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26. Completion of a fixed ratio (FR) of four consecutive variable ratio (VR) components, in which an average of 16 responses produced a brief stimulus light (S+), was required for cocaine or food delivery. This is a second-order FR 4 schedule with VR 16 components [FR 4 (VR 16:S)].
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7 March 1989; accepted 12 May 1989

Inhibition of Postsynaptic PKC or CaMKII Blocks Induction But Not Expression of LTP

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Long-term potentiation (LTP) of synaptic transmission is a widely studied cellular example of synaptic plasticity. However, the identity, localization, and interplay among the biochemical signals underlying LTP remain unclear. Intracellular microelectrodes have been used to record synaptic potentials and deliver protein kinase inhibitors to postsynaptic CA1 pyramidal cells. Induction of LTP is blocked by intracellular delivery of H-7, a general protein kinase inhibitor, or PKC(19–31), a selective protein kinase C (PKC) inhibitor, or CaMKII(273–302), a selective inhibitor of the multifunctional Ca^{2+}-calmodulin–dependent protein kinase (CaMKII). After its establishment, LTP appears unresponsive to postsynaptic H-7, although it remains sensitive to externally applied H-7. Thus both postsynaptic PKC and CaMKII are required for the induction of LTP and a presynaptic protein kinase appears to be necessary for the expression of LTP.

LONG-TERM POTENTIATION (LTP) of synaptic transmission results from tetanic stimulation of afferent fibers in the hippocampus (*1*) and is widely studied as a cellular model of learning and memory (*2*). However, despite much effort, the mechanisms responsible for LTP are incompletely understood (*2*). Pharmacological experiments have identified different aspects of LTP, referred to as induction, maintenance, and expression (*2–4*). *N*-Methyl-D-aspartate (NMDA) receptor channels are involved in inducing the enhanced transmission, but not in maintaining or expressing it (*2*, *4*). Maintenance and expression are distinguished (*3*) by the protein kinase inhibitor H-7 (*5*), which suppresses potentiated transmission in a reversible manner when applied in the bath. Thus, a persistent signal responsible for the enhanced transmission can be maintained, even though its expression is interrupted (*3*). Recently, we and others have attempted to localize these aspects of LTP to postsynaptic or presynaptic structures and to determine their molecular basis. NMDA receptor activation produces a Ca^{2+} entry into the postsynaptic cell (*6*) that is critical in inducing LTP (*7*). What happens next to achieve a persistent signal is unclear. In particular, the site of this persistent modification is not known. Some evidence supports primarily a postsynaptic locus (*8*), whereas other data point to a presynaptic change (*9*, *10*). Involvement of a protein kinase has been repeatedly suggested, with protein kinase C (PKC) (*10–12*) and Ca^{2+}-calmodulin–dependent protein kinase (CaMKII) (*13*) as the leading candidates. However, the evidence to date does not establish either of these as necessary participants in LTP (*14*).

To learn more about the role of the postsynaptic kinases in LTP, we used intracellular microelectrodes to deliver protein kinase inhibitors to postsynaptic cells. Our experiments focused on two central questions: (i) is activity of postsynaptic PKC or CaMKII required for LTP? and (ii) does the postsynaptic cell contain persistent protein

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kinase activity necessary to express LTP?

LTP was studied in the CA1 region of rat hippocampal slices (*15*). We monitored synaptic transmission with simultaneous recordings from an intracellular electrode in a pyramidal cell body and from an extracellular electrode placed in a nearby dendritic region (Fig. 1). After a conditioning tetanus, a persistent synaptic enhancement of comparable magnitude was seen with both recording techniques (Fig. 1, A and B). To test for the possible involvement of postsynaptic kinases in LTP, we used the intracellular microelectrode to introduce H-7, an inhibitor of several protein kinases (*5*). We used the same dual recording technique as for Fig. 1, A and B, monitoring transmission with an extracellular electrode (Fig. 1C) along with the H-7–containing intracellular electrode (Fig. 1D). Impalements were maintained for at least 30 min before a tetanus to allow the drug to diffuse from the microelectrode in the cell body to postsynaptic regions in dendritic spines. After a tetanus, transmission to the population of postsynaptic cells monitored with the extracellular electrode showed typical LTP. In contrast, transmission to the cell impaled with the H-7–containing microelectrode showed no persistent enhancement of the excitatory postsynaptic potential (EPSP) (*16*). After a tetanus, the initial EPSP slope decayed to the base-line level within 45 min. To ensure that the observed decay did not arise from a nonspecific synaptic depression, we also monitored transmission through an independent (nontetanized) pathway (Fig. 1E). Transmission through this pathway, monitored with the same H-7–containing electrode, remained constant for the duration of the experiment. Thus, the decay of transmission back to base-line level in the tetanized pathway (Fig. 1D) is not due to a general depressive effect of H-7 or a deterioration of the intracellular recording.

