Proc. Natl. Acad. Sci. USA
Vol. 90, pp. 8342–8346, September 1993
Neurobiology

Persistent activation of the ζ isoform of protein kinase C in the maintenance of long-term potentiation

TODD CHARLTON SACKTOR*, PAVEL OSTEN, HELEN VALSAMIS, XIAOLAN JIANG, MEGHNA U. NAIK, AND ELIZABETH SUBLETTE

Laboratory of Molecular Neuroscience, Departments of Pharmacology and Neurology, State University of New York at Brooklyn, Box 29, 450 Clarkson Avenue, Brooklyn, NY 11203

Communicated by Robert F. Furchgott, June 1, 1993 (received for review, February 10, 1993)

ABSTRACT **Long-term potentiation in the CA1 region of the hippocampus, a model for memory formation in the brain, is divided into two phases. A transient process (induction) is initiated, which then generates a persistent mechanism (maintenance) for enhancing synaptic strength. Protein kinase C (PKC), a gene family of multiple isozymes, may play a role in both induction and maintenance. In region CA1 from rat hippocampal slices, most of the isozymes of PKC translocated to the particulate fraction 15 sec after a tetanus. The increase of PKC in the particulate fraction did not persist into the maintenance phase of long-term potentiation. In contrast, a constitutively active kinase, PKM, a form specific to a single isozyme (ζ), increased in the cytosol during the maintenance phase. The transition from translocation of PKC to formation of PKM may help to explain the molecular mechanisms of induction and maintenance of long-term potentiation.**

During long-term potentiation (LTP), the tetanization of synaptic afferents initiates a sustained enhancement of synaptic transmission. Enzymes that participate in signal transduction and the regulation of synaptic strength may be persistently active during the maintenance of LTP (1) and could contribute to the underlying mechanism of memory (2). In the CA1 region of the hippocampus, the induction of LTP begins with the influx of Ca^{2+} into the postsynaptic neuron (3, 4) through the activated *N*-methyl-D-aspartate (NMDA) receptor (5, 6). Beyond that step, several protein kinases have been implicated in the induction of LTP (7–10), including protein kinase C (PKC). PKC may also have a role in the maintenance of LTP, since activators of PKC such as phorbol esters cause synaptic potentiation (11) and inhibitors of protein kinase activity such as H7 (1, 12) or pseudosubstrate peptides (13) may block expression of LTP, although their effects have not been consistent (7, 14). The multiple roles of the enzyme during induction and maintenance might be explained by the heterogeneity of PKC isozymes in brain (15).

The activation of PKC by second messengers is believed to involve the translocation of the enzyme, in which elevations of diacylglycerol or applications of phorbol esters shift the distribution of PKC to membrane (16). However, a second mechanism of PKC activation in which the kinase becomes independent of second messengers has been demonstrated, primarily *in vitro*. Proteolytic cleavage between the N-terminal regulatory domain and the C-terminal catalytic domain generates an independent constitutively active fragment, termed PKM (17) (Fig. 1*A*). When Ca^{2+} chelators are omitted during homogenization of brain tissue, a 51-kDa PKM is formed by the activation of Ca^{2+}-dependent proteases calpains (17). Endogenous PKM has been shown to occur in a tumor cell line (18). However, despite the appeal of the notion of a persistently active form of PKC in neuronal function, to our knowledge, the presence of endogenous PKM in brain has not been previously described.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

MATERIALS AND METHODS

Preparation of Hippocampal Slices. After anaesthesia with halothane, transverse 450-μm hippocampal slices from 3- to 4-week-old Sprague–Dawley rats were prepared with a McIlwain tissue slicer. For biochemical experiments or after pharmacological or electrical stimulations, the slices were frozen by contact with a metal rod cooled to −55°C. The slices were then transferred to propylene glycol/0.9 M NaCl, 1:1 (vol/vol), for dissection of CA1 regions on powdered dry ice. Pooled CA1 regions were washed twice at 4°C in homogenization buffer [50 mM Tris·HCl, pH 7.5/1 mM EDTA/1 mM EGTA/5 mM 2-mercaptoethanol/0.1 mM phenylmethylsulfonyl fluoride/aprotinin (17 kallikrein units/ml)/5 mM benzamidine/0.1 mM leupeptin (unless otherwise specified, chemicals were from Sigma)]. Four to five CA1 regions were homogenized in 300 μl of the buffer by 15 strokes of a Teflon-glass Potter–Elvehjem tissue grinder (Kontes) and centrifuged at 3000 × *g* for 5 min to remove unhomogenized material and nuclei (P1). The supernatant (S1) was centrifuged again at 100,000 × *g* for 30 min to produce a supernatant (S2, cytosolic fraction) and pellet (P2, particulate fraction). A typical yield of total protein was 50 μg from S2 and 15 μg from P2. Protein was determined by a modification of the Bradford assay (19, 20), except for slices comparing homogenization techniques with boiling in SDS/sample buffer (100°C for 10 min) without fractionation, which were determined by assay using bicinchoninic acid (Pierce).

