

Protein Synthesis-Dependent Formation of Protein Kinase M ζ in Long-Term Potentiation

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The maintenance of long-term potentiation (LTP) in the CA1 region of the hippocampus has been reported to require both a persistent increase in phosphorylation and the synthesis of new proteins. The increased activity of protein kinase C (PKC) during the maintenance phase of LTP may result from the formation of PKM ζ , the constitutively active fragment of a specific PKC isozyme. To define the relationship among PKM ζ , long-term EPSP responses, and the requirement for new protein synthesis, we examined the regulation of PKM ζ after sub-threshold stimulation that produced short-term potentiation (STP) and after suprathreshold stimulation by single and multiple tetanic trains that produced LTP. We found that, although no persistent increase in PKM ζ followed STP, the degree of

long-term EPSP potentiation was linearly correlated with the increase of PKM ζ . The increase was first observed 10 min after a tetanus that induced LTP and lasted for at least 2 hr, in parallel with the persistence of EPSP enhancement. Both the maintenance of LTP and the long-term increase in PKM ζ were blocked by the protein synthesis inhibitors anisomycin and cycloheximide. These results suggest that PKM ζ is a component of a protein synthesis-dependent mechanism for persistent phosphorylation in LTP.

Key words: phosphorylation; protein kinase C; zeta isozyme; PKM ζ ; long-term potentiation; LTP maintenance; learning and memory

A requirement for the synthesis of new proteins is a consistent observation in behavioral studies of long-term memory (for review, see Davis and Squire, 1984; Bailey and Kandel, 1993). This requirement is also shared by long-term potentiation (LTP) (Krug et al., 1984; Stanton and Sarvey, 1984; Frey et al., 1988; Otani et al., 1989), an activity-dependent, long-term increase in the efficiency of synaptic transmission, that has been used as a model to study the mechanisms of learning and memory (Bliss and Collingridge, 1993). LTP is thought to progress through several temporal stages. The induction phase of LTP (lasting seconds to several minutes) in the hippocampal CA1 region can be initiated by a high-frequency tetanus, releasing neurotransmitter that causes a strong depolarization of the postsynaptic membrane and activation of NMDA glutamate receptors (Collingridge et al., 1983; Harris et al., 1984) with the subsequent influx of calcium (Lynch et al., 1983; Malenka et al., 1988). Calcium-dependent second-messenger systems, activated downstream to these primary inductive events, include several protein kinases that can potentiate synaptic transmission, particularly the family of protein kinase C isozymes (PKC) (for review, see Nishizuka, 1988; Schwartz, 1993) and the Ca²⁺/calmodulin-dependent protein kinase II (CaM kinase II) (for review, see Lisman, 1994). In the maintenance phase of LTP (30 min and beyond), these kinases

become independent of second messengers (Klann et al., 1991; Fukunaga et al., 1993; Sacktor et al., 1993) and thus are believed to maintain an enhancement of synaptic transmission (Malinow et al., 1988; Wang and Fen, 1992; Hrabetova and Sacktor, 1995). It is in this later stage that protein synthesis inhibitors manifest their effects by returning the potentiated synaptic response to baseline.

These molecular mechanisms of potentiation, however, may be regulated further by the pattern of the afferent stimulation. In contrast to the high-frequency stimulus that produces LTP, a moderate-frequency burst of afferent activity results in only a brief synaptic enhancement, short-term potentiation (STP), without long-term alterations in synaptic transmission (Malenka, 1991). Multiple high-frequency trains produce greater potentiation than a single train, but it is not clear whether the additional potentiation results from qualitative or quantitative differences in underlying biochemical mechanisms. The protein synthesis-dependent mechanism of LTP, for example, has been postulated to be induced preferentially by stronger tetanic stimuli (Frey et al., 1993; Huang and Kandel, 1994).

A key question in LTP, therefore, is whether the mechanisms of persistent kinase activation require new protein synthesis. The activation of PKC has been studied in molecular detail and is different in LTP induction and maintenance (Sacktor et al., 1993): in the induction phase of LTP, multiple PKC isozymes are transiently activated by their translocation to membrane. In contrast, 30 min into the maintenance phase of LTP produced by a single tetanus, an increase is seen in the level of PKM, the independently active, catalytic fragment (Takai et al., 1977) of a specific PKC isozyme, ζ (Ono et al., 1989). In this article, we begin to address the issues of stimulus dependence and protein synthesis at the molecular level by examining the regulation of PKM ζ in response to varying patterns of afferent activity.

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MATERIALS AND METHODS

Stimulation and recording of hippocampal slices. Transverse 450 μ m hippocampal slices were prepared with a McIlwain tissue slicer as described previously (Sacktor et al., 1993). Slices were placed in an interface recording chamber and maintained at 32°C. The initial saline solution (125 mM NaCl, 2.5 mM KCl, 1.25 mM NaH₂PO₄, 26 mM NaHCO₃, and 11 mM glucose, pH 7.4) contained 10 mM MgCl₂ and 0.5 mM CaCl₂, equilibrated with 95% O₂/5% CO₂, and was replaced after 1 hr with solution containing 1.2 mM MgCl₂ and 1.7 mM CaCl₂ (Feig and Lipton, 1990). Test stimulation consisted of current pulses of 100 μ sec duration delivered every 15 sec to the Schaffer collateral/commissural fibers through a widely spaced, bipolar tungsten electrode. The current (typically 30–60 μ A) was set to produce 30% of the maximal response of the initial 10–40% of the field EPSP, as determined by an input–output curve for each slice. A typical baseline and maximal EPSP amplitude was 1.5–2 and 3–5 mV, respectively; a typical baseline and maximal EPSP slope was 0.4–0.5 and 1.0–1.5 mV/msec, respectively. Extracellular recordings in the CA1 stratum radiatum were made with standard glass microelectrodes, tip resistance 5–10 M Ω , that contained the saline solution. After at least 15 min of stable recordings, LTP was induced by single or multiple 100 Hz, 1 sec tetanic trains with pulses at a current set to produce 75% of the maximal EPSP slope. The patterns of trains were as follows: a single train; two trains, 20 sec intertrain interval (ITI); two trains, 10 sec ITI; three sets of two trains, 20 sec ITI, separated by 5 min; and six trains, 10 sec ITI. STP was induced by a single 50 Hz, 0.5 sec train at the current of test stimulation.

Immunoblots. Slices were frozen by contact with a metal rod cooled to –55°C and transferred to propylene glycol/0.9 M NaCl, 1:1 (v/v) for dissection of CA1 regions on powdered dry ice. Total CA1 regions or cytosolic and membrane-particulate fractions, boiled in SDS-PAGE sample buffer, were assayed for PKC isozymes as described previously (Sacktor et al., 1993) with affinity-purified antisera to PKCs α , β I, β II, γ , δ , ϵ , η (referring in this paper to the neural η -related PKC; Sublette et al., 1993), and ζ . The levels of PKC isozymes from fractions of hippocampal slices were compared by loading adjacent lanes of SDS-polyacrylamide gels with equal amounts of total protein from the fractions, as determined by a modified Bradford assay (Read and Northcote, 1981; Simpson and Sonne, 1982). Equal loading in adjacent lanes of the Western blot was confirmed by determining levels of tubulin with a monoclonal antibody (Sigma, St. Louis, MO). The densities of the protein bands were in the linear range of detection as determined with National Institutes of Health Image software on an XRS 6cx Scanner (OmniMedia, Torrance, CA).