We also tested the effects on LTP of postsynaptic injection of the peptide PKC-(19–31), a conserved region of the regulatory domain of the PKC family (*17*), which is a potent pseudosubstrate blocker of PKC activity with a median inhibition concentration (IC_{50}) of 92 n*M* (*17*, *18*). After tetanic stimulation, no persistent synaptic enhancement is seen in the cell monitored with the peptide-containing microelectrode, in contrast to the LTP recorded with an extracellular electrode from a population of nearby cells (Fig. 2). As in experiments with H-7, transmission through another untetanized pathway was constant throughout the course of the experiment (Fig. 2C). To determine if the effect of the peptide was due to its activity on PKC, we used the modified peptide [Glu^{27}]PKC(19–31) (*18*). When compared with PKC(19–31), this peptide was much less effective against PKC, equally ineffective against CaMKII, and did not block LTP when used in the intracellular recording electrode (Fig. 2D). These results suggest that PKC(19–31) acts through block of PKC (*19*).

To test for possible involvement of postsynaptic CaMKII, we used CaMKII(273–302), a synthetic peptide containing the autoinhibitory domain of CaMKII. This peptide blocks CaMKII activity at 1 μ*M*, shows no inhibition of PKC, and does not compete with calmodulin (*18*). Postsynaptic introduction of CaMKII(273–302) resulted in blockade of LTP (Fig. 2F). Again, extracellular monitoring of the conditioned pathway showed normal LTP (Fig. 2E), and intracellular monitoring of the control pathway did not show significant change over the course of the experiment (Fig. 2G). To control for effects of the peptide other than its inhibitory action on CaMKII, we used the shorter peptide CaMKII(284–302) (*18*). When compared with CaMKII(273–302), CaMKII(284–302) did not inhibit CaMKII activity, was equally ineffective against PKC, and did not block LTP (Fig. 2H). These results suggest, therefore, that the effects of CaMKII(273–302) are due to inhibition of CaMKII (*20*).

Bath-applied H-7 can suppress the expression of LTP (*3*), although it is not known whether the locus of action is pre- or postsynaptic, since it has access to all cells in the preparation. To determine whether postsynaptic inhibitors prevent expression of LTP, we impaled postsynaptic cells with H-7–containing microelectrodes after tetanic stimulation, delaying the intracellular impalement until 15 to 45 min after the tetani to be absolutely certain that no drug reached the inside of the cell before the establishment of LTP. In such experiments, we monitored transmission through two independent pathways with an extracellular electrode (Fig. 3A). After establishment of stable base lines, a tetanus was delivered, inducing LTP in one pathway. After the establishment of stable LTP (~20 min), an intracellular recording was obtained with an H-7–containing electrode (Fig. 3B), and monitoring of synaptic transmission with this intracellular electrode was commenced (*21*). If a postsynaptic H-7–sensitive process were required for the expression of LTP, one would expect to see a selective decrement of transmission through the previously potentiated pathway as H-7 from the intracellular electrode diffused into the cell. However, as shown in Fig. 3B, no decrement in transmission was detected with the H-7–containing electrode during the 30- to 45-min observation period after impalement.

