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Modified Hippocampal Long-Term Potentiation in PKCγ-Mutant Mice

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Summary

Calcium-phospholipid-dependent protein kinase (PKC) has long been suggested to play an important role in modulating synaptic efficacy. We have created a strain of mice that lacks the γ subtype of PKC to evaluate the significance of this brain-specific PKC isozyme in synaptic plasticity. Mutant mice are viable, develop normally, and have synaptic transmission that is indistinguishable from wild-type mice. Long-term potentiation (LTP), however, is greatly diminished in mutant animals, while two other forms of synaptic plasticity, long-term depression and paired-pulse facilitation, are normal. Surprisingly, when tetanus to evoke LTP was preceded by a low frequency stimulation, mutant animals displayed apparently normal LTP. We propose that PKCγ is not part of the molecular machinery that produces LTP but is a key regulatory component.

Introduction

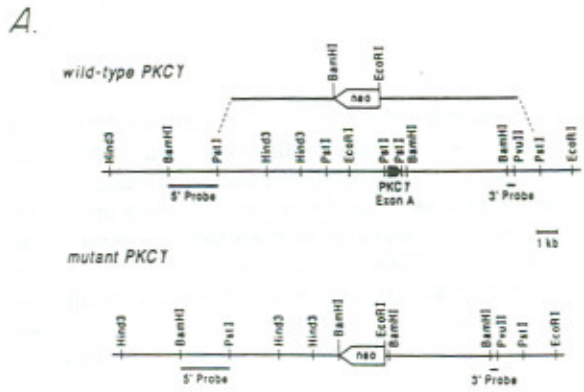
Kinases have repeatedly been implicated in the mechanism of long-term potentiation (LTP), the most intensively studied cellular model for memory (see Bliss and Collingridge, 1993). Recently, phosphatases have been found to participate in the inverse mechanism for the regulation of synaptic strength, hippocampal long-term depression (LTD) of the sort defined by Dudek and Bear (Mulkey et al., 1993; Dudek and Bear, 1992). Although the addition and removal of phosphate groups on unidentified proteins is widely believed to be important in the regulation of synaptic strength, very little evidence relates to the precise mechanisms. One possibility is that phosphorylation of some special protein(s) is an integral step in the pathway for LTP, but an alternative is that phosphorylation of this protein(s) is simply regulatory, so that the level of phosphorylation controls the likelihood, threshold, or magnitude of LTP.

Mutant mice produced by embryonic stem (ES) cell gene targeting technique to be defective in a particular gene product provide an attractive model system in which to study synaptic plasticity. We can obviate the lack of highly specific pharmacological tools to study various protein kinases with this approach; furthermore, we can investigate consequences of the mutation for behavior (for examples see Silva et al., 1992a, 1992b; Grant et al., 1992). Hippocampal LTP in particular has been suggested to play an important role in certain types of learning and memory and has attracted considerable attention (Bliss and Collingridge, 1993). The availability of knockout mice thus provides a unique opportunity to address the specific role of kinases in LTP and LTD and the relationship between these forms of synaptic plasticity and the learning and memory processes.

In an earlier study, Silva et al. (1992a) demonstrated that a null mutation in one of the kinases frequently implicated in LTP, α-CaM kinase II (αCaMKII), greatly diminishes the magnitude of LTP but seems not to eliminate it entirely. The fact that some LTP persists in the αCaMKII knockout mice was interpreted as indicating that this enzyme plays a regulatory and not an essential role. But, as Silva et al. have pointed out, the alternative possibility is that some other kinase could substitute, although not as effectively.

We have continued the program of investigating the molecular substrates of synaptic plasticity by producing mice that lack the γ isoform of Ca^{2+}–phospholipid-dependent protein kinase (PKC). PKC constitutes a family of isoenzymes involved in signal transduction pathways in diverse systems. This enzyme was chosen for study because pharmacological studies have repeatedly implicated PKC as playing a role in LTP (for reviews see Schwartz, 1993; Ben-Ari et al., 1992). For example, injection of PKC into hippocampal pyramidal cells elicits what may be aspects of LTP (Hu et al., 1987), and relatively selective inhibition of postsynaptic PKC blocks LTP induction (Malenka et al., 1989; Malinow et al., 1989; Reymann et al., 1988; Wang and Feng, 1992). PKC may also play a role in maintenance of LTP (Klann et al., 1991; Sacktor et al., 1993). In addition, redistribution of subcellular PKC in hippocampal neurons appears to coincide with LTP (Akers et al., 1986). The γ isoform was selected because it is brain specific, is richly represented in hippocampus where LTP and LTD are robustly expressed (Nishizuka, 1988), and appears to be present primarily in the dendrites and cell body of neurons (Huang et al., 1988). Moreover, the γ isoform appears late in development, so that brain defects that simply reflect abnormal neural development are less likely.

PKCγ-mutant mice are viable; their behavior in usual situations is not noticeably impaired; the brain anatomy is not apparently disturbed; and synaptic transmission appears normal. LTD is intact, but LTP is aberrant. LTP, as induced by the commonly used high frequency stimulation, is greatly diminished. When this high frequency stimulation is given after a low frequency stimulation of the sort used to produce LTD, however, LTP of approximately normal magnitude can be elicited. The production of LTP does not depend on LTD actually appearing, but just on the prior stimulation history of the synapses; we thus hypothesize that PKCγ is involved in regulating LTP but is not required for the actual process of synaptic plasticity.



Results

Generation of PKCγ-Mutant Mice

ES cell technology was used to generate PKCγ-mutant mice by homologous recombination into the germline. A homologous recombination vector was constructed containing PKCγ sequences harboring a 2.5 kb deletion. Integration of this vector would result in the loss of an exon containing the nucleotide-binding domain required for catalytic activity (Figure 1A). This vector was transfected into E14 ES cells (Thompson et al., 1989), and clones containing the desired homologous integration were identified by G418 selection and Southern blot hybridization. Five positive clones were identified, and these were injected into blastocysts to produce chimeric animals. Chimeric males were mated to C57BL/6 females and three chimeric animals transmitted the mutation through the germline. Heterozygote progeny were intercrossed, and the offspring were typed by Southern blot analysis for the PKCγ mutation (Figure 1B). PKCγ-mutant progeny are viable and can only be distinguished from wild-type littermates in the normal cage environment by mild uncoordination (Abeliovich et al., 1993 [this issue of *Cell*]).