In preliminary studies, we examined the changes in PKM ζ associated with the preparation and initial incubation of hippocampal slices. We compared the levels of PKM ζ present in the contralateral hippocampus, homogenized immediately after dissection without slicing, to slices that were incubated for 0, 15, 30, 60, 90, and 120 min in the recording chamber. No differences were observed in levels of PKM ζ for any of the conditions, in either the supernatant or the membrane-particulate fractions ($n = 4$ –6; data not shown).

We also wished to confirm that increases detected by the C-terminal PKC antisera (see Results) represented increases in the levels of isoforms, rather than alterations in binding specific to these epitopes. To our knowledge, no such change in binding to C-terminal PKC epitopes has been described; however, we were aware of the study by Klann et al. (1993), who had found in LTP a decrease in the binding of their antiserum, raised against whole PKC, which may have been attributable to a loss of epitope binding when the enzyme was phosphorylated. We compared the changes observed with our C-terminal antisera to three other antisera raised against different defined regions of PKC isozymes: the hinge region of PKC α , the hinge region of PKC β , and the pseudosubstrate region of all of the conventional isozymes, α , β I, β II, and γ (all kindly provided by M. Makowske, Department of Biochemistry, SUNY Health Science Center at Brooklyn, Brooklyn, NY). In assays of PKC 40 min after a two-train, 10 sec ITI tetanization independent from those described in Results, the increases of PKCs α and β as determined by our C-terminal antisera and by the hinge region antisera were equivalent (C-PKC α , 182 \pm 13%; hinge-PKC α , 171 \pm 20%; C-PKC β I, 155 \pm 11%; hinge-PKC β , 147 \pm 15%; $n = 6$). The increase detected by the antiserum recognizing the pseudosubstrate region (121 \pm 3%), although significant, was less than that for the other antisera, presumably because of the relatively large contribution of PKC γ , which did not increase to the extent of the other conventional isoforms (see Table 2).

Inhibition of protein synthesis. The inhibition of total protein synthesis by anisomycin and cycloheximide in hippocampal slices was determined

by incorporation of [³⁵S]methionine into trichloroacetic acid (TCA)-precipitated polypeptides. Slices, prepared as for physiology experiments, were incubated for 2 hr in beakers at interface in oxygenated saline solution at 32°C. Protein synthesis inhibitors were added for 30 min, followed by [³⁵S]methionine (15 μ Ci/ml final concentration) for 40 min. Slices were then homogenized in SDS-PAGE sample buffer, and 50 μ l of the homogenate was mixed with 50 μ l of bovine serum albumin (5 μ g/ml final concentration). The homogenate was spotted onto glass fiber filters and incubated with 10% TCA (4°C) for 30 min. The filters were washed three times for 5 min with fresh TCA, followed by 95% ethanol, and the label bound to the filter was determined in a liquid scintillation counter.

The effects of protein synthesis inhibitors on synaptic transmission and basal expression of PKM ζ were also examined. Control slices stimulated with test pulses for 70 min in our standard interface chamber were compared with slices that received test stimulation in the presence of the drugs. Anisomycin and cycloheximide had no effect on basal synaptic transmission (data not shown). Applications of anisomycin also did not affect the basal expression of PKM ζ (see Fig. 2B) or PKC isozymes ($n = 5$; data not shown). In contrast, cycloheximide, although having no effect on basal levels of PKM ζ (see Fig. 2C), caused an elevation of PKCs α and β I (α , 193 \pm 31%; β I, 180 \pm 25%; $n = 7$). These increases were presumably attributable to a decrease in proteolytic degradation. In the LTP experiments with protein synthesis inhibitors, controls were adjacent slices that received test stimulation in the presence of the inhibitors.

Statistical analysis. Differences in PKM ζ between control and tetanized slices were determined by paired Student's *t* test. Levels of multiple PKC isozymes were first analyzed by repeated-measures ANOVA. The analysis was made among the individual isozyme values, between the means of treated and nontreated isozyme values, and by the interaction of isozyme and treatment condition. In cases in which the *p* value was <0.05 for the ANOVA test, the effect of a specific treatment for each individual isozyme was then analyzed by paired *t* test. The relationship between the levels of PKM ζ and EPSP slope was analyzed by linear correlation, and significance was determined by *t* test.

RESULTS

Increases in PKM ζ correlate with the efficacy of long-term EPSP potentiation

We used different tetanization protocols to induce STP (a single 50 Hz, 0.5 sec train) and LTP (100 Hz, 1 sec tetani in one, two, or six trains; multiple trains separated by either 10 or 20 sec) (Fig. 1, Table 1). The STP stimulation resulted in transient potentiation of field EPSP responses, lasting <30 min with a $t_{1/2}$ of 7 min (Fig. 1A). In contrast, all LTP protocols showed sustained potentiations (Fig. 1B–F). Both the single-train (Fig. 1B) and the two-train, 20 sec ITI protocol (Fig. 1C) resulted in stable potentiation immediately after post-tetanic potentiation (PTP) to 165–170% of the baseline (set at 100%). Pairs of trains with 20 sec ITI, repeated three times at 5 min intervals, a protocol used by Klann et al. (1991) to achieve strong potentiation, induced an increase to ~225% (Fig. 1D). A single pair of trains separated by 10 sec ITI (Fig. 1E) also produced greater potentiation than the single pair at 20 sec ITI. Finally, six trains at 10 sec ITI, a tetanization protocol used by Stelzer et al. (1987), increased potentiation to ~200% (Fig. 1F). In both protocols using 10 sec ITIs, EPSP responses were transiently diminished from the extent obtained at PTP, then rose to a stable potentiation within 30 min.