To interpret this result, we had to estab-

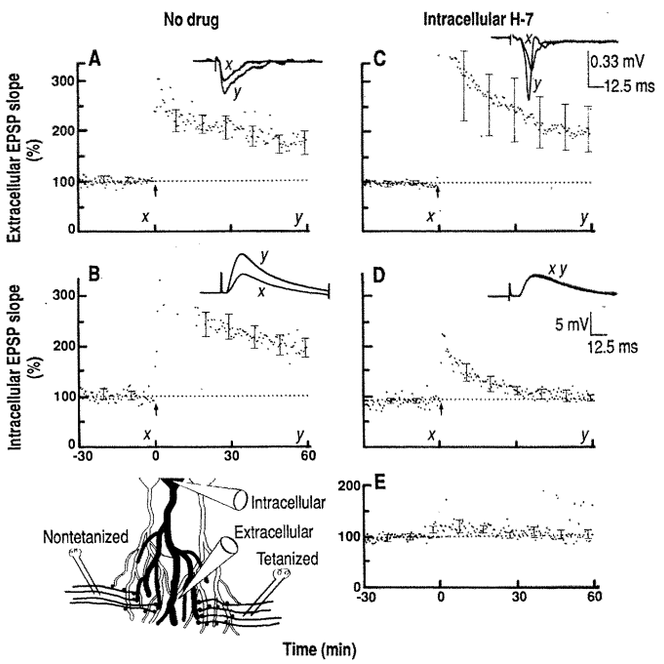

Fig. 1. H-7 in postsynaptic cell prevents LTP. (A to E) Maximal slope of rising phase of EPSPs as a function of time (*31*), given as ensemble averages of time-registered data from several slices. Tetanic stimulation is indicated by an upward arrow. Error bars indicate SEMs for representative individual time points (*3*). (Insets) Representative records of EPSPs (average of ten consecutive synaptic potentials) obtained at the time denoted by lowercase letter in graph. Simultaneous (**A**) extracellular and (**B**) intracellular recording of EPSPs show comparable percentage increases in synaptic transmission after tetanic stimulation (average of 11 slices). (C to E) As in (A) and (B) but with H-7 in the intracellular electrode (n = 11). Intracellular recording with H-7–containing electrode shows no LTP (**D**), despite LTP measured extracellularly in nearby cells (**C**). (**E**) Synaptic transmission in a nontetanized pathway, independent of the tetanized pathway, monitored with the same H-7–containing intracellular electrode (diagram). EPSP slope shows no significant change during course of experiment, indicating that intracellular H-7 does not cause a general rundown of transmission, and that the quality of recording is maintained.

