Autonomously Active Protein Kinase C in the Maintenance Phase of N-Methyl-D-aspartate Receptor-independent Long Term Potentiation*

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Abstract

In area CA1 of the hippocampus, the induction of long term potentiation (LTP) requires activation of either N-methyl-D-aspartate receptors (NMDA receptor-dependent LTP) or voltage-gated Ca\(^{2+}\) channels (NMDA receptor-independent LTP). We have investigated biochemical sequelae of NMDA receptor-independent LTP induction. We find that a persistent increase in second messenger-independent protein kinase C activity is associated with the maintenance phase of NMDA receptor-independent LTP. This increase in protein kinase C activity is prevented by blocking LTP with nifedipine, a Ca\(^{2+}\) channel antagonist, or kynurenic acid, a nonselective glutamate receptor antagonist. Additionally, we find an increase in the catalytic fragment of protein kinase C (PKM) in the maintenance phase of NMDA receptor-independent LTP, indicating that proteolytic activation of protein kinase C may account for its autonomous activation. This increase in the catalytic fragment of protein kinase C is also prevented by blocking LTP induction. These results are the first to demonstrate that persistent protein kinase C activation is a possible mechanism for the maintenance of NMDA receptor-independent LTP.

Long term potentiation (LTP)\(^1\) in area CA1 of the hippocampus has been categorized into two general types based on the pharmacology of induction: NMDA receptor-dependent LTP (NMDA LTP) and NMDA receptor-independent LTP (non-NMDA LTP) (1–4). NMDA LTP is blocked by NMDA receptor antagonists (5), and a variety of evidence suggests that Ca\(^{2+}\) influx through the NMDA receptor ionophore (6–9) is required for NMDA LTP induction (4, 5, 10–12). On the other hand, non-NMDA LTP is not blocked by NMDA receptor antagonists (1–3, 13–17). Furthermore, induction of non-NMDA LTP can be blocked in most instances by voltage-gated Ca\(^{2+}\) channel antagonists (2, 3, 15, 17), suggesting that the Ca\(^{2+}\) necessary for LTP induction enters the postsynaptic neuron via voltage-gated Ca\(^{2+}\) channels in non-NMDA LTP.

While much is known about the requirements for the induction of various forms of LTP, relatively little is understood about the biochemical mechanisms by which LTP is maintained for several hours as a stable increase in synaptic efficacy. There is, however, evidence suggesting a role for protein kinases in NMDA LTP (18–26). Moreover, recent

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\(^1\)The abbreviations used are: LTP, long term potentiation; NMDA, N-methyl-D-aspartate; PKC, protein kinase C; PKM, protein kinase M; NMDA LTP, N-methyl-D-aspartate receptor-dependent LTP; non-NMDA LTP, N-methyl-D-aspartate receptor-independent LTP; NG-(28–43), neurogranin peptide (amino acids 28–43); LTP\(_{TEA}\), non-NMDA LTP induced by tetraethylammonium application; pEPSP, population excitatory postsynaptic potential; TEA, tetraethylammonium; APY, DL-2-amino-5-phosphonovaleric acid.
biochemical studies have shown directly that a lasting increase in both protein kinase C (PKC) activity (27, 28) and Ca\(^{2+}\)/calmodulin-dependent protein kinase activity (29) is associated with the maintenance phase of NMDA LTP. The recent demonstration that LTP can be induced without NMDA receptor activation in area CA1 affords us the opportunity to compare the biochemical and physiological mechanisms of the maintenance of these two different forms of LTP in the same population of synapses. If non-NMDA LTP shares common expression mechanisms with NMDA LTP, then they should converge on common biochemical pathways. This idea gains support from studies of the interaction between NMDA LTP and non-NMDA LTP in which establishment of one form of LTP occludes to some extent the subsequent induction of the other (15, 17).