Northern blot analysis of RNA from wild-type and PKCγ-mutant mice (Figure 1C) demonstrated that PKCγ-mutant mice lack normal PKCγ RNA transcripts. Western blot analysis of total brain protein with a rabbit polyclonal antiserum specific to PKCγ indicated that no PKCγ protein could be detected in brains of mutant mice (Figure 1D). Additionally, we did not detect up-regulation of PKCβ, the major PKC isotype in the brain. Analysis of PKC activity in homogenates of mutant and wild-type brains indicated that mutant mice possess a 29% ± 4% decrease in total PKC activity (data not shown), consistent with the known distribution of PKCγ in the brain (Sae et al., 1988). Furthermore, immunoprecipitation of PKCγ activity with a mouse monoclonal antibody indicated that mutant mice were devoid of immunoprecipitable PKCγ activity (data not shown).

Histochemical analyses of PKCγ-mutant mice did not reveal any gross anatomical abnormalities in the hippocampus or elsewhere (n = 3; Figure 2). This is consistent with the late onset of PKCγ expression (Nishizuka, 1988).

Synaptic Transmission Is Unimpaired

We have examined the amplitude and time course of synaptic responses, the functioning of NMDA receptors, and paired-pulse facilitation. The only difference we could de-

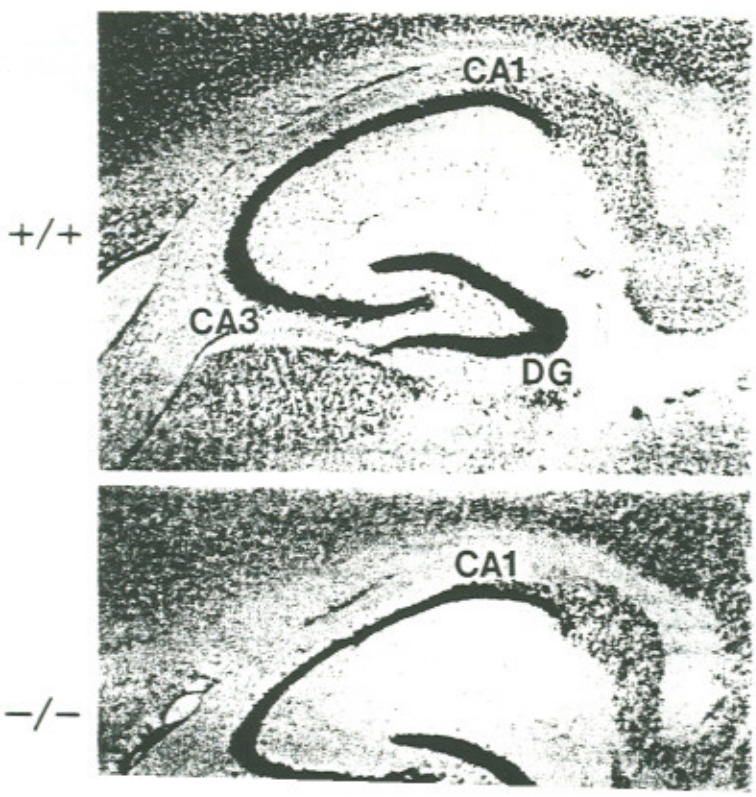


Figure 2. Horizontal Sections of Hippocampus from Wild-Type and PKCγ-Mutant Mice Stained with Cresyl Violet

Cell body layers of CA1, CA3, and dentate gyrus (DG) are indicated. Scale bar, 200 μm.

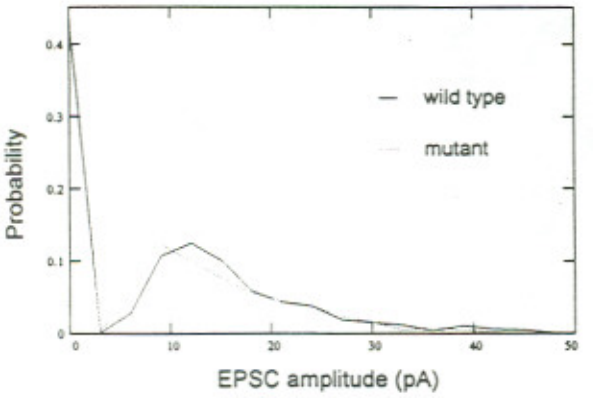


Figure 3. Amplitude Histogram of Responses to Minimal Stimulation

Responses were recorded in wild-type (solid line) and mutant slices (dashed line) in whole-cell patch clamp configuration. Stimulus intensity was adjusted such that failure rate of responses elicited was ~40%. The histogram shows the probability of observing a response as a function of response size (excitatory postsynaptic current amplitude). Bin size is 3 pA. Distributions of evoked responses are virtually indistinguishable between the wild-type (864 trials; n = 7 cells, seven slices from five mice) and the mutant (894 trials; n = 7 cells, seven slices from five mice) cells. This indicates that mutant slices display normal postsynaptic responsiveness.

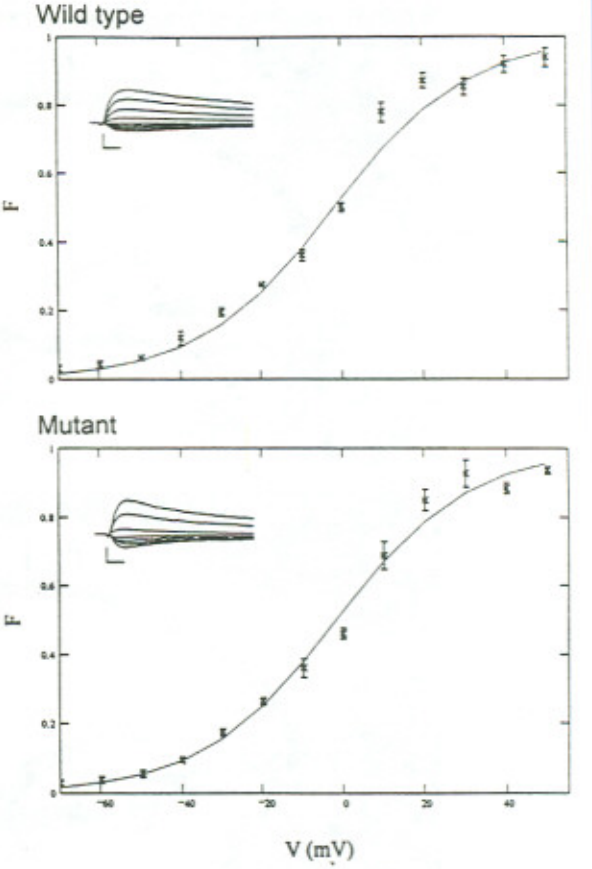


Figure 4. Voltage Dependence of NMDA Receptors

The perfusate contained 10 μM of CNQX to block non-NMDA receptor component of synaptic currents. Fraction (F) of the maximum conductance through the NMDA receptor channels is plotted as a function of holding membrane potentials. Data from four wild-type cells (top; four slices from two mice) and five mutant cells (bottom; five slices from three mice) are shown. Error bars display SEM. Insets show sample traces of synaptic currents at various holding potentials (mV): from top to bottom, +50, +40, +30, +20, −70, −50, 0, and −20 for the wild-type cell and +40, +30, +20, −60, −40, 0, and −20 for the mutant cell. Calibration bars are 10 ms and 100 pA (wild-type trace) or 200 pA (mutant trace). The smooth curves were fitted with the following equation: $g(V) = 1/[1 + \exp(-0.062V)(C/3.57)]$, where g is conductance in picosiemens, V is membrane potential in millivolts, and C is the extracellular magnesium concentration in millimoles/liter (Jahr and Stevens, 1990).