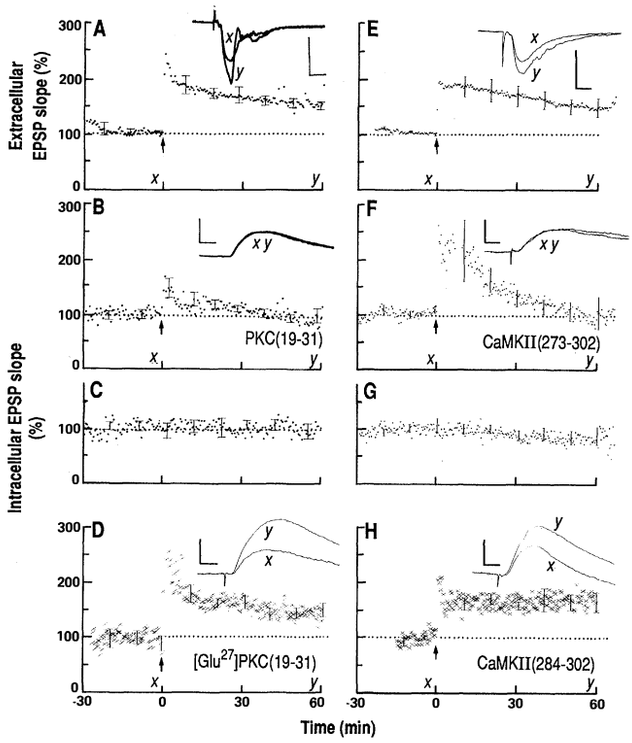

Fig. 2. Selective postsynaptic block of PKC (n = 8) or CaMKII (n = 13) prevents LTP. (**A**) Extracellular recordings of transmission in experiments testing the involvement of PKC show persistent potentiation after a tetanus (arrow). (**B**) Simultaneous monitoring of synaptic potentials with an intracellular microelectrode filled with 3 mM PKC(19–31). After the tetanus there is no persistent potentiation. (**C**) Transmission in a nontetanized pathway, monitored through the same PKC-(19–31) containing electrode, is constant throughout the experiment, indicating no nonspecific depressive effect on basal synaptic transmission. (**D**) Transmission monitored in a different set of slices, 3 mM [Glu^{27}]PKC(19–31) in the intracellular electrode, shows LTP after a conditioning tetanus (n = 6 pathways from three slices). (E to H) Recordings of transmission in experiments testing the involvement of CaMKII. (**E**) Extracellular monitoring shows LTP after tetanic stimulation. (**F**) Simultaneous monitoring of synaptic potentials with intracellular electrode containing 1.1 mM CaMKII(273–302) shows no persistent potentiation after tetanic conditioning. (**G**) Transmission in a nontetanized pathway, monitored with the CaMKII(273–302)–containing electrode, is constant throughout the experiment. (**H**) Transmission monitored in a different set of slices, using 1.1 mM CaMKII(284–302) in the intracellular electrode, shows LTP after a tetanus (n = 5 pathways from three slices). Error bars indicate SEMs for representative individual time points (*3*). (Insets) Average of ten consecutive potentials obtained at the times indicated on time axis. Scale bars for A and E: 0.33 mV, 12.5 ms; for B, D, F, and H: 5.0 mV, 12.5 ms.

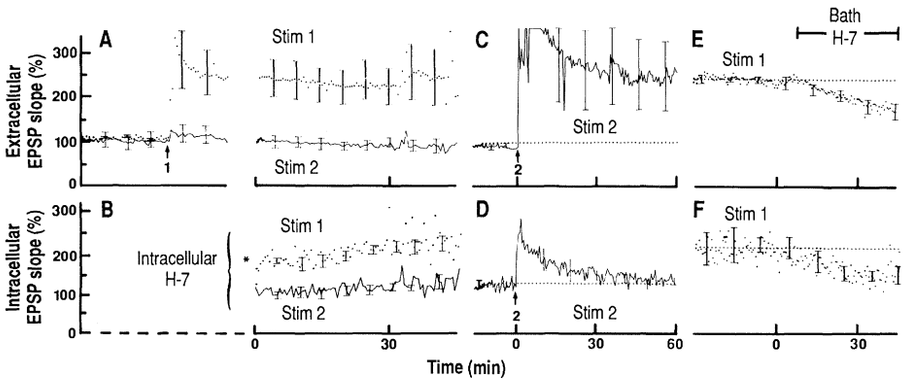

Fig. 3. Expression of LTP is insensitive to postsynaptic H-7 application. (**A**) Synaptic potentials are monitored through two independent pathways [unconnected points, stimulus 1 (stim 1); connected points, stimulus 2 (stim 2)]. Tetanic conditioning to stim 1 (arrow 1). After the establishment of stable LTP, an intracellular recording within an H-7–containing electrode is obtained (**B**) and monitoring of synaptic potentials begins within 2 min of penetration (t = 0). As monitored by the H-7–containing intracellular electrode, synaptic transmission from the potentiated pathway (stim 1, *) does not decay during the observation period and parallels transmission from the unpotentiated pathway (stim 2) (n = 13). In this panel, synaptic strength in each pathway is normalized relative to average data for the first 5 min of transmission in the untetanized pathway. (**C** and **D**) To determine whether H-7 from the intracellular electrode has reached the synaptic zone, the previously unpotentiated pathway (stim 2) is tested for the ability to generate LTP (7 of 13 slices). Delivery of a tetanus to stim 2 (arrows 2) results in no LTP as monitored with the intracellular electrode (D), despite a large persistent potentiation seen with the extracellular electrode (C). (**E** and **F**) In 8 of 13 slices, H-7 (50 to 300 μM) is subsequently applied in the bath and a comparable synaptic diminution of pathway 1 is seen with both extracellular monitoring and with the H-7–filled intracellular microelectrode. Occlusion or reduction of the effect of externally applied H-7 would have been expected if postsynaptic H-7 had already inhibited the potentiated transmission. Error bars indicate SEMs as in Fig. 1.