In the present study we tested the hypothesis that a persistent increase in second messenger-independent or autonomous PKC activity is associated with the maintenance phase of non-NMDA LTP. We have demonstrated that there is an increase in autonomous PKC activity associated with non-NMDA LTP maintenance. This effect was observed using a potent and selective PKC substrate in assays of kinase activity in vitro, and expression of the increased activity was blocked by the relatively selective PKC inhibitor peptide PKC-(19–36). We also observed on Western blots an increase in the catalytic fragment of PKC (PKM) to be associated with non-NMDA LTP maintenance, providing additional evidence for a persistent increase in autonomous PKC activity and implicating proteolytic activation as a possible mechanism.

**EXPERIMENTAL PROCEDURES**

Conventional hippocampal slices were prepared from 4–8-week-old, male, albino, Sprague-Dawley rats as described (30). Slices were perfused with physiological saline in an interface chamber at 1–2 ml/min at 33–34 °C. Physiological saline contained (in mM) NaCl (123), KCl (3.5), CaCl\(_2\) (2), MgCl\(_2\) (1.2), NaHCO\(_3\) (25), dextrose (10), pH 7.4, when saturated with 95% O\(_2\) and 5% CO\(_2\). Extracellular field recordings were made in the stratum radiatum of area CA1 using 1–10 megohm electrodes filled with 0.5 M NaCl. Slices were stimulated continuously at 0.05 Hz with a Teflon-insulated, bipolar, platinum electrode. Unless noted otherwise, 50 μM DL-2-amino-5-phosphonovaleric acid was present in all experiments for at least 1 h prior to base-line synaptic recording. LTP\(_K\) was induced with a 10-min application of TEA (25 mM). After recording from the potentiated slice for the indicated time, a control slice, which was not exposed to TEA, was recorded from briefly (<2 min) to ensure viability. Both potentiated and control slices were taken from the same slice preparation. Slices were then frozen on glass at dry ice temperature, and the region of area CA1 between the stimulating and recording electrodes was dissected from the slices under 10–20× magnification. All further manipulations to control and potentiated slices were performed simultaneously under the same conditions so that the control and potentiated slices could be directly compared.

For PKC assays tissue from control and potentiated slices from the same hippocampal slice experiment was homogenized on ice in 100 μl of buffer (in mM): Tris-HCl (20), pH 7.5, EDTA(0.5), EGTA(0.5), Na\(_4\)P\(_2\)O\(_7\) (2), phenylmethanesulfonyl fluoride (0.1), and leupeptin (25 μg/ml). Protein kinase C activity was assayed with 0.5–2.0 μg of total protein from homogenates using 10 μM NG-(28–43) as a substrate peptide. Control and potentiated slices were assayed simultaneously in the same kinase assay. Duplicate kinase assay reactions were performed for each condition at 26 °C for 2 min in a 50-μl reaction mixture consisting of (in mM, final concentration): Tris-HCl (20), pH 7.5, EDTA (0.5), EGTA (0.5, except for basal activity), Na\(_4\)P\(_2\)O\(_7\) (2), phenylmethanesulfonyl fluoride (0.1), leupeptin (25 μg/ml), MgCl\(_2\) (10), [γ\(^{32}\)P]ATP (0.1, ~2.5 μCi/reaction) (27, 28). Assays for basal PKC activity were done in an excess of EGTA (final concentration, 2.5 mM). Assays for total PKC activity
were done in the presence of Ca\(^{2+}\) (100 µM in the presence of 500 µM EGTA) and the lipid cofactors phosphatidylserine (final concentration, 320 µg/ml) and 1,2-dioctanoyl-sn-glycerol (final concentration, 30 µg/ml). Reactions were stopped by the addition of 25 µl of ice-cold ATP and EDTA (final concentrations, 33.33 mM). Duplicate 25-µl aliquots from each reaction were spotted onto P-81 phosphocellulose filter papers. The papers were washed with 150 mM H\(_3\)PO\(_4\), dried, and immersed in 2.5 ml of Aquasol-2, and radioactivity was measured by scintillation counting. For each condition, values for control reactions lacking the substrate peptide were subtracted as background. NG-(28–43) phosphorylation was linear with respect to time and protein added under these conditions (not shown) (28, 31). Data are expressed as pmol/min/µg of protein (*indicates statistical significance using unpaired Student’s t test, p < 0.05; error bars represent standard error of the mean) (see Figs. 1C, 2C, 3B, and 4B).