The time course and magnitude of the NMDA receptor components, and the ratios of non-NMDA to NMDA receptor components of synaptic currents do not differ between wild-type and mutant slices (data not shown). Further, the voltage sensitivity of the NMDA receptor that is critical for its activity, and consequently for synaptic plasticity, is unchanged by the absence of PKCγ (Figure 4).

Paired-pulse facilitation is a potentiation of the postsynaptic response to the second stimulus of the pair. It is caused by enhanced transmitter release from the presynaptic terminals (Mallart and Martin, 1968). Figure 5 presents data from experiments in which pairs of pulses were applied at varying interpulse intervals between 20–160 ms. The mutant slices showed paired-pulse facilitation that is slightly, but consistently, above that of the wild-type slices.

LTD Is Unimpaired

LTD is an important form of synaptic plasticity that shares steps with LTP; both require the initial influx of Ca^{2+} ions through NMDA receptor channels. We have examined this form of synaptic plasticity and find it to be unimpaired in induction, maintenance, and magnitude.

LTD was produced by the Dudek–Bear protocol (Dudek and Bear, 1992). Figure 6 presents histograms of the amplitude of LTD produced in 26 wild-type slices and 29 mutant slices with field potential recording. As with LTP (see below), the magnitude of the effect is quite variable from slice to slice. The mean size, shape of the distribution function, and variance of the distribution function are not significantly different for the wild-type and mutant animals. Thus, LTD is present in the mutant animals that lack PKCγ.

LTP Is Abnormal

The usual test for LTP is to examine the increase in synaptic strength produced by tetanic stimulation. We have carried out field potential experiments in 14 mutant slices to compare with 17 control slices. LTP was clearly deficient in mutant slices (Figures 7 and 8A). Identical results were obtained by an independent set of field potential recordings comparing LTP in ten mutant and four wild-type slices. Whole-cell recording is a more effective method for producing LTP than field potential recording since it allows a direct control of the postsynaptic membrane potential that is crucial for LTP induction. To test whether LTP deficiency in mutant slices can be overcome by controlling the postsynaptic membrane potential, whole-cell recordings have been carried out (7 control and 12 mutant slices). LTP clearly was attenuated or absent in the mutants (averaged trace not shown). To compare this effect quantitatively, we have made amplitude histograms (Figure 8). As usual, the amount of LTP produced follows a

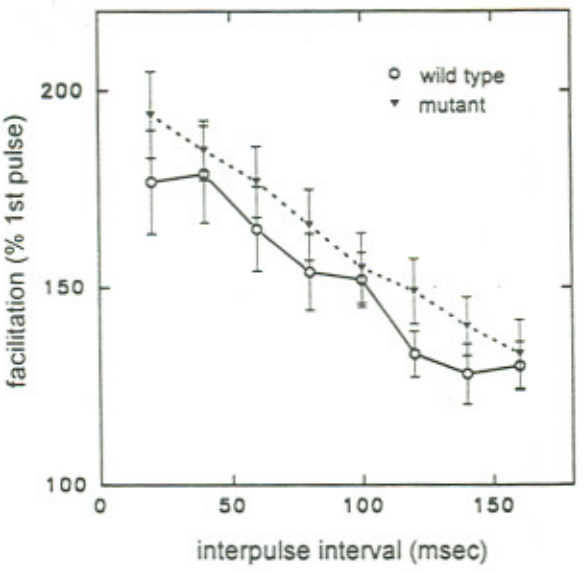


Figure 5. Paired-Pulse Facilitation in Wild-Type and Mutant Slices

Field potential responses to pairs of stimuli were monitored in wild-type (solid line; 11 slices from two mice) and mutant (dashed line; 12 slices from four mice) animals. The second response, expressed as percentage of response to the first pulse, is plotted as function of interpulse intervals. The error bars display SEM. Ca^{2+}/Mg^{2+} ratio of the perfusing medium was optimized for evoking paired-pulse facilitation and was decreased to 1 mM Ca^{2+}, 2.4 mM Mg^{2+} (from 2.5 mM Ca^{2+}, 1.3 mM Mg^{2+}).

probability distribution with a standard deviation of about 8%–30% (when LTP is expressed as percentage of the baseline). The mutant animals are clearly deficient in LTP, but the relatively large standard deviations make it difficult to decide whether LTP is entirely absent (although the mean of the distribution clearly is different between the mutant and control slices in both field potential and whole-cell recordings; for example see Figure 7).

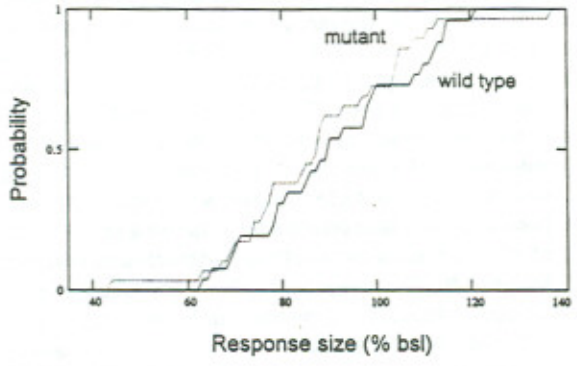


Figure 6. Cumulative Histogram of LTD

Low frequency stimulation consisting of 900 pulses at 1 Hz elicited LTD in both wild-type and mutant slices. Cumulative probability is shown as a function of mean LTD responses measured ~20–30 min after the low frequency stimulation. Response size is expressed as percentage of baseline response prior to low frequency stimulation. Field potential recordings from 26 wild-type (solid line; ten mice) and 29 mutant slices (dotted line; nine mice) are presented. Standard deviations for wild-type and mutant slices were 16.7 and 18.4, respectively. Mean LTD responses were 90% ± 3% (wild-type) and 87% ± 3% (mutant).