lish that H-7 had in fact reached the synaptic region. To do this, we delivered a tetanus to the unpotentiated pathway. No persistent potentiation was seen in the intracellular EPSPs (Fig. 3D), in contrast to the sizable LTP in the extracellular recording (Fig. 3C). Thus, H-7 reached synaptic zones and was effective. We do not believe that postsynaptic H-7 had already completed its inhibitory effect by the time synaptic monitoring commenced (less than 2 min after the impalement). If this were so, intracellular H-7 would occlude or diminish effects of externally applied H-7. On the contrary, bath application of H-7 inhibited potentiated synaptic transmission as monitored with the H-7–containing intracellular electrode (Fig. 3F), and the degree of inhibition was at least as great as that seen with extracellular recording (Fig. 3E). We conclude that once LTP is established, bath-applied H-7 acts on targets not accessible to postsynaptic intracellularly delivered H-7. Intracellular postsynaptic delivery of PKC(19–31) and CaMKII(273–302) was similarly ineffective with respect to established LTP.

These intracellular microelectrode experiments identify postsynaptic PKC and CaMKII as necessary biochemical mediators of LTP, extending previous studies that have delineated critical postsynaptic events in the induction of LTP (*22*). We attribute our results with PKC(19–31) and CaMKII(273–302) to selective inhibition of PKC and CaMKII because of the ineffectiveness of the control peptides [Glu^{27}]-PKC(19–31) and CaMKII(284–302). Postsynaptic delivery of the inhibitory peptides or H-7 selectively blocks LTP, without affecting basal transmission or slowly decaying potentiation (*3*).

Although the interactions between PKC and CaMKII in the postsynaptic cell are not known, our results suggest that both are necessary for the development of LTP, and that both are activated after Ca^{2+} entry through NMDA channels. Synergistic interactions between these kinases have been noted in purified systems (*23*). In theory, these kinases could act in parallel, although the finding that postsynaptic injection of PKC results in enhanced transmission (*11*) suggests that they act in series.

Recent experiments demonstrate that expression of LTP is associated with enhanced transmission mediated by kainate/quisqualate (K/Q) receptor channels (*8*). One interpretation is that LTP has a purely postsynaptic locus of expression. If this were true, previously potentiated transmission should be attenuated by postsynaptic delivery of H-7, a compound known to block expression of LTP when bath-applied. Contrary to this expectation, we find that post-

synaptic H-7 fails to inhibit synaptic transmission after LTP has been established (Fig. 3) (*24*).

Our results suggest that established LTP is expressed by a process that is H-7–sensitive, but in a location not accessible to postsynaptic H-7, perhaps the presynaptic terminal. This interpretation is consistent with previous results suggesting that the expression of LTP (i) involves increases in release of transmitter (*9*); (ii) is mimicked and occluded by phorbol esters (*10*; but see *25*), agents that are known to increase transmitter release in several systems (*26*); and (iii) resembles synaptic enhancement after selective presynaptic application of phorbol esters (*27*). It is possible that postsynaptic induction could lead to presynaptic expression of LTP by means of a signal traveling back from the postsynaptic neuron (*2*).

Our results and earlier studies supporting the involvement of presynaptic changes are not necessarily in conflict with the finding (*8*) that LTP is accompanied by a preferential increase in the K/Q component of the EPSP, nor with the observed increase in excitatory transmitter sensitivity in the postsynaptic cell (*28*). Several possible models for the expression of LTP could jointly involve presynaptic and postsynaptic mechanisms and thus reconcile both views. (i) Activation of a presynaptic protein kinase could enhance the response of K/Q receptors by directing the simultaneous release of an active substance, for example Zn (*29*). (ii) The selective increase of the K/Q component with LTP could result from increased transmitter release if the NMDA component is saturated in base-line transmission (*30*). (iii) The two components of the EPSP may be the result of two transmitters that are released together, each preferentially acting on either K/Q or NMDA receptors; increased release of the K/Q-activating transmitter could be responsible for LTP. (iv) There may be two populations of synapses, one of which expresses only K/Q receptors postsynaptically and which depends strongly on presynaptic protein kinase activity turned on following a tetanus. In each of these scenarios, the expression of LTP could result from persistent changes in presynaptic protein kinase activity, while manifesting itself as a selective enhancement of the K/Q response. As a last possibility, a postsynaptic kinase with an extracellular catalytic site could selectively enhance K/Q receptors.