It is important to note that control and experimental slices for one experiment were incubated simultaneously in physiological saline for the same period of time. Also, control and experimental slices from the same slice preparation were frozen, homogenized, and assayed simultaneously so that a direct comparison could be made between control and potentiated slices within the same experiment.

Western blot procedures were performed essentially as described (28, 32). Proteins from control and LTP\(_K\), homogenates (5–10 µg) from the same slice preparation were separated on 10% polyacrylamide gels. Proteins were transferred onto Immobilon-P transfer membranes. Blots were incubated with a polyclonal PKC antibody (generously provided by Freesia Huang, National Institutes of Health) diluted 1:2000, 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 is known to recognize native PKC and the catalytic fragment of PKC (PKM) in both tissue homogenates (not shown) (33–35) and purified preparations (28, 31, 33). Moreover, brief incubation of hippocampal homogenates with Ca\(^{2+}\) results in the generation of a proteolytic fragment of PKC that elutes from ion-exchange and gel filtration columns as expected for PKM and that is recognized by this antibody (35).

RESULTS

TEA-induced LTP Is Independent of NMDA Receptor Activation

Bath application of the K\(^+\) channel blocker TEA (25 mM) in conjunction with low frequency stimulation of Schaffer collaterals elicited non-NMDA LTP (LTP\(_K\)) in area CA1 of the hippocampus (3, 17) (Fig. 1A). As previously reported, LTP\(_K\) was reliably induced in the presence of the competitive NMDA receptor antagonist DL-2-amino-5-phosphonovaleric acid (50 µM, n = 31; see Figs. 1A and 4A) (see Aniksztejn and Ben-Ari (3) and Huang and Malenka (17)). The increase in population excitatory postsynaptic potential (pEPSP) slope following TEA application in the presence of 50 µM DL-2-amino-5-phosphonovaleric acid was 162 ± 9% (n = 7) measured 3 h after TEA washout (Fig. 4A). Furthermore, LTP\(_K\) induction was not blocked by prolonged application (>1 h prior to base-line synaptic recording) of the noncompetitive NMDA receptor antagonist MK-801 (10 µM) in the presence of still higher concentrations of DL-2-amino-5-phosphonovaleric acid (100–200 µM; 144 ± 6% of baseline, n = 4, not shown). These data confirm previous reports that induction of LTP\(_K\) does not require NMDA receptor activation (3, 17).

Autonomous PKC Activity in the Maintenance Phase of LTP\(_K\)

LTP\(_K\) is the most robust form of non-NMDA LTP that can be induced in a relatively homogeneous, large population of synapses in the hippocampus. We have taken advantage
of this robust form of non-NMDA LTP to test the hypothesis that a persistent increase in PKC activity is associated with the maintenance phase of non-NMDA LTP. LTP\textsubscript{K} was induced in the presence of 50 $\mu$m 2-amino-5-phosphonovaleric acid, and pEPSPs were monitored for 45 min following TEA washout (Fig. 1, A and B). For the control we recorded briefly (<2 min) from another slice from the same preparation that was not exposed to TEA. Isolated CA1 regions from the control and potentiated slices were then assayed for PKC activity using the selective PKC substrate peptide NG-(28–43) (31, 36). Autonomous, Ca\textsuperscript{2+}-independent PKC activity was significantly increased in potentiated CA1 regions 45 min following TEA washout (Fig. 1, C, solid bars; control, 0.59 $\pm$ 0.12 pmol/min/µg protein; LTP\textsubscript{K}, 0.98 $\pm$ 0.13 pmol/min/µg; $n = 14$; $p = 0.038$, unpaired Student’s $t$ test).