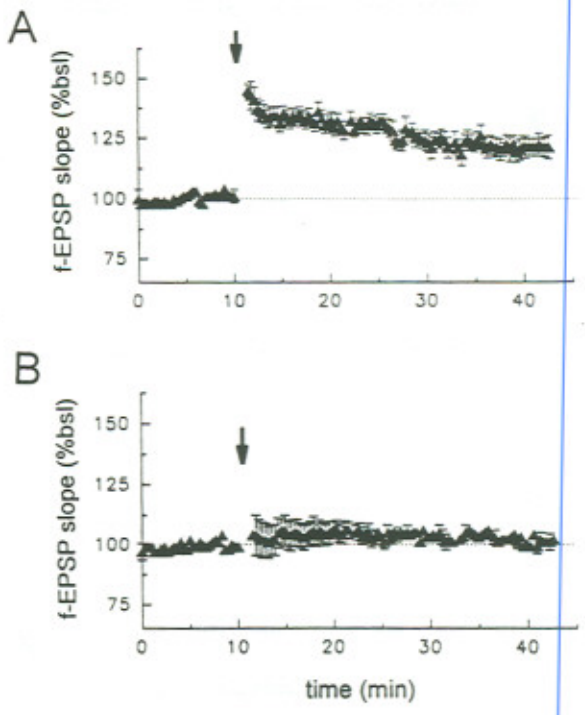


Figure 7. LTP Is Abnormal in Mutant Slices

Summary of field potential recordings from 15 wild-type (A; n = 6 mice) and 13 mutant (B; n = 5 mice) slices are shown. Initial slope of f-EPSPs is expressed as percentage of the mean baseline f-EPSP slope before tetanic stimulation (arrow). The error bars display SEM. Tetanus to evoke LTP consisted of five trains of 100 Hz stimulation, each lasting 200 ms at an intertrain interval of 10 s. Testing stimuli were given every 20 s.

In an attempt to see whether LTP is present in some cases, we determined the probability distributions of LTP amplitude for mutant and wild-type slices in the presence of 2-amino-5-phosphonopentanoate (AP5), an antagonist of NMDA receptors that disables LTP induction (Collingridge et al., 1983). The idea behind this experiment is that, if some LTP is present in the mutant slices, the probability distribution should be shifted to the left (to smaller values). Also, when LTP is inhibited by AP5 treatment, distribution of synaptic responses to tetanic stimulation should be indistinguishable between the mutant and wild-type animals. Figure 9 shows the relevant cumulative distributions of responses after high frequency stimulation, monitored in the presence of 50 μM of AP5. As is apparent, no difference can be detected between untreated mutant slices (n = 14), AP5-treated mutant slices (n = 10), and AP5-treated wild-type slices (n = 9).

We conclude that LTP either is absent or is greatly diminished under the conditions of these experiments.

LTP is a saturable phenomenon: repeated application of tetanus cannot increase the magnitude of synaptic response after a plateau is reached (Bliss and Collingridge, 1993). Because one form of synaptic plasticity (LTD) appears normal in the mutant animals, while another appears to be absent, we sought to test the hypothesis that LTP fails to appear because it is saturated in the slices when we obtain them from the animals. Under this hypothesis, PKCγ would perhaps regulate the relative balance be-

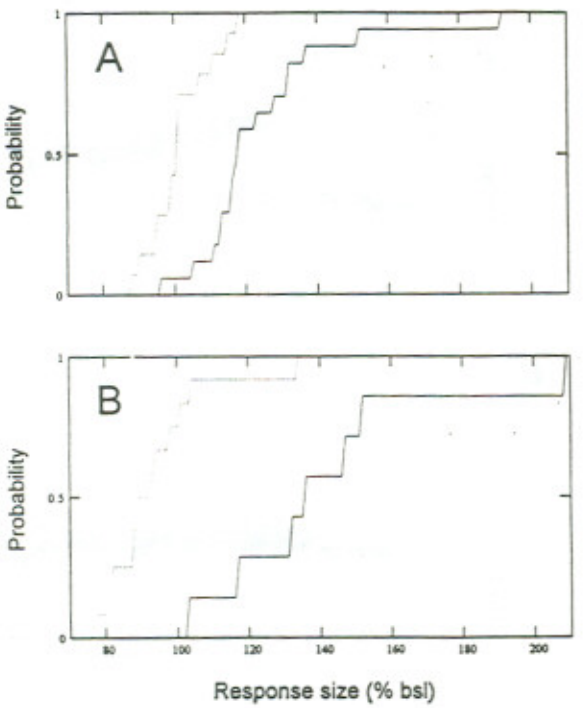


Figure 8. Cumulative Histograms of LTP

Cumulative probability is shown as a function of mean LTP response size measured between ~20–30 min after tetanus. Response size is expressed as percentage of the baseline response prior to tetanus.
(A) Field potential recordings from 17 wild-type (solid line; six mice) and 14 mutant slices (dotted line; six mice). Mean LTP responses are 124% ± 5% and 101% ± 2% for wild-type and mutant slices, respectively. Standard deviations of probability distributions are 21% (wild-type) and 8% (mutant).
(B) Whole-cell recordings from seven wild-type (solid line; three mice) and 12 mutant slices (dotted line; four mice) are shown. Mean LTP responses are 141% ± 13% for wild-type and 93% ± 4% for mutant slices. Standard deviations of probability distributions are 31% and 14% for wild-type and mutant slices, respectively.

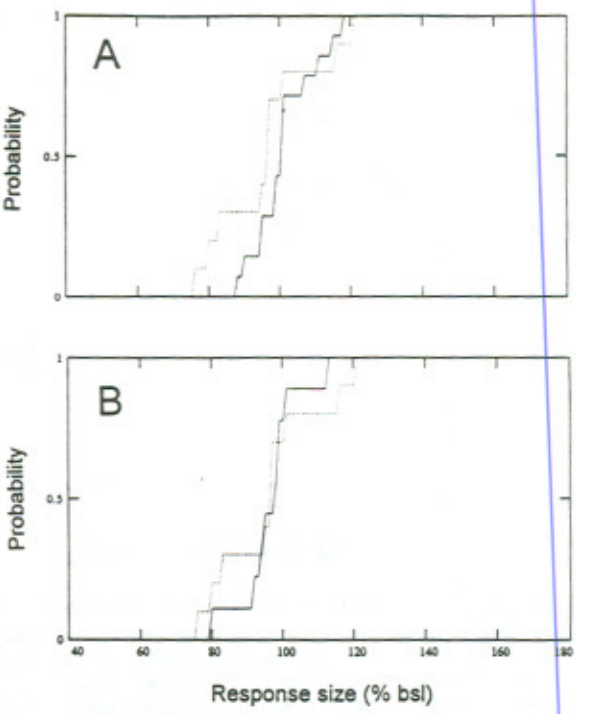


Figure 9. LTP Is Either Absent or Greatly Diminished in Mutant Slices

(A) Comparison of cumulative probability distributions of LTP amplitude between untreated mutant slices (solid line; n = 14 from six mice) and mutant slices treated with 50 μM of AP5 (dotted line; n = 10 from two mice). Mean responses are 101% ± 2% and 95% ± 5% for mutant slices without or with AP5 treatment, respectively. Cumulative distribution of LTP responses in mutant slices is indistinguishable from the distribution of responses measured in the presence of LTP inhibitor.
(B) Cumulative distributions of nine wild-type (solid line; two mice) and ten mutant slices (dotted line; two mice) that have been treated with 50 μM of AP5. Mean responses are 96% ± 3% (wild-type plus AP5) and 95% ± 5% (mutant plus AP5). When LTP induction is disabled both mutant and wild-type slices display indistinguishable cumulative probability distributions.