PKC Isozyme-Specific Antisera. Isozyme-specific rabbit antisera were prepared by injection of maleimide-activated bovine serum albumin (Pierce) conjugated to peptides corresponding to the C-terminal regions of PKC isoforms (α, PQFVHPILQSAV; βI, SYTNPEFVINV; βII, SFVNSEFLKPEVKS; γ, PDARSPTSPVPVPVM; δ, NPKYEQFLE; ε, YFGEDLMP; ζ, EYINPLLLSAEESV). The antisera were affinity-purified on columns in which the peptides had been conjugated to SulfoLink coupling gel (Pierce). Immunoblots (21) of SDS/8% polyacrylamide gels (22) were in the linear range of detection, using either alkaline phosphatase (Promega) or chemiluminescence (Amersham) detection methods. The levels of PKC isozymes from fractions of hippocampal slices were compared by loading equal amounts of total protein from the fractions on the gel. Densitometry of bands was performed with National Institutes of Health Image software on an XRS 6cx scanner (OmniMedia, Torrance, CA). For experiments demonstrating specificity of the primary antibody, the antiserum was preincubated for 2 hr at

Abbreviations: PKC, protein kinase C; LTP, long-term potentiation; EPSP, excitatory postsynaptic potential; NMDA, *N*-methyl-D-aspartate; CPP, 3-[(*RS*)-2-carboxypiperazin-4-yl]-propyl-1-phosphonic acid.

*To whom reprint requests should be addressed.

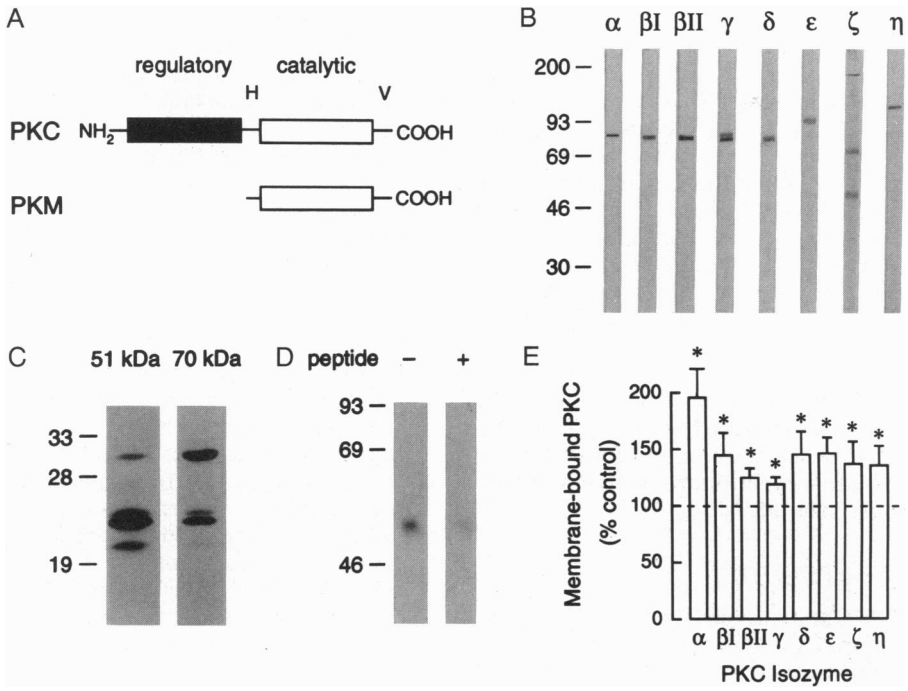

FIG. 1. PKC and PKM family of protein kinases in the hippocampus. (*A*) Schematic diagram of PKC and PKM prototypes. PKC contains a regulatory domain and a catalytic domain, separated by a hinge region (H). PKM is the independent C-terminal region generated by proteolytic cleavage at the hinge. Isozyme-specific antisera were made to the C-terminal variable regions (V) to detect both PKC and PKM. (*B*) Immunoblot of hippocampal proteins from CA1 (6 μg of protein per cm·lane) with affinity-purified PKC isozyme-specific antisera. Single bands or doublets are detected with most of the antisera. Antiserum to the C-terminal region of ζ detects three major proteins. Positions of molecular mass standards in kDa are shown on the left. (*C*) Peptide mapping. The peptide maps of the 51-kDa and 70-kDa proteins produced by CNBr cleavage are identical. Cleavage products were detected on an immunoblot with C-terminal ζ antiserum. Molecular masses of all the cleavage products are given in the text. Positions of molecular mass standards are shown at left. (*D*) Immunokinase assay. An autoradiogram shows autophosphorylation of a 51-kDa protein immunoprecipitated by the ζ antiserum. Specificity of the assay is demonstrated by preincubating the antiserum with the immunizing peptide (lane +). (*E*) Phorbol esters (5 μM phorbol 12,13-dibutyrate for 30 min) cause the translocation to the particulate fraction of all isozymes of PKC. Levels of isozymes in the particulate fraction from CA1 regions of hippocampal slices treated with phorbol are compared to levels from control slices from the same hippocampus set to 100% (mean ± SEM; $n = 8$; n values in this and other experiments represent separate animals). Levels of PKC in the particulate fraction with or without phorbol treatment were evaluated by multiple analysis of variance. The following effects were significant ($P < 0.05$): between isozymes [$F_{(7,81)} = 4.50$], between treated and nontreated [$F_{(1,81)} = 37.81$], and the interaction of isozyme and treatment condition—i.e., the patterns of change varied by isozyme [$F_{(7,81)} = 2.89$]. The effect of phorbol on individual isozymes was then evaluated by paired t test. *, For this and other figures, when experimental groups are compared to control, $P < 0.05$.

