

Inhibition of Protein Kinase C Blocks Two Components of LTP Persistence, Leaving Initial Potentiation Intact

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Protein kinase C (PKC) activity is increased following hippocampal long-term potentiation (LTP; Akers et al., 1986). A similar increase in PKC activity is measured following the induction of a long-lasting potentiation with abbreviated high-frequency stimulation (HFS) in combination with PKC-activating phorbol esters (Colley et al., 1989). Because phorbol esters have no effect on the initial potentiation produced with HFS, and because PKC activity appears to be related to the persistence of LTP and not to the initial change, we concluded that PKC regulates a post-initiation component of LTP. To define the time domain in which PKC activation is necessary for LTP, we studied the effect of the PKC inhibitors polymyxin B (PMXB) and 1-(5-isoquinolinesulfonyl)-2-methylpiperazine (H-7) micropressure ejected at different time points before and after the induction of LTP. LTP was produced in intact rats with HFS of the perforant path, and inhibitor ejections were made in the molecular layer of the dentate gyrus. PMXB, which at lower doses is a selective inhibitor of PKC, had no effect on initial potentiation, yet caused decay of the potentiated response to baseline within 2 hr. Decay occurred when PMXB was ejected 15 min before and 15 and 30 min after HFS. PMXB, at either low or high doses, was ineffective in blocking LTP persistence at time points greater than 30 min after HFS. Low doses of H-7 produced similar effects to those of PMXB. However, in contrast to a high dose of PMXB, a high dose of H-7 inhibited the persistence of LTP when delivered 240 min after HFS. A decrease in the *in vitro* phosphorylation of the PKC substrate protein F1 in animals that showed a decay of LTP following PMXB or H-7 ejection suggests that PKC was inhibited *in vivo*. We propose a model of LTP that consists of 3 separable phases: (1) initial potentiation (0–5 min), which does not require PKC activation; (2) a PKC-regulated persistence phase (5–60 min) related to synaptic modification; and (3) a second PKC-regulated persistence phase (>60 min), which is dependent on protein synthetic processes.

The Ca²⁺/phospholipid-stimulated protein kinase C (PKC), which plays a key role in transmembrane signaling (Nishizuka, 1986, 1988) has been implicated by this (Routtenberg, 1985) and other laboratories (Malenka et al., 1986) in the regulation

of hippocampal long-term potentiation (LTP),¹ a model of information storage (Bliss and Lomo, 1973). Following the induction of LTP, both an increase in membrane PKC activity (Akers et al., 1986) and *in vitro* phosphorylation of a PKC substrate, protein F1 (Lovinger et al., 1985; Routtenberg et al., 1985), are detected. These biochemical alterations are positively correlated with the persistence of LTP, but not with the initial magnitude of the potentiated response. Finally, direct activation of PKC with phorbol esters enhances the persistence of synaptic potentiation produced with abbreviated high-frequency stimulation (HFS) without having an effect on initial potentiation (Routtenberg et al., 1986; Colley et al., 1989).

We have proposed a model in which phorbol esters and HFS act synergistically to produce a long-lasting synaptic potentiation. Although a slowly developing enhancement is induced with higher doses of phorbol ester alone (Malenka et al., 1986; Colley et al., 1989), current evidence suggests that phorbol ester enhancement differs from potentiation produced with HFS (Routtenberg et al., 1986; Gustafsson et al., 1988; Muller et al., 1988; Colley et al., 1989).

To determine whether PKC activation is necessary for LTP persistence, PKC inhibitors have been used. The first of such studies indicated that PKC was essential for the regulation of LTP persistence (Lovinger et al., 1987). Potentiated responses in the intact preparation decayed to baseline values within 50 min after ejections of 3 different PKC inhibitors [polymyxin B (PMXB), mellitin, and 1-(5-isoquinolinesulfonyl)-2-methylpiperazine (H-7)]. Mellitin (50 pmol), which was effective when applied 15 min before and 10–60 min after HFS, failed to produce decay if delivered 240 min after the induction of LTP. The data indicate that PKC activation maintains LTP for 15 min, but not 240 min, after its induction.

