-tailed Student's t test. The LTP-associated increase in PKC activity is further strengthened by the fact that slices given LTP-inducing high-frequency stimulation without AP5 that were not potentiated electrophysiologically exhibited no increase in PKC activity (data not shown). (*B*) Addition of PKC-(19–36) to protein kinase assays blocks expression of the persistent increase in basal PKC activity. PKC-(19–36) was added to reaction mixtures to a final concentration of 5 μM ($n = 9$). Addition of 10 μM PKC-(19–36) results in nearly complete inhibition of basal PKC activity in control and LTP homogenates (data not shown).

basal PKC activity (98 ± 12% of control, $n = 4$; Fig. 2*A*). In addition, slices given tetanic stimulation in the presence of bath-applied AP5, an *N*-methyl-D-aspartate (NMDA) receptor antagonist (23), failed to exhibit a significant increase in basal PKC activity (113 ± 12% of control, $n = 4$). These results indicate that the induction of the persistent increase in basal PKC activity cannot be elicited by low-frequency stimulation or high-frequency stimulation without LTP and are consistent with a persistent increase in basal PKC activity being associated with LTP. The data with AP5 also indicate that activation of NMDA receptors is a necessary trigger for the persistent PKC activation.

We next posed the question: Is the increase in basal PKC activity associated solely with the maintenance phase of LTP? To answer this question, we assayed for basal PKC activity at various time points after the final tetanus. The increase in basal PKC activity appeared shortly after the final tetanus and remained elevated for at least 45 min, before decaying toward baseline 3 h after the final tetanus (Fig. 3). The time course indicates that PKC is persistently activated in the maintenance phase of LTP. In addition PKC is rapidly activated after tetanic stimulation. These data provide direct biochemical evidence for the activation of PKC immediately after LTP-inducing tetanic stimulation and complement previous studies demonstrating that protein kinase inhibitors can block the induction of LTP (1–7).

We also determined whether there were changes in total PKC activity associated with LTP. We define total PKC activity as NG-(28–43) phosphorylation assayed in a reaction mixture containing PS, DAG, and Ca^{2+}. At 2 min after the final tetanus, we observed an increase in total cofactor-dependent PKC activity (174 ± 18% of control, $n = 10$; $P < 0.02$). In contrast, we observed no change in total cofactor-dependent PKC activity at 45 min after the final tetanus or at any other time point. These results indicate that there is a rapid transient increase in total PS/DAG-stimulatable PKC activity that decays to baseline by 5 min after the final tetanus. This finding suggests that multiple mechanisms may contribute to PKC activation after LTP-inducing tetanus. Interestingly, we have observed previously that oxidative activation of PKC in hippocampal homogenates can result in an increase in PS/DAG-stimulatable activity (21).

We proceeded to determine the mechanism by which basal Ca^{2+}-independent PKC activity is increased. The mechanisms we initially considered were membrane insertion and proteolytic activation of PKC (20, 24, 25). To distinguish between these two possibilities, we determined the subcellular localization of the elevated basal PKC activity. Control and 45-min LTP CA1 homogenates were centrifuged and separated into pellet and supernatant fractions. We detected a significant increase in basal PKC activity in the supernatant fraction (177 ± 38% of control, $n = 6$; $P < 0.05$) and a statistically nonsignificant increase in the pellet fraction (132 ± 28% of control, $n = 6$; $P > 0.05$). These results indicate that the LTP-associated increase in PKC activity we observe in area CA1 cannot be solely due to membrane insertion. Interestingly, membrane insertion has been implicated as a