25°C in 50 μl of bovine serum albumin (Calbiochem; 1 mg/ml) in the presence or absence of 20 μg of immunizing peptide.

Antiserum to the C-terminal peptide of PKCη was a generous gift from Shin-ichi Osada (Yokohama University) (23). This antiserum detected the primarily lung- and skin-specific isozyme (82 kDa) at very low levels in brain. The antiserum did detect in brain, however, a prominent protein of 97 kDa that has properties of PKC (24).

Peptide Mapping. CNBr cleavage (25) was performed by excision and electroelution of proteins from an SDS/8% polyacrylamide gel and incubation for 24 hr with CNBr (3 mg/ml) in 70% (vol/vol) formic acid. After lyophilization, the C-terminal peptides were separated on a SDS/15% polyacrylamide gel and detected on immunoblots with the ζ antiserum.

Autophosphorylation. Autophosphorylation of the material immunoprecipitated by the ζ antiserum (immunokinase assay) was initiated by incubation of 50 μg of the S2 hippocampal fraction (in 200 μl of the homogenization buffer/150 mM NaCl, without 2-mercaptoethanol) and 1 μl of ζ antiserum for 2 hr at 4°C. After a 1-hr incubation with protein A-Sepharose beads (25 μl, Pharmacia), the immunocomplexes were centrifuged (1000 × *g* for 30 sec) and washed with the buffer three times. The pellet was then resuspended in 40 μl of reaction mixture (50 mM Tris·HCl, pH 7.5/10 mM $MgCl_2$/1 mM 2-mercaptoethanol/1 mM pyrophosphate), and autophosphorylation was begun by the addition of 10 μl of [γ-^{32}P]ATP (1 μCi per assay mixture at a final concentration of 1 μM; 1 Ci = 37 GBq). After 5 min at 30°C, the reaction was stopped by the addition of 50 μl of 1 mM unlabeled ATP and sample buffer. The autophosphorylated proteins, separated on an SDS/8% polyacrylamide gel and transblotted to nitrocellulose, were detected by autoradiography with X-AR5 film (Eastman Kodak).

For dilution experiments, the autophosphorylation of PKMζ was determined in solution rather than using PKMζ attached to beads. PKMζ was partially purified by loading 200 μg of the S2 hippocampal fraction (without 2-mercaptoethanol) onto a DEAE-cellulose column (Millipore) that had been equilibrated with 50 mM Tris·HCl, pH 7.5/1 mM EDTA/1 mM EGTA. The column was washed with 200 mM NaCl (to remove most of the other PKCs) and PKMζ was eluted with 400 mM NaCl. The salt was diluted in equilibration buffer and the sample was concentrated with a microconcentrator (Amicon). The 400 mM fraction was serially diluted 1:20 and autophosphorylation was performed as above, except 6.25 μCi per assay mixture was used. Autophosphorylation of the fraction enriched for PKMζ showed a single major band comigrating with PKMζ, which in parallel experiments immunoprecipitated with the ζ antiserum. Phosphate incorporation was quantitated by scintillation counting of the excised bands.

Tetanization. Slices were placed in an interface recording chamber infused initially with a saline solution (125 mM NaCl/2.5 mM KCl/1.25 mM NaH_2PO_4/26 mM $NaHCO_3$/11 mM glucose, pH 7.4) containing 10 mM $MgCl_2$ and 0.5 mM $CaCl_2$, equilibrated with 95% O_2/5% CO_2 at 32°C. This

concentration of $MgCl_2$ has been shown to help preserve CA1 pyramidal cells after slice preparation (26). After 1 hr, the divalent ion concentrations were changed to 1.2 mM $MgCl_2$ and 1.7 mM $CaCl_2$. Test stimuli of 100-μsec pulse duration were delivered every 15 sec to the Schaffer collateral/commissural fibers through a bipolar tungsten electrode. The current was set to produce 30% of the maximal population spike amplitude, typically 300–600 μA, as determined by an input–output curve for each slice. Extracellular recordings were made with standard glass electrodes containing 2 M NaCl that were placed in the CA1 pyramidal cell layer (population spikes) or stratum radiatum [field excitatory postsynaptic potentials (EPSPs)]. After at least 15 min of stable recording, a tetanus of 100 Hz for 1 sec was given. The current of the tetanic stimuli was set to produce 75% of the maximal population spike amplitude. Determination of the slope of the initial phase of the extracellular EPSP and the population spike amplitude was performed with SuperScope (GW Instruments, Somerville, MA).