No significant change in total PKC activity stimulated with Ca\textsuperscript{2+}, phosphatidylserine, and 1,2-dioctanoyl-sn-glycerol was observed at the 45-min time point (control, 2.77 $\pm$ 0.340 pmol/min/µg protein; LTP\textsubscript{K}, 3.24 $\pm$ 0.310 pmol/min/µg; $n = 9$; not shown).

We confirmed that the increase in NG-(28–43) phosphorylation associated with LTP\textsubscript{K} is due to the activity of PKC by including a relatively selective PKC inhibitor peptide PKC-(19–36) (19, 27, 28) in assays in vitro. The increase in NG-(28–43) phosphorylation was blocked by 5 $\mu$m PKC-(19–36) (Fig. 1, C, hatched bars). Therefore, as in NMDA LTP, a persistent increase in autonomous PKC activity is associated with the maintenance phase of LTP\textsubscript{K}.

**Blocking Synaptic Potentiation Prevents the Increase in Autonomous PKC Activity**

To control for possible effects of low frequency test stimulation or the solution change protocol on PKC activity, slices were stimulated at the test frequency (0.2 Hz, low frequency stimulation) for the duration of a typical LTP experiment, and a “sham” solution change protocol was performed with normal recording saline. Low frequency stimulation without TEA application caused no synaptic potentiation and no significant change in PKC activity (control, 0.656 $\pm$ 0.09 pmol/min/µg; low frequency stimulation, 0.688 $\pm$ 0.12 pmol/min/µg; $n = 5$; not shown).

To strengthen the association between LTP\textsubscript{K} and the increased PKC activity and to rule out a direct effect of TEA application on PKC activity, we blocked LTP\textsubscript{K} with the nonselective glutamate receptor antagonist kynurenic acid and assayed PKC activity. Kynurenic acid (10 $\mu$m) blocked the induction of LTP\textsubscript{K} (Fig. 2A). This result is consistent with a previous report that LTP\textsubscript{K} is blocked by the glutamate receptor antagonist CNQX (3). Kynurenic acid was used because its effects washed out within the time course of our experiments (Fig. 2B).

Blocking LTP\textsubscript{K} prevented the increase in PKC activity (Fig. 2C; control, 0.55 $\pm$ 0.07 pmol/min/µg; TEA and kynurenic acid, 0.73 $\pm$ 0.21; $n = 6$), suggesting that the requirements for LTP\textsubscript{K} induction and for the persistent increase in PKC activity are similar.

As an additional control, we blocked LTP\textsubscript{K} with the voltage-gated calcium channel antagonist nifedipine. In a series of five experiments, nifedipine (10 $\mu$m) significantly reduced LTP\textsubscript{K} (Fig. 3A; pEPSP slope, 108 $\pm$ 1% of control, $n = 5$), and this also prevented the increase in PKC activity (Fig. 3B; control, 0.369 $\pm$ 0.03 pmol/min/µg; TEA and nifedipine, 0.289 $\pm$ 0.06 pmol/min/µg; $n = 5$). The effect of nifedipine on the magnitude of LTP\textsubscript{K}, however, proved to be variable in subsequent experiments in which little or no effect of nifedipine on LTP\textsubscript{K} was observed ($n = 4$, not shown). The variable effects of nifedipine on LTP\textsubscript{K} indicate that another source of Ca\textsuperscript{2+} entry may also play a role in LTP\textsubscript{K}. Nonetheless, the associated increase in PKC activity was not observed when LTP\textsubscript{K} induction was blocked by either kynurenic acid or nifedipine. These findings support the hypothesis that the increase in PKC activity plays a role in LTP\textsubscript{K} maintenance.
Time Course of the Increase in Autonomous PKC Activity

If the increase in PKC activity is involved in the maintenance of LTP$_K$ then the increase should persist during LTP$_K$ maintenance. To test this prediction we monitored LTP$_K$ for 3 h following TEA washout (Fig. 4A) and assayed PKC activity. A significant increase in basal PKC activity was observed 3 h into LTP$_K$ maintenance (Fig. 4B; control, 0.243 ± 0.07 pmol/min/µg; LTP$_K$, 0.686 ± 0.17 pmol/min/µg; n = 7; p < 0.05). These results further indicate that the increase in PKC activity is not due to an effect of residual TEA because TEA should be completely washed out after 3 h (see Fig. 2B). Control PKC activity was lower in the 3-h LTP experiments than in the 45-min LTP experiments (Fig. 4B). The decrease in autonomous PKC activity with longer incubation times has been observed previously$^2$ and may be due to gradual deterioration of slices.