Inhibition of LTP persistence by extracellularly applied H-7 and other PKC inhibitors has also been observed in hippocampal slice preparations (sphingosine: Malinow et al., 1988; K-252b: Reymann et al., 1988b; PMXB: Reymann et al., 1988a). The degree to which these compounds can inhibit the persistence of

¹ Because there now exist differences in terminology, we wish to define key words used in this report: *LTP* has been used in the past to signify either procedure or effect. Here we refer to the procedure as *high-frequency stimulation*, or HFS and the effect after the procedure as *LTP*. The term *initial potentiation* is used here as the initial magnitude of potentiation immediately after the 4-min pattern of HFS. Posttetanic potentiation (*PTP*; McNaughton, 1982) is only a component of this initial potentiation. The *persistence* of LTP is a measure of the maintenance of initial potentiation beyond PTP and, in the present report, is first measured 15 min after HFS. Similarly, the *expression* of LTP is a measure of the enhancement of synaptic transmission; it is used in the context of experiments in which expression is blocked without affecting the underlying mechanism. The term *induction* refers to the mechanism leading to persistent synaptic enhancement, whether it is produced by electrical or chemical manipulation.

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LTP may depend on their mode of PKC inhibition; for example, sphingosine, which acts on the regulatory subunit (Hannun et al., 1986), is ineffective if delivered after HFS, whereas inhibition of the catalytic subunit by H-7 (Hidaka et al., 1984) is effective when given either before or after the induction of LTP (Malinow et al., 1988). In addition, Malinow et al. (1988) produced decay with H-7 (300 μ M) applied 3 hr after LTP induction, an effect not observed with mellitin (50 pmol; Lovinger et al., 1987). There has also been a report of H-7 having no effect on LTP, but the dose used may have been too low (Muller et al., 1988). Finally, there have been recent reports of intracellularly injected PKC and CaM-dependent kinase inhibitors blocking LTP in CA1 neurons of hippocampal slices (Malenka et al., 1989; Malinow et al., 1989).

Discrepancies concerning the dose- and time-dependent effects of PKC inhibitors on hippocampal LTP might be explained by the differences in the preparation used, drug dose, and/or method of application. To determine the time domain when PKC activation is necessary for LTP persistence, we examined the dose-response effects of the PKC inhibitors PMXB and H-7 delivered at discrete time points relative to the delivery of LTP-inducing HFS. We also measured PKC activity following inhibitor application using *in vitro* phosphorylation of the PKC substrate protein F1 as the reporter system.

Materials and Methods

Electrophysiology. Experiments were performed on urethane-anesthetized Sprague-Dawley rats. Population EPSPs evoked by perforant path stimulation were recorded in the dentate gyrus molecular layer with micropipettes filled with inhibitor solutions. Stereotaxic placement of both stimulating and recording electrodes was performed as previously described (Routtenberg et al., 1985).

Inhibitors and micropressure ejection. Stock solutions of the PKC inhibitors PMXB (Sigma) and H-7 (Seikagaku America Inc.) were prepared with 20 mM Tris (pH, 7.5) to achieve a concentration of 10 mM. Aliquots of stock solutions, stored at either 40°C (H-7) or -20°C (PMXB), were thawed and diluted as needed. Micropressure ejection of inhibitor solutions was performed using the techniques and equipment employed by McCrimmon et al. (1986), which have been previously described in detail (Lovinger et al., 1986). Ejection volumes were controlled by directly monitoring the movement of the solution meniscus in the micropipette with a 150 \times microscope (Rölyn Optics) and a fine reticule. Doses are reported as pmol drug ejected. Drug solutions were ejected in 5-nl vol unless the doses desired required concentrations greater than that of stock solutions. In these cases, equivalent ejections were achieved using 10-mM solutions ejected in vol up to 25 nl. Ejections of 5–25 nl 20 mM Tris vehicle or H-8 (Seikagaku America Inc.), an isoquinolinesulfonamide derivative that is a much more potent inhibitor of cAMP-dependent kinases than of PKC (Hidaka et al., 1984), were used for control ejections.