RESULTS

Characterization of PKC Isozymes and the PKM Form of the ζ Isozyme in Hippocampus. By immunoblot analysis, most of the antisera to the individual isozymes of PKC detected single major bands or doublets of appropriate molecular mass based upon the corresponding predicted amino acid sequences (Fig. 1*B*). The molecular masses were as follows: α, 80 kDa; βI, 77 kDa; βII, 79 kDa; γ, 79 kDa; δ, 76 kDa; ε, 89 kDa; ζ, 70 kDa; η-related isoform, 97 kDa. The antiserum to PKCζ, which has a predicted molecular mass of $\approx$68 kDa (27), however, detected three major proteins of 160, 70, and 51 kDa. Occasionally, a minor band of 80 kDa was also detected (data not shown). Antiserum binding to all three major proteins was blocked by preincubation of the antiserum with the immunizing peptide (data not shown).

To confirm that the 51- and 70-kDa proteins are structurally related, peptide maps of the two proteins were generated by CNBr cleavage. The molecular masses of the cleavage products of the two proteins, detected on immunoblot with the C-terminal ζ antiserum, were identical (Fig. 1*C*). The molecular masses of the C-terminal CNBr fragments predicted by the published amino acid sequence of PKCζ (27) are as follows: 15.3, 17.0, 17.1, 17.6, 22.3, 28.8, 29.3, and 36.1 kDa. The observed molecular masses of the fragments are as follows: 15.5, 17.0, 20.5, 22.6, 23.8, 25.2, 28.0, 29.9, 32.0, and 35.5 kDa. Variations in the sizes of the observed fragments may be due to phosphorylation. This result suggests that the 51-kDa protein is related to the C-terminal region of the 70-kDa protein.

We then characterized the catalytic activity of the 51-kDa ζ-related protein by immunokinase assay. The ζ antiserum immunoprecipitated from an extract of hippocampus a single major autophosphorylating protein, comigrating with the 51-kDa protein (Fig. 1*D*). Preincubation of the antiserum with the immunizing peptide inhibited the precipitation of the kinase. Phosphorylation of the 51-kDa substrate was linear for 30 min and was not increased by addition of phosphatidylserine or phorbol esters (data not shown). To confirm that the phosphorylation was intramolecular, the specific activity of the phosphorylation with serial dilution of the 51-kDa protein was determined at an initial reaction rate and with ATP concentration constant. The specific activity remained constant with dilution, and a plot of the logarithm of the initial velocity vs. the logarithm of the enzyme concentration (a van't Hoff's plot of the data, not shown) (28) was found to be linear with a slope of 1.00 (n = 2). These results indicate an intramolecular phosphorylation of the 51-kDa protein and, in combination with the peptide mapping, suggest that the 51-kDa protein detected by the ζ antiserum is the PKM form of the isozyme (PKMζ).

Although none of the other antisera detected major proteins of appropriate molecular mass for PKM in hippocampal slices (potential PKM forms of other isozymes were occasionally detected as minor bands, data not shown), we wished to determine whether PKMζ was formed during our homogenization and fractionation of untreated slices. Immunoblots from hippocampal slices that were homogenized in the presence of a combination of protease inhibitors and 1 mM EGTA (our usual procedure) were compared to slices that were (*i*) boiled immediately after dissection in SDS/sample buffer, which rapidly denatures cellular proteins; (*ii*) homogenized with 20 mM EGTA and protease inhibitors including 1 mM leupeptin (10 times our usual concentration); or (*iii*) preincubated for 30 min with a cell-permeable protease inhibitor, the leupeptin analogue Cbz-Val-Phe at 100 μM (where Cbz is carbobenzoxy, a gift from Shujaath Mehdi and Ekkehard Bohme, Marion Merrell Dow) (29). Relative to our normal homogenization procedure, the levels of total PKMζ using the alternative conditions were 106.6 ± 3.2%, 94.8 ± 5.9%, and 95.7 ± 8.9%, respectively (mean ± SEM; n = 3). Therefore, none of these alternative homogenization procedures significantly altered the levels of PKMζ. We also found PKMζ in hippocampus homogenized whole without slicing on a tissue chopper and in organotypic hippocampal slice cultures boiled in SDS/sample buffer (data not shown).