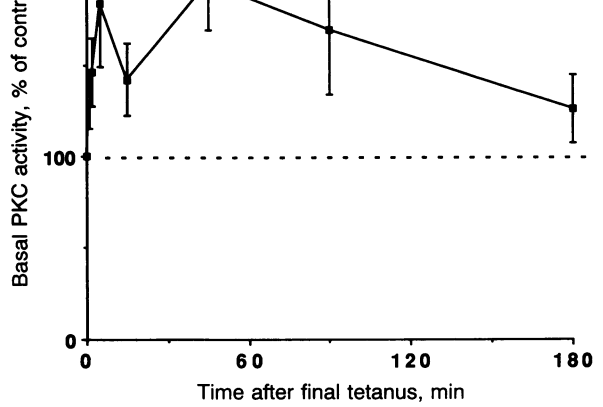

FIG. 3. Time course of LTP-associated increase in basal PKC activity. NG-(28–43) phosphorylation in homogenates of control and LTP slices was measured at various time points after the final set of tetanic stimulation. Time points include 45 sec (131 ± 16% of control, $n = 5$), 2 min (146 ± 19% of control, $n = 16$), 5 min (184 ± 35% of control, $n = 6$), 15 min (142 ± 20% of control, $n = 10$), 45 min (195 ± 26% of control, $n = 25$), 90 min (169 ± 35% of control, $n = 10$), and 180 min (126 ± 19% of control, $n = 5$). Stable LTP was present at both the 90- and 180-min time points (90 ± 23% increase in EPSP slope at 90 min; 83 ± 21% increase in EPSP slope at 180 min). Basal PKC activity was significantly elevated above control at 45 sec, 2 min, and 45 min ($P = 0.011$, one-factor ANOVA; comparisons by Fisher's protected least significant difference test). The increases observed in both basal and total PKC activity at the 2-min time point were also present after a single set of tetanic stimulation, indicating that a rapid increase in PKC activity was present in the early induction phase of LTP.

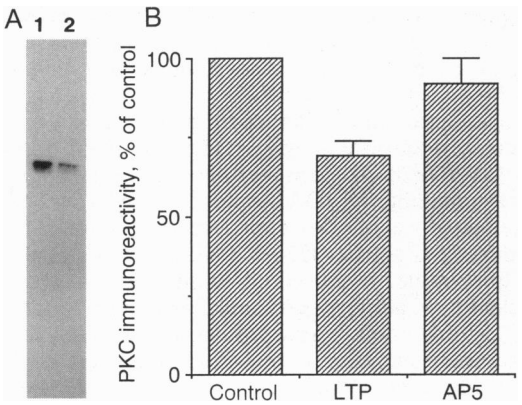

FIG. 4. LTP-associated decrease in PKC immunoreactivity. (A) Western blot for PKC of control and LTP homogenates (5 μg). Lanes: 1, control homogenate; 2, LTP homogenate. (B) Quantification of LTP-associated decrease in PKC immunoreactivity. Error bars are the SE. Data are expressed as percent control for control slices, LTP slices (n = 21), and slices given tetanic stimulation in the presence of bath-applied AP5 (50 μM; n = 4).

mechanism for PKC activation during LTP in the dentate gyrus (26).

The results of the fractionation experiments suggested the possibility that some proteolytic activation of PKC might occur during LTP, resulting in a soluble active catalytic fragment of PKC (i.e., PKM; refs. 20 and 25). To test this hypothesis, we subjected control and LTP homogenates to Western blot analysis with a polyclonal antibody against PKC. If proteolytic activation of PKC occurs, we would expect the appearance of lower molecular mass fragments of the enzyme corresponding to the constitutively active catalytic domain (≈50 kDa) and the regulatory domain (≈35 kDa), as the antibody we employed can recognize these species. We observed no proteolytic fragments of PKC on Western blots, suggesting that proteolytic activation is not responsible for the increased PKC activity.

However, these experiments revealed that a persistent modification of PKC had in fact occurred in LTP. We observed on average a 30% decrease in PKC immunoreactivity to be associated with LTP (Fig. 4). This effect was absent if the slices were given high-frequency stimulation in the presence of bath-applied AP5 (50 μM) (Fig. 4B). These results suggest that LTP is associated with a persistent modification of PKC and that NMDA receptor activation is necessary for this modification. The loss of immunoreactivity was localized to the soluble fraction of homogenates; no change in immunoreactivity was observed in the pellet fraction (data not shown). It is unlikely that the loss of immunoreactivity is simply a result of loss of the protein through down-regulation, as we observed no decrease in total PKC enzymatic activity at this time point.