Recording and data analysis. Once stable baseline EPSPs evoked with 0.1 Hz stimulation were collected, 8 trains of 400-Hz stimulation (8 pulses of 0.4 msec) were delivered to produce LTP (40–60% increase over baseline) of EPSP slopes. The persistence of the LTP was then monitored with 0.1 Hz stimulation for up to 6 hr after HFS.

Statistical analyses were performed on EPSP slopes elicited with threshold minus 2 V. Threshold was defined prior to HFS as the baseline input voltage at which a population spike was first detected. This avoided population spike contamination after HFS. Waveforms used for analysis were averages of 11 individual responses. Evoked waveforms were collected by an IBM PC-XT microcomputer and stored on disk for off-line analysis.

In vitro phosphorylation. Animals were killed 30–300 min after ejection of inhibitor as described (Lovinger et al., 1986). The area of the dorsal hippocampus surrounding the recording electrode was dissected (~30 mg) and homogenized at 4°C. Aliquots were taken for an *in vitro* phosphorylation reaction, and proteins were separated by gel electrophoresis. The degree of 32 P incorporation from γ - 32 P-ATP by the PKC substrate protein F1 was used as a measure of PKC activity. This was

analyzed either by autoradiography or scintillation counting of bands cut from 1-dimensional gels.

Results

Experiment 1: selective blockade of LTP persistence by PMXB is dose dependent

When delivered 15 min before HFS, PMXB at doses lower than 50 pmol had no effect on initial potentiation. However, 45 min after HFS, decay of the potentiated response was observed (Fig. 1). The degree of decay increased with increasing doses of inhibitor within a narrow dose range. Thirteen pmol PMXB produced a 47% decay of LTP as compared to 19% decay following Tris ejections ($t = 180$ min, paired $t = 4.93$, $df = 3$, $p < 0.02$), whereas 25 pmol PMXB produced 100% decay (paired $t = 29.09$, $df = 3$, $p < 0.0001$). With ejections of 50 and 100 pmol PMXB, the initial potentiation was reduced: 45% with ejections of 50 pmol and 87% inhibition with ejections of 100 pmol PMXB [1-way analysis of variance (ANOVA): $F = 55.21$; $df = 4, 15$; $p < 0.001$]. This observation may be explained by the ability of PMXB at higher doses to inhibit CaM-dependent kinase (Mazzei et al., 1982). Inhibitors of CaM-dependent kinase have been shown to block initial potentiation (Reymann et al., 1988b).

The inhibition of initial potentiation by higher doses of PMXB cannot be explained by an effect of inhibitor on baseline responses. In the absence of HFS, synaptic responses elicited with low-frequency stimulation remained stable for 90 min after ejection of 25, 50, or 100 pmol PMXB (data not shown).

Experiment 2: selective blockage of LTP persistence by PMXB is time dependent

The time window in which PMXB inhibits LTP persistence was assessed using a dose of PMXB (25 pmol) that did not inhibit initial potentiation. PMXB ejected 15 min before or 15 or 30 min after HFS produced a decay of the potentiated response to near baseline values within 2–3 hr (Fig. 2). PMXB delivered 60 or 120 min after HFS showed no effects on LTP persistence as compared to Tris controls seen in Figure 1 (2-way ANOVA: $p > 0.20$). It was also noted that ejections of PMXB delivered 15 min before HFS produced a more rapid decay of potentiated responses as compared to ejections delivered 15 and 30 min after HFS (2-way ANOVA/drug \times time interaction: $F = 26.7$; $df = 12, 63$; $p < 0.001$).

Experiment 3: interaction of dose- and time-dependent effects of H-7 produces selective inhibition of LTP persistence

Another PKC inhibitor, H-7, was applied with similar time and ejection parameters as PMXB. PMXB inhibits PKC by competing with diacylglycerol for a site on the regulatory subunit (Mazzei et al., 1982), whereas H-7 acts on the catalytic subunit (Hidaka et al., 1984).