In preparation for physiological experiments, applications of phorbol esters (5 μM phorbol 12,13-dibutyrate for 30 min) were shown to increase all isoforms of PKC, including PKCζ, in the particulate fractions of the CA1 regions from hippocampal slices, as compared to levels in CA1 regions from untreated slices (Fig. 1*E*). [Although purified or overexpressed PKCζ does not bind phorbol esters *in vitro* (27, 30), the agent, when applied to cells such as platelets (31) or fibroblasts (32), can result in translocation of the endogenous isozyme.]

PKC Isozymes in LTP. To determine whether PKC, or a subset of isozymes, is activated during the induction phase of LTP, we measured the shift in subcellular distribution of each isozyme in region CA1 of hippocampal slices 15 sec after a single tetanus (100 Hz for 1 sec) to Schaffer collateral/commissural fibers. [In parallel experiments, the tetanus resulted in long-lasting potentiation in 85% of slices (Fig. 2 *A* and *B*).] Fifteen seconds after the tetanization, α, βI, βII, δ, ζ, and η-related isoforms increased significantly in the particulate fraction (Fig. 2*C*).

We next examined whether the maintenance of LTP in CA1 was related to persistence of the increase of PKC in the particulate fraction. Slices that showed stable potentiations of population spike amplitude and extracellular EPSP slope (279.3 ± 20.7% and 168.3 ± 9.3% of baseline, respectively) 30 min after a tetanus were compared to adjacent slices that had stable recordings but were not tetanized. In the maintenance phase of LTP, the levels of all the isozymes of PKC in the particulate fraction of CA1 were no longer elevated (Fig. 2*D*).

PKMζ in LTP. Since PKC isozymes did not persistently translocate to membrane, we wished to know whether there was a change in the level of the PKM form of ζ that might account for the maintenance of LTP. No change in PKMζ level was observed with either applications of phorbol esters (95.4 ± 7.8% of control levels of S2; n = 8) or 15 sec after tetanization (89.3 ± 7.1%; n = 6). However, 30 min after the tetanization, PKMζ in the cytosol increased significantly (141.2 ± 14.8%; n = 11; paired t test, $P < 0.05$; Fig. 3). PKMζ could also be detected in the particulate fraction, but the level did not consistently increase 30 min after tetanization (data not shown).

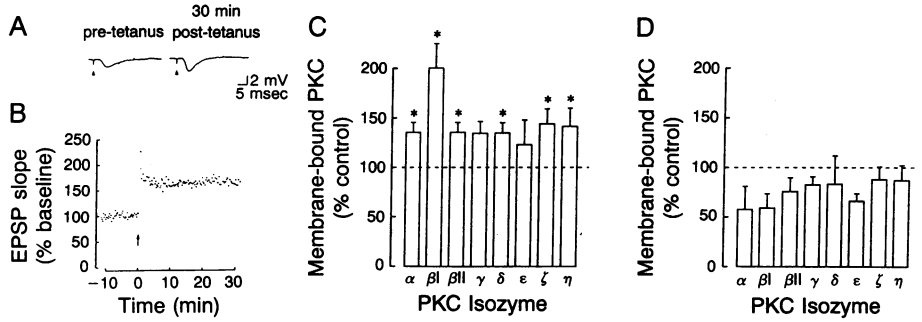

FIG. 2. Effects of tetanization on PKC in the particulate fraction from region CA1 of the hippocampus. (*A*) Representative field EPSPs, pretetanus and 30 min posttetanus. The stimulus artifacts are shown by the arrowheads. (*B*) LTP, showing a persistent increase in initial slope of extracellular EPSPs for 30 min after a tetanus (arrow). A representative experiment is shown; mean potentiation is given in text. (*C*) Increase of PKC isozymes in the particulate fraction 15 sec after tetanization. Comparisons were made to control levels of PKC in the particulate fraction from adjacent slices of the same hippocampus ($n = 6$). The translocation was analyzed by a one-way analysis of covariance of the levels of each isozyme, adjusting for the control values. The analysis showed significant differences among the isozymes [$F_{(7,49)} = 2.87$, $P < 0.02$], demonstrating that the data are not random. The changes due to tetanization were then examined separately for each isozyme by paired t test. (*D*) The increase of PKC in the particulate fraction does not persist 30 min after tetanus. Tetanized slices showed stable recordings for 15 min prior to tetanization and LTP for 30 min. Control levels of PKC in the particulate fraction were from adjacent slices that showed stable recordings for 45 min ($n = 11$).

We next examined whether the formation of PKMζ in slices showing LTP at 30 min might have occurred *in vitro* during homogenization and fractionation by a protease activated by tetanization, despite the presence of protease inhibitors adequate for untetanized slices. We first found that the yield of PKMζ by our usual homogenization and fractionation of tetanized slices was identical [98.0 ± 9.7% ($n = 3$)] to that from other tetanized slices from the same hippocampus that had been boiled (without fractionating) in SDS/sample buffer. We next boiled both tetanized and untetanized slices in SDS and found that the level of PKMζ in the tetanized slices was significantly greater than in the controls (123.6 ± 10.1%; $n = 6$; paired t test, $P < 0.05$). Since whole tissue boiled in SDS contains the sum of the proteins in the subcellular fractions (P1, P2, and S2), this increase in PKMζ is consistent with the larger increase seen in the S2 fraction alone.