We hypothesized that phosphorylation of PKC could account for the change in immunoreactivity associated with LTP. In preliminary experiments, we observed a decrease in immunoreactivity of purified PKC when the enzyme was autophosphorylated (64 ± 13% of control, n = 3). Additionally, we observed that incubation of homogenates with alkaline phosphatase led to a small increase in PKC immunoreactivity (134 ± 3% of control, n = 4). These preliminary data indicated that the phosphorylation state of the enzyme had an effect on its ability to bind the antibody.

Therefore, we determined whether incubation of control and LTP homogenates with phosphatase could reverse the LTP-associated loss in PKC immunoreactivity. A 45-min phosphatase incubation led to a 33% reversal of the loss in immunoreactivity while a 90-min incubation led to a 64% reversal (Table 1). These data indicate that a persistent increase in PKC phosphorylation is associated with the maintenance phase of LTP and suggest this as a possible mechanism contributing to the persistent activation of PKC observed.

An additional observation in these experiments suggested that at least part of the phosphorylation occurred in the catalytic domain of the enzyme. In the control 90-min incubation experiments (incubation without phosphatase), a 47-kDa fragment of PKC (presumably PKM) appeared in control samples but not in LTP samples (data not shown). This band was most likely an artifact of PKC proteolysis during the long incubation at 37°C. Incubation with phosphatase for 90 min caused a corresponding band to appear in the LTP samples but did not increase the band in the control samples, suggesting a reversal of the loss in immunoreactivity on the 47-kDa fragment in the LTP samples (data not shown). These results are consistent with a persistent phosphorylation of the catalytic domain of PKC. There was no difference in the amount of 47-kDa immunoreactivity between control and LTP samples after the 45- or 90-min phosphatase treatment, which is consistent with the data presented above suggesting that generation of PKM is not associated with LTP.

DISCUSSION

A role for PKC in LTP induction and maintenance has been hypothesized in recent years. A prediction of this hypothesis is an increase in activity (i.e., persistent activation of the enzyme) during LTP. The development of the substrate NG-(28–43), which is a selective and potent substrate for PKC (Fig. 1 and ref. 16), has permitted us to address this hypothesis directly. The results described in this report illustrate that PKC is indeed activated during the early maintenance stages of LTP (Figs. 2 and 3) and suggest a role for the enzyme in maintaining the long-lasting increase in synaptic strength associated with LTP. We also observed PKC activation in the induction stage of LTP, which strongly supports the hypothesis that activation of PKC contributes to the induction of LTP. These results are in good agreement with prior observations that PKC-(19–36), a selective peptide inhibitor of PKC, could block LTP induction (2) or maintenance (7). Our investigations complement previous studies using protein kinase inhibitors to block LTP induction and

Table 1. Phosphatase treatment reverses the LTP-associated decrease in PKC immunoreactivity

Incubation time, min	LTP PKC immunoreactivity, % of control		% reversal of LTP-associated loss in PKC immunoreactivity
	− phosphatase	+ phosphatase	
45 (n=5)	52 ± 6	68 ± 4	33
90 (n=7)	56 ± 5	84 ± 5	64

Percent reversal was calculated by dividing the average percent decrease in PKC immunoreactivity in phosphatase-treated samples by the average percent decrease in samples without phosphatase and subtracting that number from 100%. If this calculation is done for each individual experiment, the percent reversal for the 45-min and 90-min phosphatase incubations is 27 ± 12% and 66 ± 8%, respectively. Error bars are the SE for the indicated number of determinations.

maintenance (1–7) as inhibitors can implicate the involvement of protein kinases but do not directly demonstrate protein kinase activation.

One interesting question we have not addressed in these studies is the localization of the persistently activated PKC. However, consideration of the existing literature suggests the possibility that persistently activated PKC could reside both pre- and postsynaptically. For example, studies on phosphorylation of the presynaptic protein neuromodulin/growth-associated protein 43/F1/B50 (6, 27–29) have implicated PKC activation in the presynaptic terminal. In addition, injection of selective peptide inhibitors of protein kinases, including an inhibitor of PKC, into the postsynaptic neuron can reverse the maintenance of LTP (7). These data strongly suggest that persistently activated PKC that is necessary for the expression of LTP is located postsynaptically.