We examined the relationship between long-term EPSP potentiation and the formation of PKM ζ . Equal amounts of total protein from control and tetanized CA1 regions were loaded in adjacent lanes on Western blots, and the accuracy of pipetting and the efficiency of nitrocellulose transfer were confirmed in each experiment by immunostaining with antisera to tubulin (Table 1 and data not shown). There was no increase in PKM ζ 40 min after the 50 Hz, 0.5 sec protocol, which produced only STP (Table 1, Fig. 2A). In contrast, every LTP protocol pro-

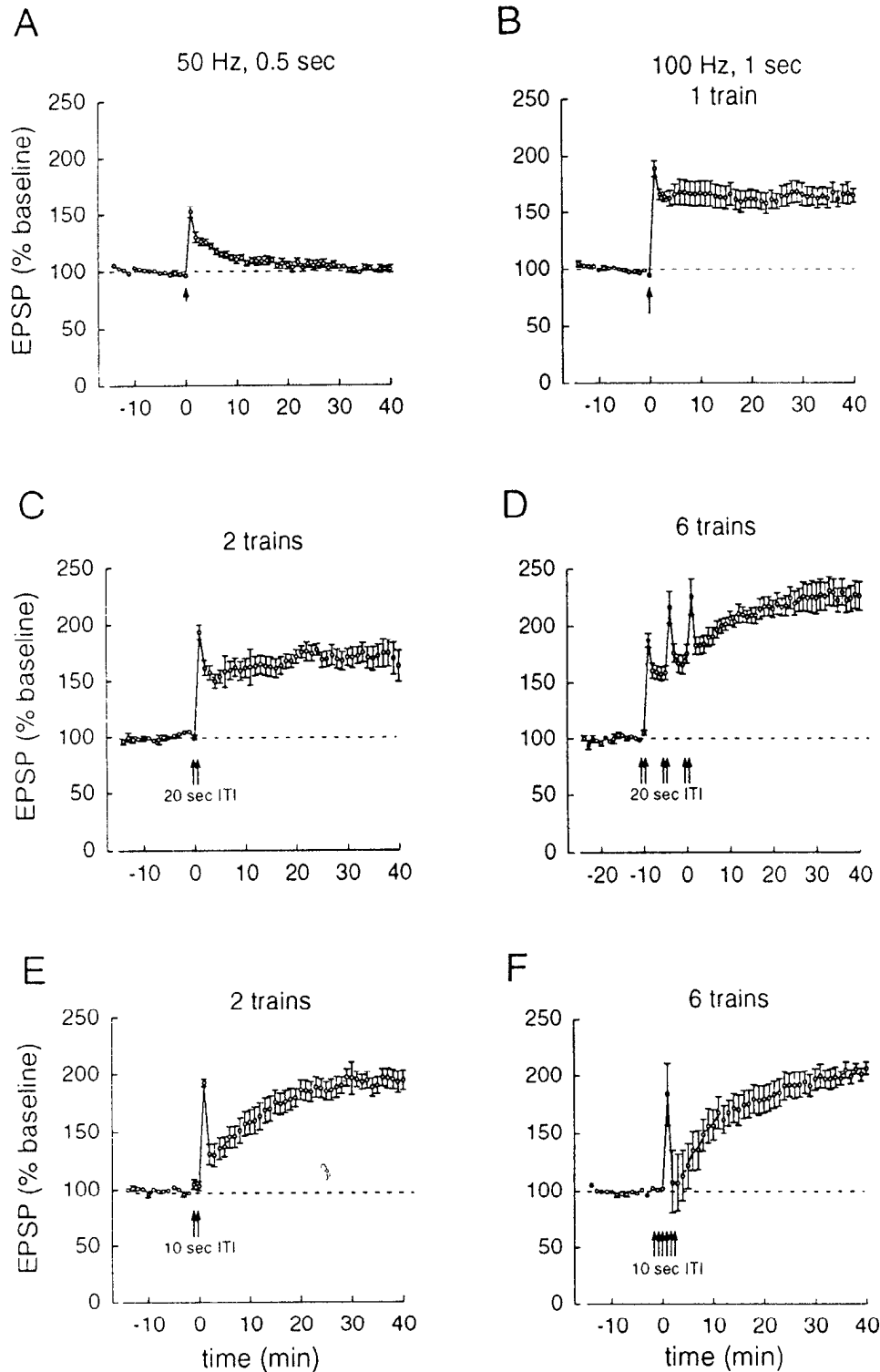


Figure 1. Time courses of EPSP potentiation in CA1 after different tetanization procedures (tetanic trains shown by arrows). Pretetanus baseline EPSP responses were set at 100% (mean \pm SEM). *A*, A single 50 Hz, 0.5 sec tetanus induced STP, which lasted <30 min. *B–F*, In all other tetanization protocols, we used 100 Hz, 1 sec trains and induced stable LTP (see Table 1 for mean potentiations). *B*, A single train; *C*, two trains, 20 sec ITI; *D*, three sets of 20 sec ITI pairs, separated by 5 min; *E*, two trains, 10 sec ITI; *F*, six trains, 10 sec ITI.

duced increases in PKM ζ (Table 1, Fig. 2*A*). Because tetanization effectiveness varied within each protocol, we examined the relationship between the increases in EPSP and PKM ζ in LTP based on responses in individual slices. Despite the variabilities in both LTP effectiveness and increases of PKM ζ ,

EPSP potentiation and PKM ζ formation were found to be linearly correlated (Pearson's $r = 0.55$, $p = 0.012$; Fig. 3). However, because of the variability observed, these data could also be consistent with more complex, nonlinear relationships between PKM ζ levels and EPSP slope.

Table 1. PKM ζ levels and EPSP potentiation after various tetanization protocols

Tetanus	PKM ζ	EPSP potentiation
1 train, 50 Hz 0.5 sec	106 \pm 6 (107 \pm 4)	102 \pm 3
1 train, 100 Hz 1 sec	133 \pm 8* (129 \pm 7*)	165 \pm 6*
2 trains, 100 Hz 1 sec, 20 sec ITI	129 \pm 7* (134 \pm 11*)	168 \pm 17*
6 trains, 100 Hz 1 sec, 20 sec ITI	142 \pm 10* (139 \pm 14*)	226 \pm 14*
2 trains, 100 Hz 1 sec, 10 sec ITI	136 \pm 13* (142 \pm 15*)	196 \pm 12*
6 trains, 100 Hz 1 sec, 10 sec ITI	141 \pm 10* (138 \pm 8*)	204 \pm 12*

Mean percent changes \pm SEM for PKM ζ levels and EPSP responses 40 min after different tetanic stimulation procedures. PKM ζ increased after all LTP protocols, but not after STP (1 train, 50 Hz 0.5 sec). Controls, set at 100%, were either PKM ζ levels in adjacent control slices or pretetanus EPSP responses. The densitometric values for PKM ζ were also normalized to the levels of tubulin in each lane to control for protein loading, producing equivalent results (within parentheses). Asterisks denote significance ($p < 0.05$) by paired t test ($n = 6$ –8 for PKM ζ determinations). Mean values of PKM ζ also included some experiments in which population spike amplitudes were recorded.

The increase in PKM ζ persists during the maintenance of LTP

A time course of PKM ζ formation was obtained for the two-train, 10 sec ITI LTP protocol, which showed stable EPSP potentiation of 185% for 2 hr (Fig. 4A). The increase in PKM ζ was first observed 10 min after the tetanus and lasted for 2 hr, the latest time point examined (Fig. 4B), paralleling the persistence of LTP.