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15. We studied LTP in the CA1 region of transverse hippocampal slices [B. E. Alger and R. A. Nicoll, *J. Physiol. (London)* **328**, 105 (1982)]. Synaptic transmission was elicited by stimulating Schaffer collateral/commissural inputs with bipolar stainless steel electrodes. Extracellular field EPSPs were recorded from stratum radiatum region with glass microelectrodes filled with 3*M* NaCl. Intracellular recordings were obtained from cell bodies with glass microelectrodes averaging 100- to 150-megohm resistance. Intracellular electrode tips contained one of following solutions: 3*M* $KMeSO_4$ with 1% AcCOOH, as the control solution; 3*M* $KMeSO_4$, 1% AcCOOH with 200 m*M* H-7; or 3*M* $KMeSO_4$ with one of the following peptides: 3 m*M* or 100 μM PKC(19–31), 3 m*M* PKC(19–36), 3 m*M* [Glu^{27}]-PKC(19–31), 1.1 m*M* CaMKII(273–302), or 1.1 m*M* CaMKII(284–302). All intracellular electrodes were back-filled with 3*M* $KMeSO_4$. Intracellular recordings were deemed acceptable if membrane potentials were greater than 55 mV and action potentials were greater than 65 mV in amplitude. A tetanus consisted of four 200-Hz trains, each lasting 100 ms and delivered 20 s apart. Picrotoxin (5 μM) was routinely used in superfusate to reduce inhibitory transmission. Synaptic strength was assessed by measuring the initial slope of the synaptic potential for both intracellular and extracellular recordings. Early action potentials often prevented accurate measurements of synaptic transmission immediately after tetanic stimulation, and such records were not included in ensemble averages.
16. In some experiments with intracellular kinase blockers, as well as with control solutions, a tetanus induced a complex EPSP, the later components of which remained persistently potentiated. We take these components to reflect polysynaptic pathways that are potentiated [R. Miles and R. Wong, *Nature* **329**, 72 (1987)] at inaccessible synapses. We assume the initial EPSP slope to reflect pure monosynaptic transmission and thus obviate the problem of polysynaptic contamination (*31*).
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18. The peptides used to study possible involvement of PKC were as follows:

RFARKGALRQKNVHEVKN PKC(19–36)
RFARKGALRQKNV PKC(19–31)
RFARKGALEQKNV [Glu^{27}]PKC(19–31).

These were kindly provided by J. Nestor, B. Kemp, and K. Tatemoto, respectively. The underlined basic residues are important for inhibitory funciton; glutamic acid was substituted for one of these to produce a control peptide. Peptides used to test for participation of CaMKII were made with a solid-phase peptide synthesizer (Applied Biosystems), purified by reversed-phase high-performance liquid chromatography, and confirmed by automated sequencing. Their sequences are as follows: HRS-TVASCMH**RQET**VDCLKKFNAR**RKLKGA**, CaMKII(273–302); **QET**VDCLKKFNAR**RKL-KGA**, CaMKII(284–302); and C**RKLKGAILTT-MLA**TR, [Cys^{296}]CaMKII(296–311). The residues shown in boldface are crucial for inhibition of the catalytic activity of CaMKII (**RQET**) and for calmodulin binding (**RKLKGAILTTMLA**). The autoinhibitory peptide lacks an essential portion of the calmodulin-binding domain, whereas the control peptide lacks an arginine essential for inhibition of the catalytic site. The effect of the peptide inhibitors and H-7 on purified rat brain CaMKII was determined with a synthetic peptide substrate [P. Hanson, M. S. Kapiloff, L. L. Lou, M. G. Rosenfeld, H. Schulman, *Neuron* **3**, 59 (1989)]. The effect of H-7 and peptide inhibitors on PKC was determined with Ac-YHRSRKRVA-NH_2 as substrate.

Inhibitor	CaMKII	PKC
	IC_{50} (μM)	
PKC(19–31)	60	0.09*
PKC(19–36)	100	0.15*
[Glu^{27}]PKC(19–31)	>100	>200
CaMKII(273–302)	1.0	>200
CaMKII(284–302)	>80	25
[Cys^{296}]CaMKII(296–311)	0.1	5
	K_i (μM)	
H-7 (Sigma)	7	6

*Values obtained from (*17*).