The onset of the increase in PKC activity was also examined. No significant increase in basal PKC activity was observed 5 or 20 min following the washout of TEA (Fig. 4B). Therefore, the increase in PKC activity is gradual or delayed in onset relative to the presumed induction phase of LTP$_K$.

No significant change in total PKC activity stimulated with Ca$^{2+}$, phosphatidylserine and 1,2-dioctanoyl-sn-glycerol was observed at any time point tested (5 min, 20 min, 45 min, 3 h; data not shown).

Posttranslational Modification of PKC Is Associated with LTP$_K$ Maintenance

To provide an additional line of evidence for an increase in autonomous PKC activity that does not rely on substrate selectivity or inhibitor selectivity, we tested for posttranslational modification of PKC in the maintenance phase of LTP$_K$. The best characterized mechanism for generating an increase in autonomous PKC activity is proteolytic activation (37–40). Proteolytic activation of PKC occurs when the regulatory domain is removed, leaving the 45–50-kDa catalytic domain (PKM), which is active in the absence of second messenger activators (37–40). To test the hypothesis that the persistent increase in PKC activity associated with LTP$_K$ is due to proteolytic activation of PKC, a Western blot analysis was performed using a polyclonal antibody that recognizes multiple PKC isoforms as well as proteolytically activated PKC (33–35) and preferentially binds to unphosphorylated PKC (28). A significant increase in a 45-kDa immunoreactive PKC fragment was observed (Fig. 5A; LTP$_K$, 119 ± 6% of control; n = 10; p = 0.009, unpaired Student’s t test). Previous characterization of this fragment indicates that its immunoreactivity, molecular weight, and charge character are consistent with that of proteolytically activated PKC (PKM) (31, 33–35, 37–40). To demonstrate that the increase in PKM is specifically associated with LTP$_K$, we blocked LTP$_K$ with kynurenic acid (see Fig. 2A). Blocking LTP$_K$ completely eliminated the increase in PKM (Fig. 6). Thus, we have provided additional evidence for an increase in autonomous PKC activity associated with LTP$_K$, and this increase in PKC activity may involve proteolytic activation of PKC.

In NMDA LTP, Klann et al. (28) observed a decrease in native PKC immunoreactivity that was reversed by treatment of homogenates with phosphatases, indicating an increase in phosphorylation of PKC. Using the same antibody and blotting conditions, we saw no evidence for a change in phosphorylation of PKC as no change in native PKC immunoreactivity was observed in LTP$_K$ (Fig. 5B; LTP$_K$, 125 ± 15% of control, n = 10). This result complements our finding of no change in total PKC activity in LTP$_K$. Also, in contrast to Klann et al. (28), we observed an increase in the catalytic fragment of PKC. An increase in a catalytic fragment of PKC isoform $\zeta$ has been previously described in NMDA.

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$^2$E. Klann and J. D. Sweatt, personal communication.
LTP using affinity-purified antibodies against PKCζ (41). The antibody used in the present studies, however, does not appear to recognize PKCζ in the hippocampus in a side by side comparison with the PKCζ antibody used in the study of Sacktor et al. (41) (data not shown, PKCζ antibody generously provided by Dr. Todd Sacktor). These findings suggest differences in the posttranslational modifications of PKC observed in NMDA LTP and LTPK.

DISCUSSION

We have demonstrated that a persistent increase in protein kinase activity is associated with the early maintenance phase of LTPK. The evidence that this increase in kinase activity is due to protein kinase C is compelling.