These increases of PKM ζ during LTP were measured in CA1 regions that had been boiled in SDS-PAGE sample buffer, a procedure that sums all intracellular compartments. Thus, although the changes in PKM ζ were on average modest (20–40%), the increases in the appropriate cellular compartment might be considerably larger. In our previous study of single-train LTP (Sacktor et al., 1993), for instance, the increase in PKM ζ 30 min after a single train was selective to the cytosolic compartment. We found that the change in PKM ζ 40 min after the stronger two-train, 10 sec ITI stimulation was similarly compartmentalized, increasing in the cytosol (159 \pm 12% of controls; $n = 6$, $p < 0.05$), but not changing in the membrane-particulate fraction (103 \pm 9%). The NMDA receptor antagonist 3-[(RS)-2-carboxypiperazin-4-yl]-propyl-1-phosphonic acid (or CPP; Tocris Cookson, St. Louis, MO), blocked both the increases in EPSP responses (data not shown) and the levels of total PKM ζ (105 \pm 7%; $n = 4$) 40 min after the two-train LTP, as was observed previously for single-train LTP (Sacktor et al., 1993).

Protein synthesis inhibitors block both LTP and the increase of PKM ζ

We examined the effect of anisomycin, a protein synthesis inhibitor that has been used extensively to block the protein synthesis-dependent mechanisms of LTP (Krug et al., 1984; Frey et al., 1988; Otani et al., 1989; Huang et al., 1994). The inhibition of total protein synthesis in hippocampal slices was confirmed by measuring the ability of anisomycin to block incorporation of [³⁵S]methionine into TCA-precipitated proteins. Anisomycin (10 μ M) inhibited the incorporation of label into polypeptides by 96 \pm 4% ($n = 4$). The blockade of LTP by anisomycin was also confirmed in the potentiation induced by two-train, 10 sec ITI tetanization. Whereas LTP was maintained for at least 2 hr without decrement in the absence of inhibitors (Fig. 4A, open circles), potentiation

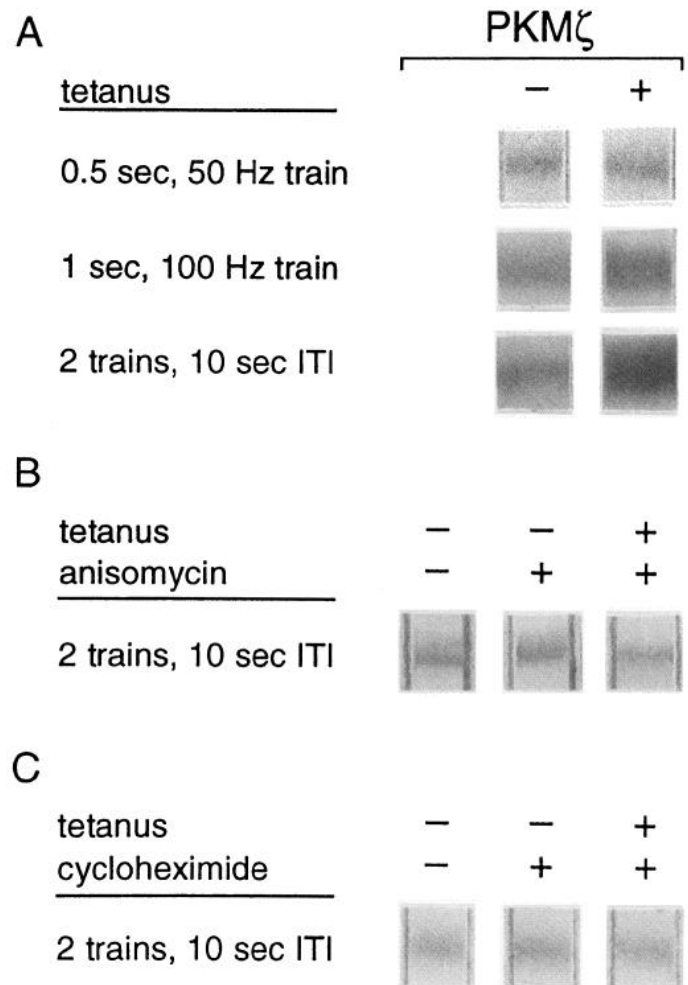


Figure 2. The increase in PKM ζ is specific to tetanization protocols that produce LTP and is dependent on new protein synthesis. *A*, Representative immunoblots of CA1 regions, showing total PKM ζ 40 min after a tetanus (+), and in adjacent control slices (-). STP (0.5 sec, 50 Hz train) produced no change. Stimuli that caused LTP (1 sec, 100 Hz train; and two trains, 10 sec ITI) increased PKM ζ . *B*, Anisomycin (10 μ M), although not affecting basal levels of PKM ζ , blocked the increase of PKM ζ after two-train, 10 sec ITI tetanization. In these experiments, three adjacent slices, each stimulated by test pulses, were treated: (1) without anisomycin, (2) in the presence of anisomycin, and (3) tetanized with two trains, 10 sec ITI, in the presence of anisomycin. No differences in PKM ζ were observed among the three treatment groups. *C*, Equivalent results were obtained with applications of cycloheximide (60 μ M).

began to decrease after 10–15 min in the presence of anisomycin, with a $t_{1/2}$ of 32 min (Fig. 4A, closed circles). Applications of anisomycin did not affect the basal levels of PKM ζ (levels of PKM ζ in slices not treated with the drug were 95 \pm 12% of the levels in anisomycin-treated slices, $n = 5$; Fig. 2B). The protein synthesis inhibitor, however, blocked the increase of PKM ζ 40 min after the strong tetanus (levels of PKM ζ in anisomycin-treated tetanized slices were 105 \pm 7% of the anisomycin-treated controls, $n = 5$; Figs. 2B, 4B). A second protein synthesis inhibitor, cycloheximide (30–60 μ M, inhibiting total protein synthesis by 82 \pm 3%), also blocked the maintenance of LTP (data not shown) and the formation of PKM ζ after tetanization (100 \pm 4%, $n = 7$; Fig. 2C), without affecting basal levels of the kinase (95 \pm 6%, $n = 7$; Fig. 2C).

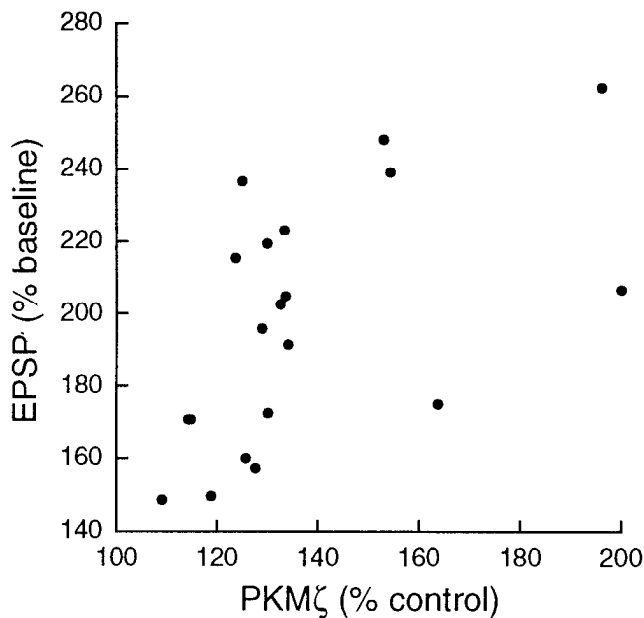


Figure 3. Scatter chart showing relationship between increases of PKM ζ and EPSP potentiation in LTP maintenance 40 min after tetanization. Analysis by linear correlation showed Pearson's $r = 0.55$, with $p = 0.012$ by t test. LTP was analyzed without STP to eliminate the contribution of nonpotentiated responses at 40 min.