To determine the role of NMDA-receptor activation in the formation of PKMζ, we applied the NMDA-receptor antagonist, 3-[(*RS*)-2-carboxypiperazin-4-yl]-propyl-1 phosphonic acid (CPP, 10 μM; Tocris Neuramin, Bristol, U.K.) during the experiment. CPP prevented the long-lasting synaptic potentiation (98 ± 2.9%; Fig. 4*A*) and the increase in PKMζ (99.5 ± 9.1%, $n = 4$; Fig. 3). No change was seen in levels of PKC in the particulate fraction at 30 min (Fig. 4*B*).

DISCUSSION

PKC appears to be activated during the induction and maintenance phases of LTP, but by different molecular mechanisms. A transient translocation of PKC isozymes occurs in the induction phase, followed by a persistent increase in the PKM form of a single isozyme, ζ, in the maintenance phase. In the induction phase, both Ca^{2+}-dependent and Ca^{2+}-independent isozymes are responding to second messengers, presumably Ca^{2+} and lipid second messengers produced by phospholipases (33). This initial translocation could prime the enzyme for the formation of constitutively active PKM, since membrane-bound PKC is particularly sensitive to proteolysis (34) (Fig. 4*C*). We speculate that the NMDA-mediated activation of proteases such as calpains (35, 36) may have contributed to a loss of PKC at the membrane and a proteolytic formation of PKMζ that we observed at 30 min. According to this model, the catalytic domain of the ζ isoform would be selectively stable, protected from complete proteolysis that affects the other PKC isoforms. Alternatively, PKMζ may be a gene product related to PKCζ that increases during the maintenance phase of LTP. Although the mechanism of PKMζ formation could be pre- or posttranslational, the latter is supported by evidence that applications of protease inhibitors, particularly those that affect calpains, inhibit the formation of LTP maintenance (37).

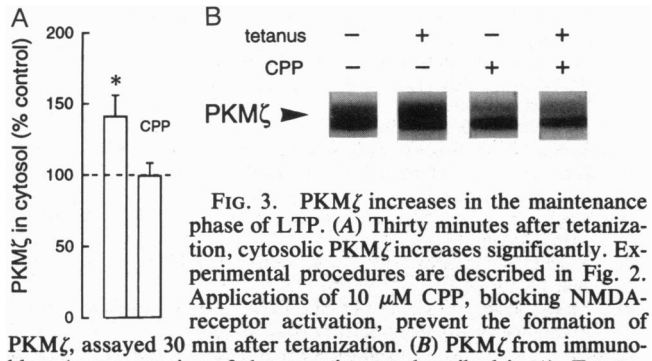

FIG. 3. PKMζ increases in the maintenance phase of LTP. (*A*) Thirty minutes after tetanization, cytosolic PKMζ increases significantly. Experimental procedures are described in Fig. 2. Applications of 10 μM CPP, blocking NMDA-receptor activation, prevent the formation of PKMζ, assayed 30 min after tetanization. (*B*) PKMζ from immunoblots (representative of the experiments described in *A*). Tetanus lanes: −, from control CA1 that was recorded at low frequency but not tetanized; +, from CA1 30 min after tetanization. CPP lanes: −, not added; +, added. CPP blocks the formation of PKMζ.

While the mechanism of its formation remains to be elucidated, the increase in PKMζ is consistent with previous reports that a persistent kinase activity, likely to be related to PKC, is present during (38) and necessary for (1, 12, 13) the maintenance of LTP. Although purified PKCζ has been reported to have some activity even in the absence of lipids (27), the level of constitutive phosphotransferase activity of PKCζ is ≈15% of the activity of the PKM form of the isozyme (39). Therefore, a conversion from PKCζ to PKMζ would result in a large increase in constitutive activity persisting in the maintenance phase of LTP. Our findings are consistent with those of Klann *et al.* (38), who report that the persistent kinase activity toward exogenous PKC substrates does not require diacylglycerol, Ca^{2+}, or phosphatidylserine. Furthermore, the transition from translocation of PKC to formation of PKM may explain why inhibitors of hydrophobic interactions (e.g., sphingosine and polymyxin B), which affect regulatory domains of some enzymes, including PKC, become ineffective in blocking LTP after the initial phase of the potentiation (1, 12).