First, we measured kinase activity using a potent and selective PKC substrate, NG-(28–43). Phosphorylation of NG-(28–43) is increased by addition of PKC activators (Ca2+, phosphatidylserine, 1,2-dioctanoyl-sn-glycerol) to purified PKC preparations (31) or hippocampal homogenates (28). NG-(28–43) is phosphorylated by proteolytically activated PKC (PKM) both in purified preparations (31) and in hippocampal homogenates (35). The K_m of NG-(28–43) is approximately 150 nM for purified PKC, proteolytically activated PKC (PKM) (31, and crude hippocampal homogenate stimulated with PKC activators (36). Thus, NG-(28–43) is clearly a PKC substrate in both purified preparations and crude hippocampal homogenates. NG-(28–43) is not readily phosphorylated by purified preparations of Ca2+/calmodulin-dependent protein kinase, AMP-dependent protein kinase (31)) or phosphorylase kinase (36) under conditions where they exhibited relatively high phosphotransferase activity using other substrates. Thus, the increase in NG-(28–43) phosphorylation associated with LTPK is not likely to be caused by any of these major hippocampal serine/threonine kinases whose substrate specificities are known to overlap to some extent with that of PKC. NG-(28–3) cannot be phosphorylated by tyrosine kinases as it contains no tyrosine residues (31). Also, the relatively selective PKC inhibitor peptide PKC-(19–36) at a concentration of 10 µM blocks ~95% of NG-(28–43) phosphorylation by purified PKC (31), hippocampal homogenates stimulated with PKC activators, and hippocampal homogenates under basal assay conditions used in this study (28). Furthermore, the IC50 of PKC-(19–36) is remarkably similar for purified PKC, hippocampal homogenates stimulated with PKC activators, and hippocampal homogenates under basal conditions using 10 µM NG-(28–43) as substrate (IC50 ~0.5 µM when kinase or homogenate was preincubated with PKC-(19–36) prior to onset of assay) (28, 31), indicating that phosphorylation of NG-(28–43) in hippocampal homogenates is largely due to PKC even under basal conditions. While these data provide strong evidence that the majority of NG-(28–43) phosphorylation in hippocampal homogenates is due to PKC, we cannot rule out the possibility that some protein kinase, other than PKC, Ca2+/calmodulin-dependent protein kinase, PKA, or phosphorylase kinase, which has a similar susceptibility to PKC-(19–36) inhibition, contributes to NG-(28–43) phosphorylation in hippocampal homogenates.

Second, the PKC inhibitor peptide, PKC-(19–36), completely blocked the increase in NG-(28–43) phosphorylation associated with LTPK. The concentration of PKC-(19–36) used in these studies (5 µM) was chosen to be submaximal for PKC inhibition to increase selectivity. This result supports our interpretation that the increase in NG-(28–43) phosphorylation in LTPK is due to phosphorylation by PKC.

Finally, we observed an increase in an approximately 45-kDa fragment of PKC associated with the maintenance phase of LTPK. A variety of evidence suggests that this 45-kDa band is the constitutively active, catalytic fragment of PKC (PKM). A persistent increase in autonomous PKC activity is associated with the increase in the 45-kDa fragment following

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LTP<sub>K</sub>, as expected for an increase in PKM. The PKC antibody we used has been well characterized and is known to recognize native PKC and the catalytic fragment of PKC (PKM) in both tissue homogenates, including hippocampus (33–35) (not shown) and purified preparations (28, 31, 33). Also, brief incubation of hippocampal homogenates with Ca<sup>2+</sup> results in the generation of a 45–50-kDa proteolytic fragment of PKC recognized by the same antibody (35) (not shown) and a large increase in basal PKC activity (35) (not shown). Both the immunoreactivity and the enzyme activity elute from ion-exchange chromatography as expected for PKM (35). These data strongly suggest that the 45-kDa PKC fragment is the constitutively active, catalytic fragment of PKC, PKM. An increase in a PKC fragment of the appropriate molecular weight for proteolytically activated PKC (PKM) alone provides evidence for an increase in basal PKC activity.

These results provide strong evidence that PKC is persistently activated in the maintenance phase of LTP<sub>K</sub>. Also, the present studies provide the first direct evidence suggesting persistent PKC activation as a possible mechanism for the maintenance of a form of NMDA receptor-independent LTP.