In our experiments to determine the mechanism for the persistent PKC activation, we found that neither membrane insertion nor proteolytic activation of PKC could account for the persistent activation of PKC. However, we did observe a persistent modification of PKC—decreased immunoreactivity—to be associated with LTP (Fig. 4). The persistent modification is due to phosphorylation, as treatment of LTP homogenates with phosphatase can reverse the effect (Table 1). While our results do not prove that this modification causes the persistent increase in PKC activity, previous reports have shown that PKC autophosphorylation leads to an increase in activity, an increased sensitivity to Ca^{2+}, and altered membrane binding and proteolytic susceptibility of the enzyme (22, 30–33). However, it also is possible that the increased phosphorylation of PKC in LTP is due to phosphorylation of PKC by another protein kinase(s), as a result of activation of a cascade of protein kinases (34).

The results of our studies suggest, in agreement with previous studies, that the activation of PKC is due to a persistent intrinsic modification of the enzyme rather than a sustained increase in a second messenger (1). It is unlikely that a persistent increase in a second messenger is responsible for the increase in basal PKC activity we observe because of the manner in which our samples are prepared. Tissue [20–40 μg of protein (the volume of the tissue is such that it is barely visible to the naked eye)] from a control or LTP slice is homogenized in 100 μl of buffer. Only 5 μl of this homogenate is used in a 50-μl protein kinase assay reaction. Therefore, it is likely that our sample is diluted 1:1000 before each protein kinase assay is performed. Expression of increased enzyme activity under such dilute conditions suggests the likelihood of an intrinsic modification of the enzyme. Additionally, assays for levels of the second messenger DAG performed at 2 and 45 min indicated no significant difference between control and LTP samples (data not shown).

The subsequent result of activation of PKC during LTP may be the increased phosphorylation of target substrates such as neuromodulin and neurogranin (27–29, 35), regulation of NMDA receptors (36, 37), and increased neurotransmission (38, 39). In addition, very long-term increases in synaptic efficacy may be the result of PKC phosphorylating other downstream effectors such as transcription factors or proteins involved in protein synthesis, processes likely to be involved in very long-term changes in nerve cells (40).