Our results differ, however, from a previous study by Akers *et al.* (40) on LTP by perforant-path stimulation, in which a persistent translocation of PKC was reported. The difference may be due to the method of induction, the

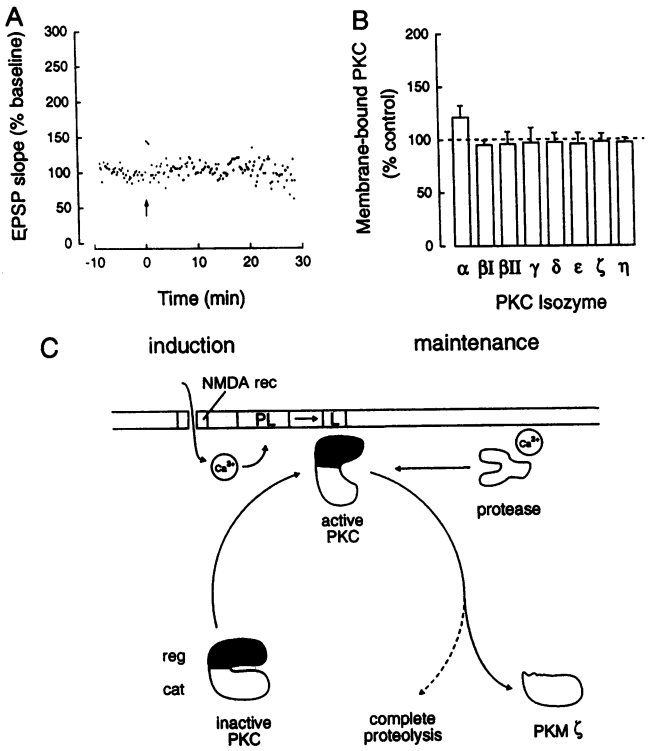

FIG. 4. NMDA-receptor activation is necessary for the persistence of PKC activation. (*A*) CPP (10 μM) in the bath blocks LTP of field EPSPs after a tetanus (arrow). A representative experiment is shown. (*B*) Thirty minutes after tetanization, PKC levels in the particulate fraction are equal to control levels from untetanized slices ($n = 4$). (*C*) Schematic representation of roles of PKC and PKM in LTP. After an initial translocation of multiple PKC isozymes to membrane, a persistently active form of the enzyme, PKM, specific to a single isozyme, ζ, increases in the maintenance phase. We speculate that the intermediate signal transduction events may occur as follows: (*i*) During induction, Ca^{2+} entering through NMDA receptors (rec) activates phospholipases (PL), which stimulate the production of lipid second messengers (L). Inactive cytosolic PKC, containing a regulatory (reg) and a catalytic (cat) domain, translocates to membrane during the induction phase immediately after synaptic activation. (*ii*) The translocation serves to activate the enzyme, priming it for proteolysis. (*iii*) In the maintenance phase of LTP, most of the membrane-bound PKCs are proteolytically degraded. The ζ isoform undergoes limited proteolysis of its regulatory domain to form PKMζ, which is independent of second messenger stimulation.

synaptic locus of the potentiation, or the region that was assayed (the entire dorsal hippocampus in Akers' compared to the isolated CA1 in our experiments).

Persistence of protein kinase activity through the loss of regulation by second messengers has been proposed as a molecular mechanism for memory (2). Examples are the persistence of cAMP-dependent protein kinase activity during long-term sensitization in *Aplysia* through proteolysis of the enzyme's regulatory subunit (41) and the autoactivation of the Ca^{2+}/calmodulin-dependent protein kinase by autophosphorylation (42, 43). Studies of LTP may provide insight into the role such a molecular mechanism plays in physiological long-term synaptic enhancement in the hippocampus. For PKC, our results indicate that the molecular mechanism for persistence is specific to the maintenance phase of the potentiation and to a single isoform of the enzyme.

We thank Drs. W. Sossin and M. Osman for their assistance in the production of antisera; S.-i. Osada for antiserum to PKCη; P. Bergold for organotypic hippocampal slice cultures; M. Makowske for helpful discussions; I. Kupferman for reading the manuscript; R. Margolis for statistical analysis; M. Stewart, C. Scheffey, and R. K. S. Wong for advice on recording from hippocampal slices; and A. Geller for his technical assistance. This study was supported by National Institutes of Health Grant KO8 NS01515-03 to T.C.S. and Postdoctoral Fellowship T5 32 NS07117-15 to H.V.