The increase in basal PKC activity, associated with non-NMDA LTP, like PKM, is independent of typical second messenger activators of PKC. The increased PKC activity is Ca<sup>2+</sup>-independent because basal PKC activity is measured in the presence of an excess of the Ca<sup>2+</sup> chelator EGTA. Also, it is unlikely that a persistent increase in a second messenger is responsible for the increase in basal PKC activity because of the manner in which our samples are prepared. A fraction of the CA1 region (20–30 µg of protein; volume, ≤1 µl) from LTP or control slices is homogenized in 100 µl of buffer and often diluted further. Only 5 µl of this homogenate is added to a 50-µl kinase assay reaction. Therefore, our sample is diluted at least 1:1000 before each kinase assay reaction is performed. Increased kinase activity under such dilute conditions suggests an intrinsic modification of the enzyme such as proteolytic activation. Thus, the increase in PKC activity is due to an autonomously active form of the enzyme, and the most parsimonious explanation is that the increased PKC activity is a result of the increase in PKM.

Considering this finding with previous results, NMDA LTP and LTP<sub>K</sub> have in common persistent activation of PKC in their maintenance phase. The increase in basal PKC activity is of similar magnitude in both forms of LTP (Fig. 1B) (27, 28) and is prevented by blocking LTP (Figs. 2 and 4) (27, 28). If we assume that the effector substrates of PKC are similar in the two forms of LTP, then these results complement the finding that NMDA LTP can partially occlude subsequent LTP<sub>K</sub> expression and vice versa (17). It is interesting that two forms of LTP with distinct induction mechanisms may converge on a common effector mechanism, persistent activation of PKC, in the maintenance phase.

Previous studies, using the same antibody and protocols used in the present study, indicate that the mechanism of PKC activation in NMDA LTP is increased phosphorylation of PKC (28) and not proteolytic activation because no change in the PKM fragment was observed on Western blots in NMDA LTP (28) (but see Sacktor et al. (41)). In the present studies, no increase in PKC phosphorylation was observed while an increase in PKM was observed. Thus, the two distinct means of Ca<sup>2+</sup> entry in NMDA LTP and LTP<sub>K</sub> appear to cause different posttranslational modifications of PKC. Differences in subcellular localization of Ca<sup>2+</sup> influx or in intracellular Ca<sup>2+</sup> concentrations between the two forms of LTP are possible explanations for this observation. Also, the activation of two distinct signaling pathways by Ca<sup>2+</sup> influx through NMDA receptors or through voltage-gated Ca<sup>2+</sup> channels in hippocampal neurons has been recently described (42).
An increase in a proteolytic fragment of PKC has been previously observed in NMDA LTP using an antibody specific for the PKC ζ isoform (41). The antibody used in the present study and the study of Klann et al. (28) does not appear to recognize this PKC ζ fragment or native PKC ζ in direct comparisons using Western blots of hippocampal homogenates with both antibodies (data not shown). Thus, the increase in PKM that we observe in LTPK is different from that observed in NMDA LTP.

In conclusion, we have found that, as in NMDA LTP, a persistent increase in basal PKC activity is associated with LTPK, a form of NMDA receptor-independent LTP. The mechanism of persistent PKC activation in LTPK may involve generation of the catalytically active fragment of PKC, PKM. Although differences in posttranslational modification of PKC occur in the two forms of LTP, these results indicate that persistent activation of PKC may play a role in the expression of both NMDA receptor-dependent and NMDA receptor-independent forms of LTP, suggesting a general role for PKC activity in LTP. It will be interesting to examine whether PKC activity is strictly required for the expression of LTPK and other forms of NMDA receptor-independent LTP.