The inhibitory actions of the peptides on CaMKII were independent of calmodulin concentration with the exception of [Cys^{296}]CaMKII(296–311), which appears to act by competing with calmodulin (tested against CaMKII with 100 n*M* calmodulin). Abbreviations for the amino acid residues are: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.
19. When at least 30 min was allowed for drug delivery and diffusion to intracellular targets, block of LTP was observed in seven of eight experiments with intracellular microelectrodes containing 3 m*M* PKC(19–31), and six of seven experiments with 3 m*M* PKC(19–36), a slightly less potent blocker of PKC. With 100 μM PKC(19–31) in the intracellular microelectrode, LTP was blocked in three experiments in which tetanic stimulation was applied 40 to 110 min after impalement, but not in two experiments in which only 20 min was allowed for drug delivery. This would be consistent with the slow spread of the peptide to its site of action.
20. We also used the calmodulin-binding peptide [Cys^{296}]CaMKII(296–311) and saw a block of LTP in five of six experiments, suggesting that calmodulin is required to activate CaMKII, which is then required for LTP.
21. Monitoring of synaptic transmission began within 2 min of impalement. Recordings not stabilized within 2 min of penentration were not included in the study.
22. H. Wigstrom, B. Gustafsson, Y.-Y. Huang, W. C. Abraham, *Acta Physiol. Scand.* **126**, 317 (1986); R. Malinow and J. P. Miller, *Nature* **320**, 529 (1986); B. R. Sastry, J. W. Goh, A. Auyeung, *Science* **232**, 988 (1986); S. R. Kelso *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **83**, 5326 (1986).
23. As one possibility, it has been noted that PKC-dependent phosphorylation of the calmodulin-binding growth-associated protein neuromodulin (also known as GAP-43, B-50, F1, pp46, and P-57) results in release of its bound calmodulin. At sites where calmodulin availability was limited, CaMKII activation could require simultaneous PKC activa-

tion [K. A. Alexander, B. M. Cimler, K. E. Meier, D. R. Storm, *J. Biol. Chem.* **262**, 6108 (1987)].
24. One interpretation is that a weakly H-7 responsive (but postsynaptic) mechanism is involved in the expression of LTP. However, this seems unlikely since, if anything, expression of LTP is more sensitive to H-7 than to induction of LTP. H-7 at 50 μ*M* is sufficient to affect established LTP (*3*), whereas 50 to 100 μ*M* H-7 fails to block induction of LTP [D. Muller, J. Turnbull, M. Baudry, G. Lynch, *Proc. Natl. Acad. Sci. U.S.A.* **85**, 6997 (1988); R. Malinow and R. W. Tsien, unpublished results].
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29. Zn, ubiquitous to central synapses, has been shown to enhance K/Q responses while depressing NMDA responses; S. Peters, J. Koh, D. W. Choi, *Science* **236**, 589 (1987); G. L. Westbrook and M. L. Mayer, *Nature* **328**, 640 (1987).
30. The increased NMDA component seen during PTP or paired pulse facilitation (*8*) may be due to increased probability of transmitter release from individual presynaptic boutons, each producing an already saturated response of its postsynaptic NMDA receptors.
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32. We thank A.-B. Jefferson for kindly performing some of the peptide inhibition assays and D. Madison and R. Y. Tsien for helpful discussions and suggestions about the manuscript. Supported by PHS grants GM30179 (H.S.) and NS24067 (R.W.T.).

9 May 1989; accepted 24 July 1989

Imaging of Memory-Specific Changes in the Distribution of Protein Kinase C in the Hippocampus

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Activation of protein kinase C (PKC) can mimic the biophysical effects of associative learning on neurons. Furthermore, classical conditioning of the rabbit nictitating membrane (a form of associative learning) produces translocation of PKC activity from the cytosolic to the membrane compartments of the CA1 region of the hippocampus. Evidence is provided here for a significant change in the amount and distribution of PKC within the CA1 cell field of the rabbit hippocampus that is specific to learning. This change is seen at 1 day after learning as focal increments of [^{3}H]phorbol-12,13-dibutyrate binding to PKC in computer-generated images produced from coronal autoradiographs of rabbit brain. In addition, 3 days after learning, the autoradiographs suggest a redistribution of PKC within CA1 from the cell soma to the dendrites.