Protein synthesis-dependent increases in cytosolic PKCs are observed during specific LTP protocols using multiple tetanic trains with short intertrain intervals

In parallel with the measurements of PKM ζ , we examined changes in PKC isoforms in the maintenance of LTP induced by different tetanic stimulations. There were no changes in the total levels of PKC isoforms 40 min after STP, single-train LTP, or the 20 sec ITI LTP protocols (data not shown). In contrast, after both 10 sec ITI protocols, multiple PKC isoforms increased at 40 min (Fig. 5, Table 2, and data not shown). The increases observed with the two-train, 10 sec ITI protocol were blocked by both anisomycin and cycloheximide (Fig. 5, Table 2). A time course for PKCs was determined in parallel with that described for PKM ζ . The isoforms were first observed to increase 40 min after the tetanus, and most had returned to baseline by 2 hr (data not shown). PKCs α and β I were exceptions, remaining elevated at 2 hr (α , $177 \pm 21\%$; β I, $184 \pm 26\%$; $n = 8$). Thus, in contrast to the increase in PKM ζ , which appeared early and was stable for 2 hr, most of the PKCs rose after an initial delay and were elevated only transiently.

To address whether the increases in PKCs observed specifically after the two-train, 10 sec ITI protocol contributed to the persistent activation of the enzyme by distribution to membrane, the subcellular location of the increase in PKC isoforms was determined. Forty minutes after the tetanus, the levels of all isoforms were increased in the cytosol, but were unchanged in the membrane-particulate fraction (data not shown).

DISCUSSION

The increase in PKM ζ has the activity-dependent and temporal properties of LTP maintenance

Our findings indicate that PKM ζ is a molecular correlate of LTP maintenance. Increases of PKM ζ occurred after all tetanization protocols that produced LTP, but not after a weak stimulus that produced only STP. Both LTP and the formation of PKM ζ were

blocked by NMDA receptor antagonists and protein synthesis inhibitors. Forty minutes after strong tetanization that produced LTP, the levels of PKM ζ were correlated with the increases of the EPSP response. During LTP, the increase in PKM ζ began 10 min after the tetanus and persisted for at least 2 hr. This delay in onset of PKM ζ formation is appropriate for the kinetics of proteolysis by calpains, in which proteolytic products are typically observed several minutes after a rise in Ca^{2+} (Suzuki et al., 1992). The persistence of the increase in PKM ζ during LTP is consistent with the observation that continuously elevated PKC catalytic activity is essential for the maintenance of synaptic potentiation (Malinow et al., 1988; Wang and Feng, 1992; Hrabetova and Sacktor, 1995).

Because increases in PKM ζ were observed with all tetanization protocols producing LTP, the kinase may be a component of the molecular mechanism of maintenance for both single- and multiple-train LTP protocols. At first consideration, these results could be viewed as contrasting with the proposition that distinct forms of LTP, termed "early" and "late" LTP, are differentially produced by the number of tetanic trains (Frey et al., 1988, 1993; Reymann et al., 1988; Huang and Kandel, 1994). Our results, however, may not conflict with this proposal. In the study by Huang and Kandel (1994), early LTP, produced by a single train using pulses set at the same intensity as test recordings, was relatively weak and decremental (121% by 1 hr). This early LTP was protein synthesis-independent, in contrast to the more stable "late" LTP induced by multiple trains. In our study, a single tetanus, with pulses set at a current intensity giving 75% of the maximal EPSP response, resulted in a large potentiation (165%) that did not diminish in the period examined (Fig. 1B). Thus, our single tetanus may be initiating the signal transduction mechanisms associated with late LTP. It will be interesting to determine whether STP (produced by a weak stimulus), early LTP (produced by a moderate stimulus), and the residual potentiation in the presence of protein synthesis inhibitors (produced by a strong stimulus) share a single underlying protein synthesis-independent mechanism of potentiation.

PKM ζ is a component of a protein synthesis-dependent mechanism for sustained activation of PKC

Although there is substantial evidence that LTP requires the synthesis of new proteins, the nature of these proteins and their roles in the mechanisms of potentiation are largely unknown (but see Fazeli et al., 1993; Qian et al., 1993; Thomas et al., 1994). We have observed that the increase of PKM ζ in the maintenance phase of LTP was blocked by protein synthesis inhibitors. We have not yet addressed, however, which steps in the formation of the kinase require the synthesis of new proteins. Because the inhibitors were applied 30 min before the tetanus, these steps could include the following: (1) the initial translocation of PKC ζ to membrane (Sacktor et al., 1993) in the induction phase of LTP (placing the isoform in a conformational state accessible to cleavage; Kishimoto et al., 1989); (2) the proteolysis at the hinge region of the isoform (rendering the enzyme autonomous); or (3) the formation of the proteolytic substrate PKC ζ . Protein synthesis requirements have been observed for the first two mechanisms during learning-related modifications of protein kinases in *Aplysia*. For example, inhibitors of protein synthesis block both the persistent translocation of PKC (Sossin et al., 1994) and the proteolysis of the regulatory subunit of the cAMP-dependent protein kinase (Bergold et al., 1990; Hegde et al., 1993). The third mechanism postulates a preferential cleavage of newly synthesized PKC ζ to PKM ζ . A recent study, however, using *in situ*