tween LTP and LTD. Were this true, the mutants should reveal LTP if preceded by the production of LTD: if we unsaturated LTP, we should then be able to elicit it.

Figure 10A displays the summary of recordings in which LTD was elicited prior to the production of LTP. After the LTD stimulation protocol, as is clear from this set of experiments, normal appearing LTP can be elicited in the mutant slices. Cumulative histograms from 21 mutant and 9 control slices (Figure 10B) reveal that significant LTP can be consistently produced in the mutant slices. When LTP was elicited in the presence of 50 μM of AP5 after the LTD stimulation protocol no potentiation was observed in the mutant slices; the mean response after the tetanus (81% ± 6%; n = 9) was not significantly different from the mean LTD response (87% ± 3%; n = 29).

Although the prior LTD stimulation protocol does enable the subsequent production of LTP, the mechanistic explanation appears not to be that LTP was saturated: the magnitude of LTP is unrelated to the magnitude of the preceding LTD, whereas the saturation hypothesis would require that greater LTD would permit greater LTP. Throughout this paper, LTD and LTP responses have been calculated as percentage of the very initial baseline before application of tetanus or low frequency stimulation, with the exception of Figure 11A. In Figure 11A, LTP response subsequent to LTD is displayed as percent potentiation relative to the mean response after LTD, and is compared with LTD response expressed as percent depression relative to the initial baseline to test the following prediction: if the magnitude of subsequent LTP correlates with the magnitude of prior LTD then data points should fall on a line with slope of +1. Our results demonstrate that there is no correlation between the two magnitudes. Thus, the quantity of LTP produced appears independent of the extent of LTD.

To test further the possible role of prior LTD in enabling LTP production, we applied 50 μM of AP5 during the LTD stimulation protocol, and then washed out the AP5 for 30 min before using the standard tetanic stimulus to produce LTP. In these experiments, the LTD was, as we would expect, effectively blocked by AP5 (Figure 11B, compare lanes a and c), but the amount of LTP produced was not significantly different from that of control slices subjected to the same procedures in the absence of AP5 (Figure 11B, compare lanes b and d).

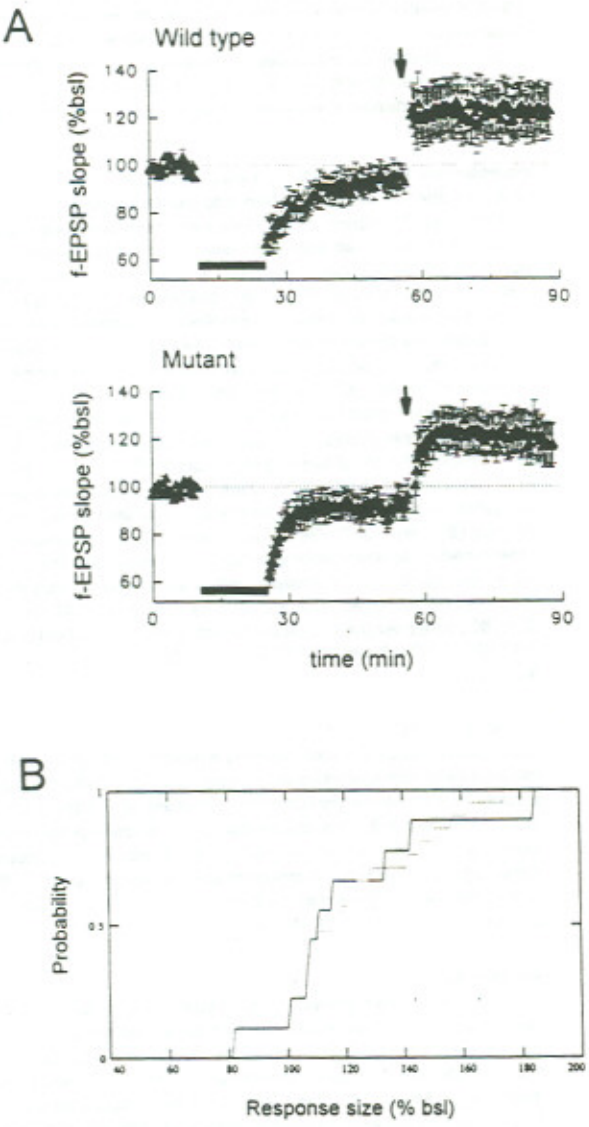


Figure 10. LTP Can Be Elicited in Mutant Slices after the LTD Protocol

(A) Summary of field potential recordings from 8 wild-type (five mice) and 15 mutant slices (nine mice) in which LTD protocol (solid bar) was followed by a tetanus to evoke LTP (arrow). Responses are expressed as percentage of the baseline response (f-EPSP slope) prior to low frequency stimulation. The error bars display SEM.

(B) Cumulative probability distributions of LTP responses from nine wild-type (solid line; five mice) and 21 mutant slices (dotted line; nine mice) in which LTD protocol was given prior to tetanus to elicit LTP. Average LTP responses measured between ~20–30 min after tetanus is expressed as percentage of the average baseline response obtained before the application of LTD protocol. Wild-type and mutant slices displayed mean LTP responses of 120% ± 10% and 113% ± 8%, respectively.