PROTEIN KINASE C (PKC) IS ENriched within the pyramidal cells of the hippocampus (*1, 2*) and may be a critical intracellular second messenger in associative learning (*3, 4*). There is an increase of membrane-associated PKC in the CA1 region of hippocampi after classical conditioning in rabbits (*5*), an effect that has been attributed to translocation of the enzyme (*6, 7*). To determine how the observed conditioning-induced translocation of PKC activity is distributed among and within neurons of the hippocampus, we have employed in vitro quantitative autoradiography (*8*). [^{3}H]-Phorbol-12,13-dibutyrate ([^{3}H]PDBU), a highly specific radioligand for membrane-associated PKC, was used to determine the spatial localization of brain PKC in classically conditioned rabbits both 1 day and 3 days after the behavioral experience.

In the initial set of experiments, rabbits were randomly assigned to one of three groups. Group C (conditioned) received a 400-ms 1-kHz tone (CS), which terminated at the same time as a 100-ms periorbital electric shock (UCS), for 80 trials per day for 3 days. Group UP (unpaired) animals also received an identical number of CSs and UCSs per day over 3 days, but in an explicitly unpaired fashion. Group N (naïve) remained in their home cages for 3 days. Conditioned and unconditioned responses were measured as a retraction of the nictitating membrane as had been described (*5, 9*). All group C animals showed reliable conditioned extension of the nictitating membrane (>90% conditioned responses) by the end of the third day of conditioning. After the animals were killed on day 4 of the experiment, their brains were processed for quantitative autoradiography by standard methods (*10*).

The use of [^{3}H]PDBU as a quantitative autoradiographic radioligand for PKC has been well established (*11, 12*). This phorbol ester has a much higher affinity for PKC associated with the membrane than for the cytosolic enzyme and is relatively soluble in the aqueous phase (*13*). The relatively low lipid solubility of PDBU compared with other phorbol esters results in low nonspecific binding in autoradiographic assays (*11, 12*). Film autoradiograms were analyzed on a computerized imaging system (Imaging Research) based on a charge-coupled device; the system quantified tissue radioactivity from measured autoradiographic optical densities.

We validated the autoradiography assay by means of an exogenous pharmacological challenge. Muscarinic agonists such as carbachol activate and increase membrane-associated PKC (*6, 7*). To assess the extent to which activation and translocation is manifest by an increase in autoradiographically determined [^{3}H]PDBU binding, we prepared from naïve animals hippocampal slices (400 μm thick) of the type used in the "electrophysiological slice" preparation (*14*). The slices were then exposed to either 10 m*M* carbachol or artificial cerebrospinal fluid (ACSF) for 20 min and then processed for autoradiography (*10*). Carbachol-incubated slices showed a 19 ± 3% increase in [^{3}H]-PDBU binding over controls ($P < 0.01$ Student's t test, $n = 20$ slices each group). The [^{3}H]PDBU binding assay revealed a carbachol-induced increase of membrane-associated PKC and therefore provided evidence that the technique itself did not translocate all the PKC and that amounts of PKC associated with membranes as measured by other methods (*6, 7*) were comparable to amounts measured with our assay. This result demonstrates the sensitivity of the [^{3}H]-PDBU autoradiographic method to the exogenous activation or membrane-association of PKC, or both.

Representative quantitative autoradiographic images from each of the three groups in the initial experiment are shown in Fig. 1. For each coronal section through the dorsal hippocampus, a region of interest (ROI) was produced (in a manner that was blind with respect to the experimental groups; see legend to Fig. 1) around the CA1 cell field. Multiple sections from each animal were analyzed for specific binding in this ROI, and these values for each section were averaged for each animal. Group C animals showed a 49 ± 7% and 43 ± 13% increase in [^{3}H]PDBU specific binding over the UP and N groups, respectively, representing statistically significant differences [$F = 8.716$, $P < 0.01$, one-way analysis of variance (ANOVA), $n = 5$ in each group]. There was no significant difference between the binding values for UP and N groups. In all sections, nonspecific binding was less than 8%. These quantitative images demonstrate statistically significant focal increases in [^{3}H]PDBU binding within the selected ROI (which included the stratum pyrimidale and stratum oriens of CA1 cell fields)

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