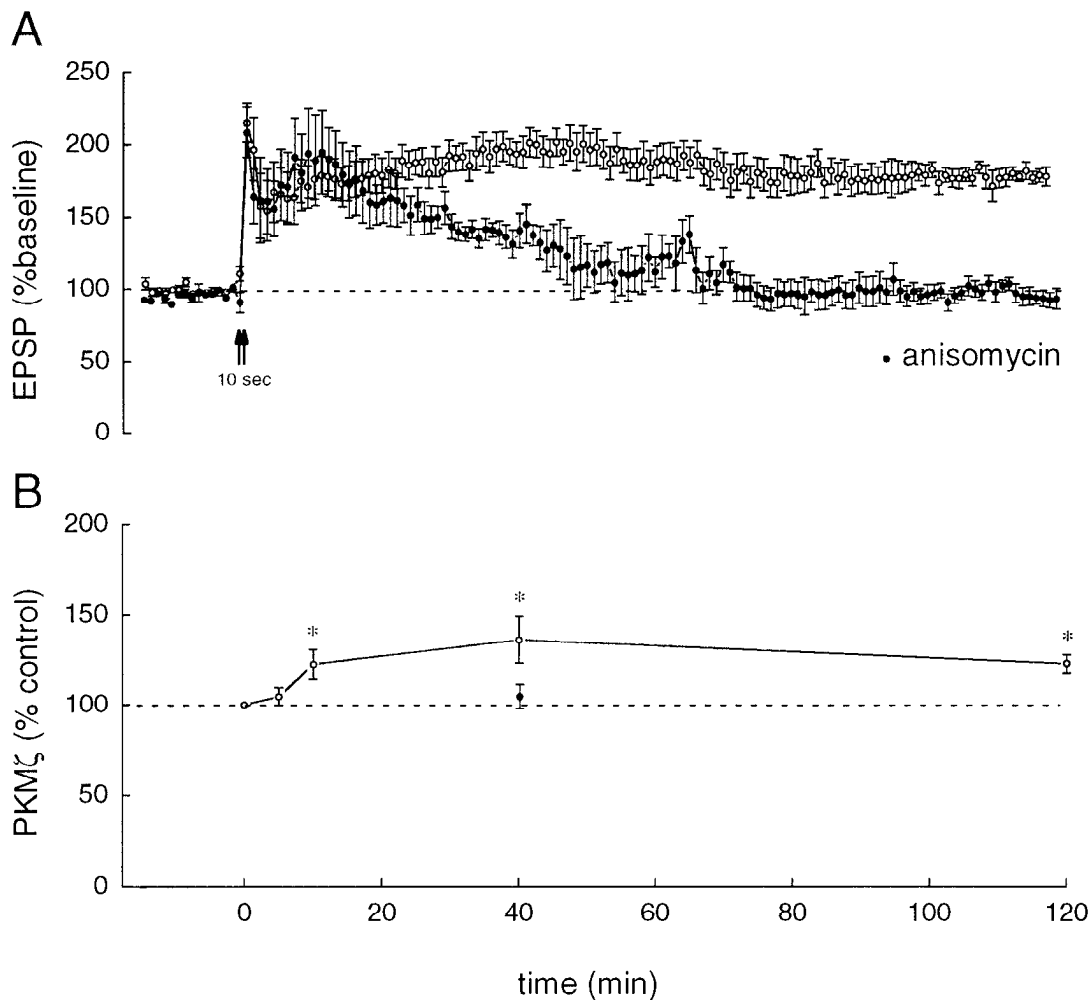


Figure 4. Increases in EPSP and PKM ζ are parallel with respect to time and are dependent on protein synthesis. *A*, Time course of EPSP potentiation after two-train, 10 sec ITI tetanization, in the absence (*open circles*) and presence (*closed circles*) of anisomycin (10 μ M, applied to the bath 30 min before the tetanus). In the absence of inhibitors, potentiation lasted at least 2 hr without decrement ($n = 4$, tetanic trains shown by *arrows*). In the presence of anisomycin, EPSP potentiation showed a $t_{1/2}$ of 32 min ($n = 4$). *B*, Time course of the increase in PKM ζ in LTP (*open circles*). PKM ζ from tetanized CA1 regions were compared with adjacent control regions that showed stable recordings for equivalent periods of time. PKM ζ was increased 10 min ($122 \pm 8\%$, $n = 7$), 40 min ($136 \pm 13\%$, $n = 8$), and 120 min ($124 \pm 6\%$, $n = 7$) after the tetanus; each $p < 0.05$, paired t test (*asterisks*). Anisomycin blocked the increase at 40 min (*closed circle*).

hybridization failed to observe increases in ζ RNA after LTP (Thomas et al., 1994). Nonetheless, new synthesis of ζ may be attributable to translational, rather than to transcriptional regulation. The identification of the newly synthesized proteins and the protease that regulate the formation of PKM ζ will be important future areas for investigation.

PKM formation versus PKC translocation in LTP maintenance

In this study, we also attempted to address whether different tetanization procedures could account for a discrepancy in the literature concerning the mechanism for the persistent activation of PKC in LTP. Specifically, three previous studies (Klann et al., 1993; Sacktor et al., 1993; Angenstein et al., 1994) have been unable to confirm the persistent translocation of PKC, originally reported by Akers et al. (1986) for LTP *in vivo* using multiple-train tetanization. Differences between *in vitro* and *in vivo* preparations do not appear to account for the inconsistency, because Angenstein et al. (1994) also examined LTP *in vivo*. Persistent PKC translocation occurs, however, in other forms of long-term

synaptic plasticity related to memory formation: presynaptic facilitation in *Aplysia* (Sossin et al., 1994), eye-blink conditioning in the rabbit (Bank et al., 1988), and LTP in the CA3 region of the hippocampus (Son et al., 1994). Because we observed an increase in total PKC with short ITI interval protocols, we examined the possibility that some of the enzyme might have partitioned into membrane. Although we found the increase of PKC restricted to the cytosolic compartment, it may yet be possible that a different time point or stimulation protocol would show an increase of membrane-bound PKCs in the maintenance of LTP. Such an increase, however, would not appear to correlate with LTP maintenance over all tetanization protocols.

PKM ζ formation, protein synthesis, and the molecular mechanisms of memory

Short-term memory is widely considered to be mediated by post-translational modifications of synaptic proteins, and long-term memory to require new proteins for permanent changes in synaptic structure (Davis and Squire, 1984; Montarola et al., 1986; Bailey and Kandel, 1993). This scenario is supported by studies in