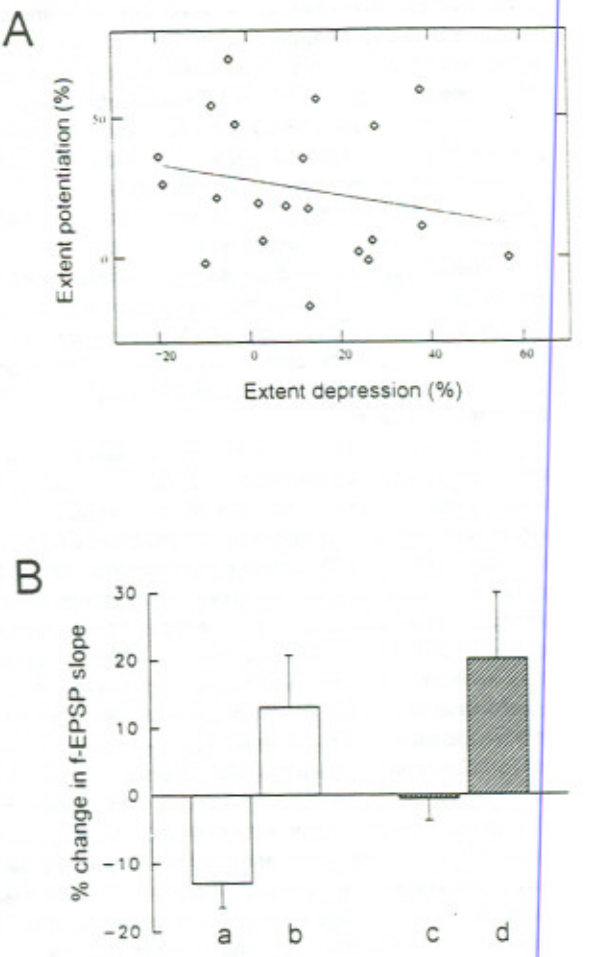


Figure 11. LTP Produced in Mutant Slices Does Not Depend on the Actual LTD but on the Prior Stimulation History of Synapses

(A) Extent of LTP produced (percentage of potentiation of the response to LTP tetanus relative to response after the LTD protocol) is plotted as a function of extent of initial LTD (percentage of depression relative to the baseline response). If amplitude of LTP is proportional to the extent of initial LTD then data points should fall on a line with slope of +1. Our results (n = 21) demonstrate that there is no correlation between the extent potentiation and extent depression. Linear regression of data points yields the line shown with a slope of −0.28 and correlation coefficient of −0.23.

(B) Field potential recordings of mutant slices (n = 21 from nine mice) where LTD protocol was followed by a tetanus (lanes a and b) are compared to AP5-treated mutant slices (n = 10 from six mice; lanes c and d). AP5 (50 μM) was applied 30 min before and during the LTD protocol and washed out for 30 min before the subsequent application of tetanus. Control experiments in wild-type slices demonstrated that 50 μM of AP5 was sufficiently washed out in 30 min to enable LTP production (data not shown). Mean percent change in responses relative to mean baseline response (f-EPSP slope) before the LTD protocol are shown. Lanes a and c display LTD produced in the absence (a) or in the presence (c) of AP5. Lanes b and d are mean potentiation observed when tetanus was given after the LTD protocol in the absence (b) or in the presence of AP5 (d).

We conclude, then, that the history of low frequency stimulation somehow does enable the slices from mutant animals to express LTP, but that the mechanism by which this priming effect works is not through the actual production of LTD.

Discussion

The absence of the γ isoform of PKC has little detectable effect on baseline synaptic transmission. We could find only a very slight increase in paired-pulse facilitation. Syn-

aptic plasticity, however, is modified but not eliminated because LTD is apparently normal, and LTP, which is absent under the usual test circumstances, can be elicited if preceded by a period of low frequency stimulation.

Clearly, the action of PKCγ is not necessary for the production of LTP. We propose that this kinase is required for some regulatory role in LTP, although we cannot exclude the possibility that PKCγ action in fact is essential for LTP, but that some other kinase is replacing it. This hypothesis may be tested by making multiple deletions. For example, it is now possible to produce mice that are doubly deficient in PKCγ and αCaMKII. In such mutant animals would LTP be completely abolished? If kinases are regulatory then we should observe occasional LTP, albeit less frequently.

The fact that LTP is enabled by low frequency stimulation, but that the appearance of LTD or calcium influx through NMDA receptor channels seems irrelevant for this effect, surprised us. This observation indicates a complexity in the control of LTP-inducing mechanisms that, as far as we are aware, has not been revealed in earlier studies, although descriptions of similar effects have appeared earlier (Huang et al., 1992; Coan et al., 1989; Fujii et al., 1991). Interestingly, however, the earlier investigations reported what seems to be, on the surface at least, an effect in the opposite direction: in several studies, low frequency stimulation or weak tetani that elicit short-lasting potentiation have been found to inhibit the subsequent triggering of LTP for a period of time. Perhaps this inhibition is mediated through PKCγ action, and removing this regulatory pathway unmasks another priming mechanism. In any case, we propose that the use history of a synapse modifies the state of LTP regulatory systems in ways that have not previously been apparent. Presumably, such mechanisms have functional significance, but our study reveals too little about the properties of the postulated regulation to permit speculation about what these functional consequences might be.

An interesting observation is that the usual protocol for LTP production does not, in every circumstance, accurately assess the potential for a slice to produce LTP. In our PKCγ-mutant mice one would conclude from the usual methods that the slices lack the capacity to produce LTP; our study, however, demonstrates that the hippocampal slices from the mutant mice retain the capacity to express LTP of approximately normal magnitude when an alternative stimulation protocol is used. It remains to be seen whether such a stimulation protocol and LTP induced by it have any physiological relevance in an intact animal.

Experimental Procedures

Mapping and Cloning of Mouse PKCγ

PKCγ was cloned from a 129/Ola mouse genomic cosmid library (Stratagene) with a rat PKCγ cDNA probe. The exon structure was determined in a region of PKCγ that encodes the adenosine triphosphate-binding motif, a required component of the kinase catalytic domain (Freisewinkel et al., 1991). Exon A corresponds to amino acids 363–426 of the rat protein (Knopf et al., 1986). The homologous recombination vector p21 was assembled in a tetramolecular ligation reaction, using a 4.5 kb PstI fragment of cosmid 3C 5′ of exon A (subcloned into Bluescript II [Stratagene] and excised with BamHI and XhoI), a 1.8 kb XhoI–EcoRI fragment containing *neo* driven by the PGKI promoter (a gift from Dr. M. Rudnick), a 5.5 kb fragment of cosmid 3C 3′ of exon A (subcloned into Bluescript II and excised with EcoRI and XbaI), and XbaI–BamHI restriction-digested Bluescript II vector. This construct was designed to delete a 2 kb fragment of the PKCγ gene, including exon A.