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1. Malinow, R., Madison, D. V. & Tsien, R. W. (1988) *Nature (London)* **335**, 820–824.
2. Malinow, R., Schulman, H. & Tsien, R. W. (1989) *Science* **245**, 862–866.
3. Lovinger, D. M., Wong, K., Murakami, K. & Routtenberg, A. (1987) *Brain Res.* **436**, 177–183.
4. Reymann, K. G., Frey, U., Jork, R. & Matthies, H. (1988) *Brain Res.* **461**, 388–392.
5. Malenka, R. C., Kauer, J. A., Perkel, D. J., Mauk, M. D., Kelly, P. T., Nicoll, R. A. & Waxham, M. N. (1989) *Nature (London)* **340**, 554–557.
6. Colley, P. A., Sheu, F.-S. & Routtenberg, A. (1990) *J. Neurosci.* **10**, 3353–3360.
7. Wang, J.-H. & Feng, D.-P. (1992) *Proc. Natl. Acad. Sci. USA* **89**, 2576–2580.
8. Silva, A. J., Stevens, C. F., Tonewaga, S. & Wang, Y. (1992) *Science* **257**, 201–206.
9. Grant, S. G. N., O'Dell, T. J., Karl, K. A., Stein, P. L., Soriano, P. & Kandel, E. R. (1992) *Science* **258**, 1903–1910.
10. Klann, E., Chen, S.-J. & Sweatt, J. D. (1991) *J. Biol. Chem.* **266**, 24253–24256.
11. Baudier, J., Bronner, C., Kligman, D. & Cole, D. R. (1989) *J. Biol. Chem.* **264**, 1824–1828.
12. Watson, J. B., Battenberg, K. K., Wong, F. E., Bloom, F. E. & Sutcliffe, J. G. (1990) *J. Neurosci. Res.* **26**, 397–408.
13. Baudier, J., Deloume, J. C., Van Dorsselaer, A., Black, D. & Matthes, H. W. D. (1991) *J. Biol. Chem.* **266**, 229–237.
14. Ocorr, K. A. & Schulman, H. (1991) *Neuron* **6**, 907–914.
15. Kemp, B. E., Benjamini, E. & Krebs, E. G. (1976) *Proc. Natl. Acad. Sci. USA* **73**, 1038–1042.
16. Chen, S.-J., Klann, E., Gower, M. C., Powell, C. M., Sessoms, J. S. & Sweatt, J. D. (1993) *Biochemistry* **32**, 1032–1039.
17. Towbin, H., Staehelin, T. & Gordon, J. (1979) *Proc. Natl. Acad. Sci. USA* **76**, 4350–4354.
18. Huang, K.-P. & Huang, F. L. (1986) *J. Biol. Chem.* **261**, 14781–14787.
19. Aquino, A., Hartman, K. D., Knode, M. C., Grant, S., Huang, K.-P., Niu, C.-H. & Glazer, R. I. (1988) *Cancer Res.* **48**, 3324–3329.
20. Sessoms, J. S., Chen, S.-J., Chetkovich, D. M., Powell, C. M., Roberson, E. D., Sweatt, J. D. & Klann, E. (1993) *Second Messengers Phosphoproteins*, in press.
21. Palumbo, E. J., Sweatt, J. D., Chen, S.-J. & Klann, E. (1992) *Biochem. Biophys. Res. Commun.* **187**, 1439–1445.
22. Mochly-Rosen, D. & Koshland, D. E., Jr. (1987) *J. Biol. Chem.* **262**, 2291–2297.
23. Collingridge, G., Kehl, S. J. & McLennan, H. (1983) *J. Physiol. (London)* **334**, 33–46.
24. Bazzi, M. D. & Nelsestuen, G. (1988) *Biochem. Biophys. Res. Commun.* **152**, 336–343.
25. Inoue, M., Kishimoto, A., Takai, Y. & Nishizuka, Y. (1977) *J. Biol. Chem.* **252**, 7610–7616.
26. Akers, R. F., Lovinger, D. M., Colley, P. A., Linden, D. J. & Routtenberg, A. (1986) *Science* **231**, 587–589.
27. Routtenberg, A. & Lovinger, D. M. (1985) *Behav. Neural Biol.* **43**, 3–11.
28. Eichberg, J., deGraan, P. N. E. & Gispen, W. H. (1986) *Biochem. Biophys. Res. Commun.* **136**, 1007–1016.
29. Gianotti, C., Nunzi, M. G., Gispen, W. H. & Corradetti, R. (1992) *Neuron* **8**, 843–848.
30. Borner, C., Filipuzzi, I., Wartmann, M., Eppenberger, U. & Fabbro, D. (1989) *J. Biol. Chem.* **264**, 13902–13909.
31. Huang, K.-P., Chan, K.-F. J., Singh, T. J., Nakabayashi, H. & Huang, F. L. (1986) *J. Biol. Chem.* **261**, 12134–12140.
32. Wolf, M., Cuatrecasas, P. & Sayhoun, N. (1985) *J. Biol. Chem.* **260**, 15718–15722.
33. Ohno, S., Konno, Y., Akita, Y., Yano, A. & Suzuki, K. (1990) *J. Biol. Chem.* **265**, 6296–6300.
34. Charriaut-Marlangue, C., Otani, S., Creuzet, C., Ben-Ari, Y. & Loeb, J. (1991) *Proc. Natl. Acad. Sci. USA* **88**, 10232–10236.
35. Klann, E., Chen, S.-J. & Sweatt, J. D. (1992) *J. Neurochem.* **58**, 1576–1579.
36. Chen, L. & Huang, L. Y. (1991) *Neuron* **7**, 319–326.
37. Chen, L. & Huang, L. Y. (1992) *Nature (London)* **356**, 521–523.
38. Malenka, R. C., Madison, D. V. & Nicoll, R. A. (1986) *Nature (London)* **321**, 175–177.
39. Hu, G.-Y., Kvalby, F., Walaas, S. I., Albert, K. A., Skjeflo, P., Anderson, P. & Greengard, P. (1987) *Nature (London)* **328**, 426–429.
40. Goelet, P., Castellucci, V. F., Schacher, S. & Kandel, E. R. (1986) *Nature (London)* **322**, 419–422.