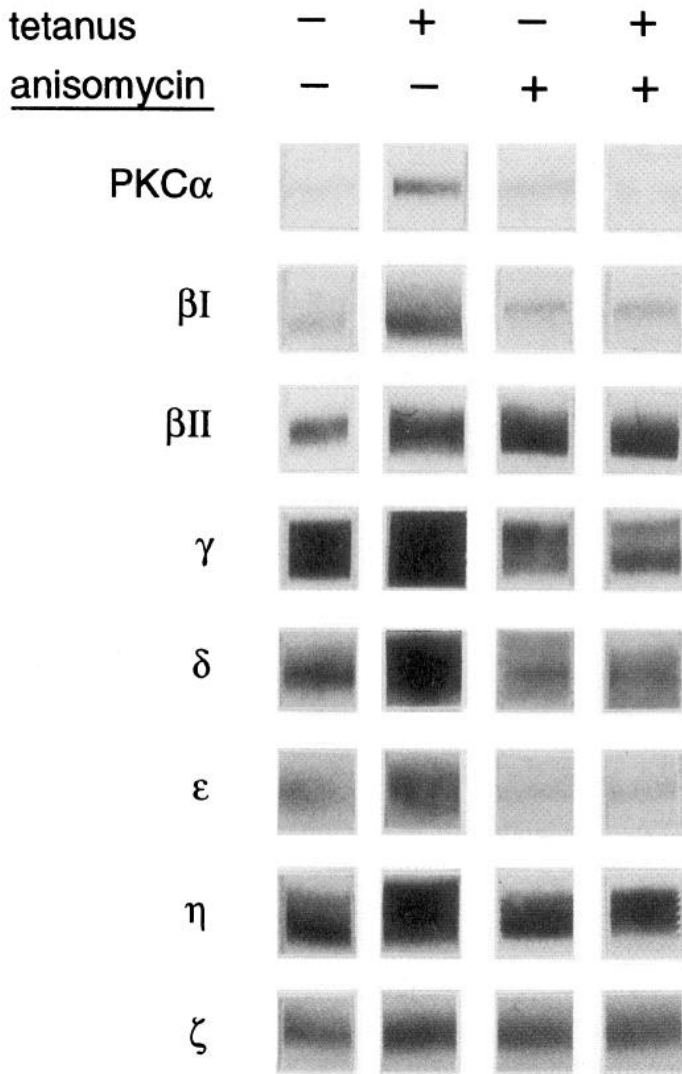


Figure 5. The two-train, 10 sec ITI tetanization protocol results in an increase of all PKC isoforms, which is dependent on new protein synthesis. Representative immunoblots are from CA1 regions, showing PKC isoforms after test stimulation (*tetanus* -) or 40 min after two-train tetanization (*tetanus* +). The increases are blocked by anisomycin (10 μ M). The tetanization experiments with and without anisomycin are from separate animals and, therefore, the basal levels of PKC isoforms in each experimental pair are different. There were no direct effects of anisomycin on the basal levels of PKC isoforms (see Materials and Methods).

which protein synthesis inhibitors appear to block long-term memory, while sparing short-term memory. In this context, the current molecular explanation for LTP maintenance—persistent phosphorylation by autonomous protein kinases—appears inconsistent with, or at least redundant for, the requirement for protein synthesis. Our findings demonstrate that, contrary to this expectation, persistent phosphorylation and new protein synthesis converge in the maintenance of PKC. The observation that PKM ζ is downregulated in the maintenance of homosynaptic long-term depression in CA1 (Hrabetova and Sacktor, 1994), contributing to dephosphorylation in this form of plasticity (Mulkey et al., 1993), further supports the notion that states of phosphorylation in neurons are stable, yet dynamic, and may participate in the bidirectional regulation of synaptic transmission. These findings do not imply that structural synaptic modifications are unnecessary for LTP; on the contrary, the structure of synapses

Table 2. Protein synthesis-dependent increases of PKC isoforms after two-train, 10 sec ITI tetanization

Isozyme	Tetanus	Tetanus + anisomycin	Tetanus + cycloheximide
α	207 \pm 19*	86 \pm 12	105 \pm 20
β I	147 \pm 23*	96 \pm 11	80 \pm 6
β II	127 \pm 8*	105 \pm 2	88 \pm 6
γ	126 \pm 9*	98 \pm 11	107 \pm 9
δ	185 \pm 38*	103 \pm 14	73 \pm 11
ϵ	182 \pm 19*	97 \pm 17	108 \pm 8
η	162 \pm 21*	85 \pm 11	91 \pm 10
ζ	134 \pm 8*	111 \pm 18	94 \pm 5

Protein synthesis-dependent increases of PKC isoforms 40 min after the two-train, 10 sec ITI tetanization protocol. Significant differences among the individual PKC isoforms were determined by ANOVA ($F_{(7,28)} = 2.84, p < 0.05$). The change for each isoform was then determined by paired *t* test ($n = 8, p < 0.05$ denoted by asterisks). Both anisomycin (10 μ M) and cycloheximide (30–60 μ M) prevented the increases in PKC isoforms.

(Hosokawa et al., 1995) may be the site of expression maintained by phosphorylation. Although the precise site of LTP expression remains controversial at this time, the mechanism for the regulation of PKC ζ in LTP maintenance unifies the observations of a persistent activation of kinases with the requirement for new protein synthesis.

REFERENCES

- Akers RF, Lovinger DM, Colley PA, Linden DJ, Routtenberg A (1986) Translocation of protein kinase C activity may mediate hippocampal long-term potentiation. *Science* 231:587–589.
- Angenstein F, Riedel G, Reymann KG, Staak S (1994) Hippocampal long-term potentiation *in vivo* induces translocation of protein kinase C γ . *NeuroReport* 5:381–384.
- Bank B, DeWeer A, Kurizan AM, Rasmussen H, Alkon DL (1988) Classical conditioning induces long-term translocation of protein kinase C in rabbit hippocampal CA1 cells. *Proc Natl Acad Sci USA* 85:1988–1992.
- Bailey CH, Kandel ER (1993) Structural changes accompanying memory storage. *Annu Rev Physiol* 55:397–426.
- Bergold PJ, Sweatt JD, Winicov I, Weiss KR, Kandel ER, Schwartz JH (1990) Protein synthesis during acquisition of long-term facilitation is needed for the persistent loss of regulatory subunits of the *Aplysia* cAMP-dependent protein kinase. *Proc Natl Acad Sci USA* 87:3788–3791.
- Bliss TVP, Collingridge GL (1993) A synaptic model of memory: long-term potentiation in the hippocampus. *Nature* 361:31–39.
- Collingridge GL, Kehl SJ, McLennan H (1983) Excitatory amino acids in synaptic transmission in the Schaffer collateral-commissural pathway of the rat hippocampus. *J Physiol (Lond)* 334:34–46.
- Davis HP, Squire LR (1984) Protein synthesis and memory: a review. *Psychol Bull* 96:518–559.
- Fazeli MS, Corbet J, Dunn MJ, Dolphin AC, Bliss TV (1993) Changes in protein synthesis accompanying long-term potentiation in the dentate gyrus *in vivo*. *J Neurosci* 13:1346–1353.
- Feig S, Lipton P (1990) *N*-methyl-D-aspartate receptor activation and Ca²⁺ account for poor pyramidal cell structure in hippocampal slices. *J Neurochem* 55:473–483.
- Fukunaga K, Stoppini L, Miyamoto E, Muller D (1993) Long-term potentiation is associated with an increased activity of Ca²⁺/calmodulin-dependent protein kinase. *J Biol Chem* 268:7863–7867.
- Frey U, Krug M, Reymann KG, Matthies H (1988) Anisomycin, an inhibitor of protein synthesis, blocks late phases of LTP phenomena in the hippocampal CA1 region *in vitro*. *Brain Res* 452:57–65.
- Frey U, Huang Y-Y, Kandel ER (1993) Effects of cAMP simulate a late stage of LTP in hippocampal CA1 neurons. *Science* 260:1661–1664.
- Harris EW, Ganong AH, Cotman CW (1984) Long-term potentiation in the hippocampus involves activation of *N*-methyl-D-aspartate receptors. *Brain Res* 323:132–137.