1. Malinow, R., Madison, D. V. & Tsien, R. W. (1988) *Nature (London)* **335**, 820–824.
2. Schwartz, J. H. & Greenberg, S. M. (1987) *Annu. Rev. Neurosci.* **10**, 459–476.
3. Lynch, G., Larson, J., Kelso, S., Barrionuevo, G. & Schottler, F. (1983) *Nature (London)* **305**, 719–721.
4. Malenka, R. C., Kauer, J. A., Zucker, R. S. & Nicoll, R. A. (1988) *Science* **242**, 81–84.
5. Collingridge, G. L., Kehl, S. J. & McLennan, H. (1983) *J. Physiol. (London)* **334**, 34–46.
6. Harris, E. W., Ganong, A. H. & Cotman, C. W. (1984) *Brain Res.* **323**, 132–137.
7. Malinow, R., Schulman, H. & Tsien, R. W. (1989) *Science* **245**, 862–866.
8. 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.
9. O'Dell, T. J., Kandel, E. R. & Grant, S. G. N. (1991) *Nature (London)* **353**, 558–560.
10. Silva, A. J., Stevens, C. F., Tonegawa, S. & Wang, Y. (1992) *Science* **257**, 201–206.
11. Malenka, R. C., Madison, D. V. & Nicoll, R. A. (1986) *Nature (London)* **321**, 175–177.
12. Colley, P. A., Sheu, F.-S. & Routtenberg, A. (1990) *J. Neurosci.* **10**, 3353–3360.
13. Wang, J.-H. & Feng, D.-P. (1992) *Proc. Natl. Acad. Sci. USA* **89**, 2576–2580.
14. Muller, D., Turnbull, J., Baudry, M. & Lynch, G. (1988) *Proc. Natl. Acad. Sci. USA* **85**, 6997–7000.
15. Nishizuka, Y. (1992) *Science* **258**, 607–614.
16. Kraft, A. & Anderson, W. (1983) *Nature (London)* **301**, 621–623.
17. Inoue, M., Kishimoto, A., Takai, Y. & Nishizuka, Y. (1977) *J. Biol. Chem.* **252**, 7610–7616.
18. Baxter, G., Oto, E., Daniel-Issakani, S. & Strulovici, B. (1992) *J. Biol. Chem.* **267**, 1910–1917.
19. Simpson, I. A. & Sonne, O. (1982) *Anal. Biochem.* **119**, 424–427.
20. Read, S. M. & Northcote, D. H. (1981) *Anal. Biochem.* **116**, 53–64.
21. Towbin, H., Staehelin, T. & Gordon, J. (1979) *Proc. Natl. Acad. Sci. USA* **76**, 4350–4354.
22. Laemmli, U. K. (1970) *Nature (London)* **227**, 680–685.
23. Osada, S.-i., Mizuno, K., Saido, T. C., Akita, Y., Suzuki, K., Kuroki, T. & Ohno, S. (1990) *J. Biol. Chem.* **265**, 22434–22440.
24. Sublette, E., Naik, M. U., Jiang, X., Osten, P., Valsamis, H., Osada, S.-i., Ohno, S. & Sacktor, T. C. (1993) *Neurosci. Lett.*, in press.
25. Gross, E. (1967) *Methods Enzymol.* **11**, 238–255.
26. Feig, S. & Lipton, P. (1990) *J. Neurochem.* **55**, 473–483.
27. Ono, Y., Fujii, T., Ogita, K., Kikkawa, U., Igarashi, K. & Nishizuka, Y. (1989) *Proc. Natl. Acad. Sci. USA* **86**, 3099–3103.
28. Huang, K.-P., Chan, K.-F. J., Singh, T. J., Nakabayashi, H. & Huang, F. L. (1986) *J. Biol. Chem.* **261**, 12134–12140.
29. Mehdi, S., Angelastro, M. R., Wiseman, J. S. & Bey, P. (1988) *Biochem. Biophys. Res. Commun.* **157**, 1117–1123.
30. Nakanishi, H. & Exton, J. H. (1992) *J. Biol. Chem.* **267**, 16347–16354.
31. Crabos, M., Imber, R., Woodtli, T., Fabbro, D. & Erne, P. (1991) *Biochem. Biophys. Res. Commun.* **178**, 878–883.
32. Borner, C., Guadagno, S. N., Fabbro, D. & Weinstein, I. B. (1992) *J. Biol. Chem.* **267**, 12892–12899.
33. Linden, D. J., Sheu, F.-S., Murakami, K. & Routtenberg, A. (1987) *J. Neurosci.* **7**, 3783–3792.
34. Kishimoto, A., Kajikawa, N., Shiota, M. & Nishizuka, Y. (1983) *J. Biol. Chem.* **258**, 1156–1164.
35. Seubert, P., Larson, J., Oliver, M., Jung, M. W. & Lynch, G. (1988) *Brain Res.* **460**, 189–194.
36. Simian, R. & Noszek, J. C. (1988) *Neuron* **1**, 279–287.
37. Del Cerro, S., Larson, J., Oliver, M. W. & Lynch, G. (1990) *Brain Res.* **530**, 91–95.
38. Klann, E., Chen, S.-J. & Sweatt, J. D. (1991) *J. Biol. Chem.* **266**, 24253–24256.
39. Nakanishi, H., Brewer, K. A. & Exton, J. H. (1993) *J. Biol. Chem.* **268**, 13–16.
40. Akers, R. F., Lovinger, D. M., Colley, P. A., Linden, D. J. & Routtenberg, A. (1986) *Science* **231**, 587–589.
41. Greenberg, S. M., Castelluci, V. F., Bayley, H. & Schwartz, J. H. (1987) *Nature (London)* **329**, 62–65.
42. Saitoh, T. & Schwartz, J. H. (1985) *J. Cell Biol.* **100**, 835–842.
43. Miller, S. G. & Kennedy, M. B. (1986) *Cell* **44**, 861–870.