Homologous Recombination in Mouse ES Cells and Introduction of the Deletion into the Germline

E14 ES cells (Thompson et al., 1989) were transfected with 50 μg of plasmid P21 (linearized with NotI restriction enzyme) by electroporation. (Biorad gene pulser set at 800 V and 3 μF). G418 selection (150 μg/ml) was applied 24 hr after transfection, and G418-resistant colonies were isolated on days 5–7 of selection. Genomic DNA isolated from approximately 200 colonies was digested with BamHI restriction enzyme, Southern blotted, and hybridized with a 1.2 kb BamHI–PstI fragment of cosmid 3C 5′ of the flanking region in p21 (Figure 1). Six clones were identified that harbored the predicted homologous integration. These clones were confirmed by digestion with a BamHI–PvuII probe within the 3′ flanking region of p21 (Figure 1), or an NcoI–EcoRI fragment of cosmid 3C 3′ of the flanking sequences in plasmid p21 (data not shown). Chimeric mice were generated as described by Bradley (Bradley, 1987). Germline transmission of the deletion was determined by Southern blotting of tail DNA as described above. Later, mice were typed by PCR analysis with a set of *neo* primers (5′-CTTGGGTGGAGAGGCTATTC-3′ and 5′-AGGTGAGATGACAGGAGATC-3′, a 280 bp fragment) and a set of primers to exon A (5′-GGATGACGATGTAGACTGCA-3′ and 5′-CTCTTACCACTGGTCACATC-3′, a 200 bp fragment).

Analysis of PKCγ RNA

RNA was isolated from total brain by extraction with guanidine HCl followed by cesium chloride gradient centrifugation. RNA (200 μg) was electrophoresed in the presence of formaldehyde, Northern blotted, and probed with a 200 bp polymerase chain reaction product within exon A of PKCγ (see above) or a human glyceraldehyde 3-phosphate dehydrogenase (*hG3PDH*; Clonetech) control probe. Human *G3PDH* is a housekeeping gene that cross-hybridizes with the mouse gene and serves to control for RNA quantity.

Western Blot

Partially purified brain extract (5 μg) (Yasuda et al., 1990) was denatured in sodium dodecyl sulfate loading buffer and separated by sodium dodecyl sulfate–polyacrimide gel electrophoresis. Gels were then transferred to Hybond–enhanced chemiluminescence nylon membranes (Amersham), and membranes were incubated with anti-PKCγ or anti-PKCβ antibody (rabbit anti-rat peptide antibodies at 0.2 μg/ml, GIBCO BRL). Membranes were subsequently incubated with a goat anti-rabbit horseradish peroxidase-labeled antibody (Southern Biotechnology Associates, Incorporated) Antigen–antibody complexes were detected by enhanced chemiluminescence (ECL, Amersham).

Histochemistry

Mice were perfused with 2% paraformaldehyde in PIPES buffer under deep sodium pentobarbitol anesthesia (anesthesia performed under the guidelines of the Massachusetts Institute of Technology Division of Comparative Medicine), and their brains were postfixed for up to 8 hr at 4°C and then soaked overnight in phosphate buffer containing 30% sucrose and 2 mM $MgCl_2$ at 4°C. Horizontal sections were cut at 30 μm or 40 μM on a sliding microtome or with a cryostat and were stained with cresyl violet.

Electrophysiology

Standard procedures were used to prepare transverse hippocampal slices from wild-type or mutant mice (male or female, mostly 1 to 3 months old; younger animals were typically used for whole-cell recordings). Slices (350 μm) were transferred to an incubation chamber and were allowed to recover for at least 1 hr before recording. During recording, each slice was submerged under a continuously perfusing medium that was saturated with 95% O_2, 5% CO_2. The perfusate contained: 120 mM NaCl, 3.5 mM KCl, 1.25 mM NaH_2PO_4, 26 mM $NaHCO_3$, 1.3 mM $MgCl_2$, and 2.5 mM $CaCl_2$. Whole-cell patch clamp experiments

were performed in the presence of 50 μM of picrotoxin. Experiments were carried out at room temperature (23°–25°C).

Schaffer collateral–commissural fibers were stimulated by bipolar tungsten electrodes that delivered 100 ms pulses. Field potentials were recorded in field CA1 with glass electrodes filled with perfusing medium. Whole-cell patch clamp recordings were carried out according to standard techniques. Excitatory postsynaptic currents were recorded with glass electrodes (~4MΩ) filled with 130 mM cesium gluconate, 5 mM CsCl, 5 mM NaCl, 10 mM HEPES–CsOH (pH 7.2), 0.5 mM EGTA, 1 mM $MgCl_2$, 2 mM Mg–adenosine triphosphate and 0.2 mM Li–guanosine triphosphate. Stimulus intensity was adjusted to evoke baseline responses of similar sizes for all recordings. For minimal stimulation recordings in whole-cell mode, stimulus intensity was adjusted such that failure rate of responses elicited was ~40%. Tetanus to evoke LTP consisted of 5 trains of 100 Hz stimulation, each lasting 200 ms at an intertrain interval of 10 s. In whole-cell recordings, the membrane potential of postsynaptic cell was held at −70 mV except during tetanus, when it was −30 mV. LTD protocol was 900 pulses of 1 Hz stimulation as described (Dudek and Bear, 1992). Recordings were performed with axopatch 200 (Axon Instruments, Incorporated). Signals were filtered at 2 kHz, digitized at 5 kHz, and analyzed with programs written in AXOBASIC. The initial slopes of field excitatory postsynaptic potentials (f-EPSPs) and the peak amplitudes of excitatory postsynaptic currents were measured for field potential and whole-cell recordings, respectively. CNQX was from Cambridge Research Biochemicals and D(−)-AP5 was from Research Biochemicals, Incorporated.

Time course and magnitude of the NMDA receptor components, and the ratios of non-NMDA to NMDA receptor component of synaptic currents were obtained from whole-cell patch clamp recording experiments in which excitatory postsynaptic currents were monitored before and after application of 10 μM of CNQX from the same cell at holding potential of −50 mV in 1.3 mM Mg^{2+}. Charge carried by NMDA receptor component measured in the presence of CNQX was compared to the charge of synaptic current before CNQX application that represents both non-NMDA and NMDA components. The ratios of NMDA component to the sum of NMDA and non-NMDA components did not differ between the wild-type and mutant neurons: for ten wild-type neurons (n = 10 slices, four mice) this ratio was 40% ± 4% (± SEM), and for ten mutant neurons (n = 10 slices, four mice) it was 36% ± 3% (± SEM). Consequently, the ratios of non-NMDA to NMDA components of synaptic currents are also indistinguishable between the mutant and the wild-type cells.

Not all the data used to prepare cumulative histograms shown in Figures 8A and 10B were included in the averaged traces displayed in Figures 7 and 10A, since a few of the data files were not compatible with the analysis program that was used for averaging data. Cumulative histograms of responses obtained only from those data used for the averaged trace are indistinguishable from the cumulative histograms presented in the paper (data not shown).