- Hegde AN, Goldberg AL, Schwartz JH (1993) Regulatory subunits of cAMP-dependent protein kinases are degraded after conjugation to ubiquitin: a molecular mechanism underlying long-term synaptic plasticity. *Proc Natl Acad Sci USA* 90:7436–7440.
- Hosokawa T, Rusakov DA, Bliss TVP, Fine A (1995) Repeated confocal imaging of individual dendritic spines in the living hippocampal slice: evidence for changes in length and orientation associated with chemically induced LTP. *J Neurosci* 15:5560–5573.
- Hrabetova S, Sacktor T (1994) Down-regulation of protein kinase M ζ in maintenance of homosynaptic long-term depression in hippocampal CA1. *Soc Neurosci Abstr* 20:446.
- Hrabetova S, Sacktor T (1995) Evidence that constitutive PKC activity is necessary for both LTP and LTD maintenance. *Soc Neurosci Abstr* 21:601.
- Huang Y-Y, Kandel ER (1994) Recruitment of long-lasting protein kinase A-dependent long-term potentiation in the CA1 region of hippocampus requires repeated tetanization. *Learn Memory* 1:74–82.
- Kishimoto A, Mikawa K, Hashimoto K, Yasuda I, Tanaka S-i, Tominaga M, Kuroda T, Nishizuka Y (1989) Limited proteolysis of protein kinase C subspecies by calcium-dependent neutral protease (calpain). *J Biol Chem* 264:4088–4092.
- Klann E, Chen S-J, Sweatt JD (1991) Persistent protein kinase activation in the maintenance phase of long-term potentiation. *J Biol Chem* 266:24253–24256.
- Klann E, Chen S-J, Sweatt JD (1993) Mechanism of protein kinase C activation during the induction and maintenance of long-term potentiation probed using a selective peptide substrate. *Proc Natl Acad Sci USA* 90:8337–8341.
- Krug M, Lossner B, Ott T (1984) Anisomycin blocks the late-phase of long-term potentiation in the dentate gyrus of freely moving rats. *Brain Res Bull* 13:39–42.
- Lisman J (1994) The CaM kinase II hypothesis for the storage of synaptic memory. *Trends Neurosci* 17:406–412.
- Lynch G, Larson J, Kelso S, Barrionuevo G, Schottler F (1983) Intracellular injections of EGTA block induction of hippocampal long-term potentiation. *Nature* 305:719–722.
- Malenka RC (1991) Postsynaptic factors control the duration of synaptic enhancement in area CA1 of the hippocampus. *Neuron* 6:53–60.
- Malenka RC, Kauer JA, Zucker RS, Nicoll RA (1988) Postsynaptic calcium is sufficient for potentiation of hippocampal synaptic transmission. *Science* 242:81–84.
- Malinow R, Madison DV, Tseini RW (1988) Persistent protein kinase activity underlying long-term potentiation. *Nature* 335:820–824.
- Montarola PG, Goelet P, Castellucci VF, Morgan J, Kandel ER, Schacher S (1986) A critical period for macromolecular synthesis in long-term heterosynaptic facilitation in *Aplysia*. *Science* 234:1249–1254.
- Mulkey RM, Herron CE, Malenka RC (1993) An essential role for protein phosphatases in hippocampal long-term depression. *Science* 261:1051–1055.
- Nishizuka Y (1988) The molecular heterogeneity of protein kinase C and its implication for cellular recognition. *Nature* 334:661–665.
- Ono Y, Fujii T, Ogita K, Kikkawa U, Igarashi K, Nishizuka Y (1989) Protein kinase C ζ subspecies from rat brain: its structure, expression, and properties. *Proc Natl Acad Sci USA* 86:3099–3103.
- Otani S, Marshall CJ, Tate WP, Goddard GV, Abraham WC (1989) Maintenance of long-term potentiation in rat dentate gyrus requires protein synthesis but not messenger RNA synthesis immediately post-tetanzation. *Neuroscience* 28:519–526.
- Qian Z, Gilbert ME, Colicos MA, Kandel ER, Kuhl D (1993) Tissue-plasminogen activator is induced as an immediate-early gene during seizure, kindling and long-term potentiation. *Nature* 361:453–457.
- Read SM, Northcote DII (1981) Minimization of variation in the response to different proteins of the Coomassie Blue G dye-binding assay for protein. *Anal Biochem* 116:53–64.
- Reyman KG, Frey U, Jork R, Matthies H (1988) Polymyxin B, an inhibitor of protein kinase C, prevents the maintenance of synaptic long-term potentiation in hippocampal CA1 neurons. *Brain Res* 440:305–314.
- Sacktor TC, Osten P, Valsamis H, Jiang X, Naik MU, Sublette E (1993) Persistent activation of the ζ isoform of protein kinase C in the maintenance of long-term potentiation. *Proc Natl Acad Sci USA* 90:8342–8346.
- Schwartz JH (1993) Cognitive kinases. *Proc Natl Acad Sci USA* 90:8310–8313.
- Simpson IA, Sonne O (1982) A simple, rapid, and sensitive method for measuring protein concentration in subcellular membrane fractions prepared by sucrose density ultracentrifugation. *Anal Biochem* 119:424–427.
- Son II, Madelian V, Carpenter DO (1994) The translocation and involvement of protein kinase C in mossy-fiber-CA3 long-term potentiation in hippocampus of the rat brain. *Soc Neurosci Abstr* 20:264.
- Sossin WS, Sacktor TC, Schwartz JH (1994) Persistent activation of protein kinase C during the development of long-term facilitation in *Aplysia*. *Learn Memory* 1:189–202.
- Stanton PK, Sarvey JM (1984) Blockade of long-term potentiation in rat hippocampal CA1 region by inhibitors of protein synthesis. *J Neurosci* 4:3080–3088.
- Stelzer A, Slater NT, ten Bruggencate G (1987) Activation of NMDA receptors blocks GABAergic inhibition in an *in vitro* model of epilepsy. *Nature* 326:698–701.
- Sublette E, Naik M, Jiang X, Sacktor TC (1993) Evidence for a new isoform of protein kinase C in rat hippocampus. *Neurosci Lett* 159:175–178.
- Suzuki K, Saido TC, Hirai S (1992) Modulation of cellular signals by calpain. *Ann NY Acad Sci* 674:218–227.
- Takai Y, Kishimoto A, Inoue M, Nishizuka Y (1977) Studies on a cyclic nucleotide-independent protein kinase and its proenzyme in mammalian tissue I. Purification and characterization of an active enzyme from bovine cerebellum. *J Biol Chem* 252:7603–7609.
- Thomas KL, Laroche S, Errington ML, Bliss TVP, Hunt SP (1994) Spatial and temporal changes in signal transduction pathways during LTP. *Neuron* 13:737–745.
- Wang J-H, Feng D-P (1992) Postsynaptic protein kinase C essential to induction and maintenance of long-term potentiation in the hippocampal CA1 region. *Proc Natl Acad Sci USA* 89:2576–2580.