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Fig. 1. Basal PKC activity is increased 45 min into the maintenance phase of LTP<sub>K</sub>

A, time course of LTP<sub>K</sub>. pEPSP slope is plotted as a function of time. Each point represents the slope of the average of four consecutive traces normalized to the base-line average of each experiment in this and all subsequent pEPSP time course figures. TEA (25 mM) application in the presence of 50 µM DL-2-amino-5-phosphonovaleric acid (APV) caused a lasting potentiation (172 ± 12%, n = 14). B, example pEPSPs taken before (a), during (b), and 45 min after (c) TEA washout. C, increase in basal, Ca<sup>2+</sup>-independent PKC activity 45 min into LTP<sub>K</sub> (solid bars). Addition of PKC-(19–36) (5 µM) to kinase assays <em>in vitro</em> blocks the increase in PKC activity associated with LIT<sub>K</sub> (hatched bars).
Fig. 2. Blocking LTPK with kynurenic acid prevents the increase in PKC activity
A, LTPK was completely blocked when TEA was applied in the presence of 10 mM kynurenic acid (n = 5). B, physiological effects of kynurenic acid (10 mM) are completely reversible (n = 4). C, no significant increase in PKC activity was observed in slices exposed to TEA in the presence of kynurenic acid. Control slices were similarly perfused with 10 mM kynurenic acid.
Fig. 3. Blocking LTP with 10 µM nifedipine prevents the increase in basal PKC activity
A, time course of pEPSP slope before and after TEA application (25 mM) in the presence of 10 µM nifedipine and 50 µM APV. Potentiation is significantly reduced to 108 ± 1% of baseline 45 min after TEA washout (n = 5). B, no change in basal PKC activity occurs after TEA application in the presence of 10 µM nifedipine and 50 µM APV (n = 5). Control slices were similarly perfused with nifedipine.
Fig. 4. Time course of the increase in basal PKC activity associated with LTP<sub>K</sub> maintenance
A. 3-h time course of LTP<sub>K</sub>. pEPSP slope is plotted as a function of time. TEA (25 mM) application in the presence of 50 µM APV caused a lasting potentiation (144 ± 6%, n = 7). Inset numbers represent times in minutes where PKC activity was measured in B. B, the increase in PKC activity associated with LTP<sub>K</sub> persists for at least 3 h following TEA application. Basal PKC activity was measured in separate experiments at various time intervals following onset of TEA washout (5 min, n = 5; 20 min, n = 7; 45 min, n = 14; 180 min, n = 7). A significant increase in basal PKC activity was observed 45 and 180 min following TEA washout (* indicates statistical significance using an unpaired Student’s t-test).
test, \( p < 0.05 \). No significant change in total \( \text{Ca}^{2+} \)/phosphatidylserine/oleoylacylglycerol-stimulated PKC activity was observed at any time point (not shown).
Fig. 5. Proteolytic activation of PKC is associated with the increase in PKC activity

A, a significant increase in the proteolytically activated fragment of PKC occurs 45 min into 
LTPK maintenance. Left, example Western blot analysis reveals a statistically significant 
increase in the 45-kDa immunoreactive protein (PKC Fragment) 45 min into LTPK 
maintenance. Right, grouped densitometry data from LTPK Western blot experiments reveal 
a statistically significant increase in PKM immunoreactivity (119 ± 6% of control, n = 10, p 
= 0.009). B, no significant change in native PKC immunoreactivity occurs during LTPK. 
Left, example Western blot analysis (shorter exposure than in A) using a polyclonal antibody 
raised against classical PKC isoforms demonstrates no change in native PKC
immunoreactivity (PKC). Right, grouped densitometry data from LTPK Western blot experiments reveal no significant change in the immunoreactivity of native PKC (n = 10, 45-min time point).
Fig. 6. Blocking LTP$_K$ with kynurenic (kyn.) acid prevents the increase in PKM associated with LTP$_K$ maintenance

*Left,* example Western blot analysis using a polyclonal antibody raised against classical PKC isoforms demonstrates no significant increase in PKM when LTP$_K$ is blocked by kynurenic acid. *Right,* grouped densitometry data from kynurenic acid block experiments show no increase in PKM when LTP$_K$ is blocked by kynurenic acid (89 ± 8% of control, $n = 6$).