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References

Abelivich, A., Paylor, R., Chen, C., Kim, J., Wehner, J., and Tonegawa, S. (1993). PKCγ-mutant mice exhibit moderate deficits in contextual learning. Cell *75*, this issue.

Akers, R. F., Lovinger, D. M., Colley, P. A., Linden, D. J., and Routtenberg, A. (1986). Translocation of protein kinase C activity may mediate hippocampal long-term potentiation. Science *231*, 587–589.

Ben-Ari, Y., Aniksztejn, L., and Bregestovski, P. (1992). Protein kinase C modulation of NMDA currents: an important link for LTP induction. Trends Neurosci. *15*, 333–339.

Bliss, T. V. P., and Collingridge, G. L. (1993). A synaptic model of memory: long-term potentiation in the hippocampus. Nature *361*, 31–39.

Bradley, A. (1987). Production and analysis of chimeric mice. In Teratocarcinomas and Embryonic Stem Cells: A Practical Approach, E. J. Robertson, ed. (Oxford: IRL Press), pp. 113–151.

Coan, E. J., Irving, A. J., and Collingridge, G. L. (1989). Low-frequency activation of the NMDA receptor system can prevent the induction of LTP. Neurosci. Lett. *105*, 205–210.

Collingridge, G. L., Kehl, S. J., and McLennan, H. (1983). Excitatory amino acids in synaptic transmission in the Schaffer collateral–commisural pathway of the rat hippocampus. J. Physiol. London *334*, 33–46.

Dudek, S. M., and Bear, M. F. (1992). Homosynaptic long-term depression in area CA1 of hippocampus and effects of N-methyl-D-aspartate receptor blockade. Proc. Natl. Acad. Sci. USA *89*, 4363–4367.

Freisewinkel, I., Riethmacher, D., and Stabel, S. (1991). Downregulation of protein kinase C-γ is independent of a functional kinase domain. FEBS Lett. *280*, 262–266.

Fujii, S., Saito, K., Miyakawa, H., Ito, K., and Kato, H. (1991). Reversal of long-term potentiation (depotentiation) induced by tetanus stimulation of the input to CA1 neurons of guinea pig hippocampal slices. Brain Res. *555*, 112–122.

Grant, S. G., O'Dell, T. J., Karl, K. A., Stein, P. L., Soriano, P., and Kandel, E. R. (1992). Impaired long-term potentiation, spatial learning, and hippocampal development in *fyn* mutant mice. Science *258*, 1903–1910.

Hu, G. Y., Hvalby, Ø., Walaas, S. I., Albert, K. A., Skjeflo, P., Andersen, P., and Greengard, P. (1987). Protein kinase C injection into hippocampal pyramidal cells elicits features of long term potentiation. Nature *328*, 426–429.

Huang, F. L., Yoshida, Y., Nakabayashi, H., Young, W. S., and Huang, K.-P. (1988). Immunocytochemical localization of protein kinase C isozymes in rat brain. J. Neurosci. *8*, 4734–4744.

Huang, Y. Y., Colino, A., Selig, D. K., and Malenka, R. C. (1992). The influence of prior synaptic activity on the induction of long-term potentiation. Science *255*, 730–733.

Jahr, C. E., and Stevens, C. F. (1990). Voltage dependence of NMDA-activated macroscopic conductances predicted by single-channel kinetics. J. Neurosci. *10*, 3178–3182.

Klann, E., Chen, S. J., and Sweatt, J. D. (1991). Persistent protein kinase activation in the maintenance phase of long-term potentiation. J. Biol. Chem. *266*, 24253–24256.

Knopf, J. L., Lee, M.-H., Shultzman, L. A., Kriz, R. W., Loomis, C. R., Hewick, R. M., and Bell, R. M. (1986). Cloning and expression of multiple protein kinase C cDNAs. Cell *46*, 491–502.

Malenka, R. C., Kauer, J. A., Perkel, D. J., Mauk, M. D., Kelly, P. T., Nicoll, R. A., and Waxham, M. N. (1989). An essential role for postsynaptic calmodulin and protein kinase activity in long-term potentiation. Nature *340*, 554–557.

Malinow, R., Schulman, H., and Tsien, R. W. (1989). Inhibition of postsynaptic PKC or CaMKII blocks induction but not expression of LTP. Science *245*, 862–866.

Mallart, A., and Martin, A. R. (1968). The relation between quantum content and facilitation at the neuromuscular junction of the frog. J. Physiol. *196*, 593–604.

Mulkey, R. M., Herron, C. E., and Malenka, R. C. (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 implications for cellular regulation. Nature *334*, 661–665.

Raastad, M., Storm, J. F., and Andersen, P. (1992). Putative single quantum and single fiber excitatory postsynaptic currents show similar amplitude range and variability in rat hippocampal slices. Eur. J. Neurosci. *4*, 113–117.

Reymann, K. G., Brödemann, R., Kase, H., and Matthies, H. (1988). Inhibitors of calmodulin and protein kinase C block different phases of long-term potentiation. Brain Res. *461*, 388–392.

Sacktor, T. C., Osten, P., Valsamis, H., Jiang, X., Naik, M. U., and 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.

Sae, K., Saito, N., Shearman, M. S., Kikkawa, U., Ono, Y., Igarashi, K., Tanaka, C., and Nishizuka, Y. (1988). Distinct cellular expression of β1 and β2 subspecies of protein kinase C in rat cerebellum. J. Neurosci. *8*, 3850–3856.

Schwartz, J. H. (1993). Cognitive kinases. Proc. Natl. Acad. Sci. USA *90*, 8310–8313.

Silva, A., Stevens, C. F., Tonegawa, S., and Wang, Y. (1992a). Deficient hippocampal long-term potentiation in α-calcium-calmodulin kinase II-mutant mice. Science *257*, 201–206.

Silva, A., Paylor, R., Wehner, J., and Tonegawa, S. (1992b). Impaired spatial learning in α-calcium-calmodulin kinase II-mutant mice. Science *257*, 206–211.

Thompson, S., Clarke, A. R., Pow, A. M., Hooper, M. L., and Melton, D. W. (1989). Germ line transmission and expression of a corrected HPRT gene produced by gene targeting in embryonic stem cells. Cell *56*, 313–321.

Wang, J. H., and 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.

Yasuda, I., Kishimoto, A., Tanaka, S., Tominaga, M., Sakurai, A., and Nishizuka, Y. (1990). A synthetic peptide substrate for selective assay of protein kinase C. Biochem. Biophys. Res. Commun. *166*, 1220–1227.