There was no effect of 50 or 250 pmol H-7 on baseline synaptic activation (data not shown) or initial potentiation (Fig. 3; 1-way ANOVA: $p > 0.20$). However, both 50 and 250 pmol H-7 delivered 15 min before HFS produced decay of potentiated responses to baseline values (Fig. 3; 2-way ANOVA/drug \times time interaction: $F = 4.7$ and 7.3 , respectively; $df = 30, 144$; $p < 0.001$). Fifty pmol H-7 was also effective in inhibiting the persistence of LTP when ejected 15 or 30 min after HFS (Fig. 3). Although H-7 was much less effective when ejected 30 min after HFS than when ejected at earlier time points, it still produced a significantly greater decay of LTP than ejections of Tris vehicle

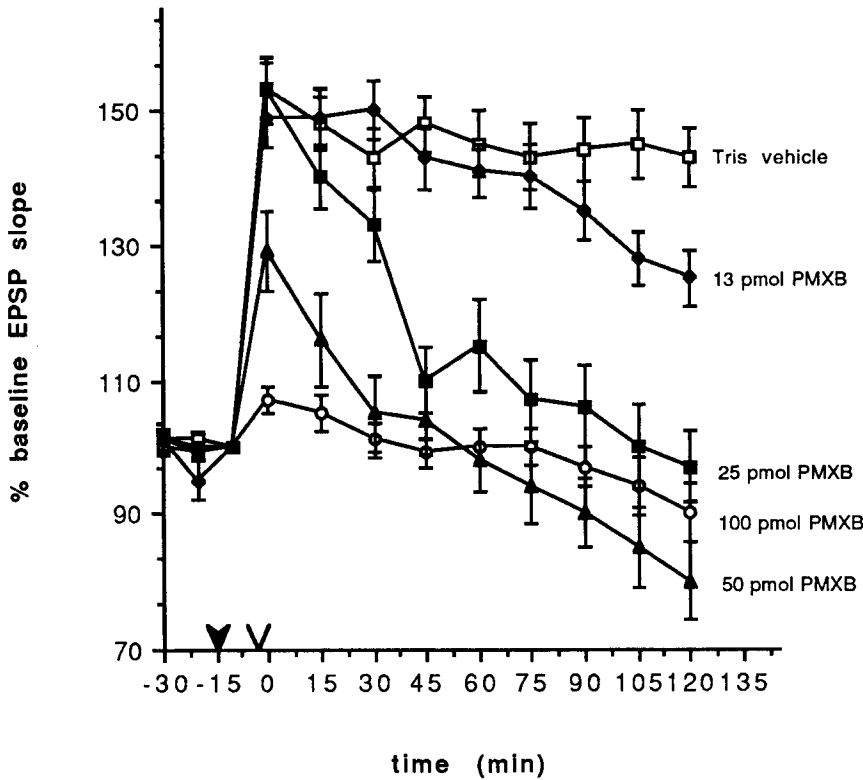


Figure 1. Dose-dependent effects of PMXB on LTP. Ejections (*thick arrowhead*) of PMXB 15 min before HFS (*thin arrowhead*) produced dose-dependent inhibition of LTP. Lower doses (13 and 25 pmol) inhibited the persistence of LTP but not initial potentiation, causing decay of LTP to baseline by 120 min after HFS (2-way ANOVA/drug \times time interaction of 25 pmol PMXB: $F = 3.9$; $df = 12,78$; $p < 0.001$). Higher doses (50 and 100 pmol) attenuated initial potentiation, as well ($N = 4$ per group). Error bars represent SEM values.

or the A kinase inhibitor H-8 (Fig. 3; 2-way ANOVA/drug effect: $F = 4.8$; $df = 2,108$; $p < 0.025$).

Ejections of 50 pmol H-7 given 60, 120, and 240 min after HFS had no effect on LTP persistence (Fig. 4; $p > 0.20$). However, 250 pmol H-7 delivered 240 min after HFS was capable of producing a decay of potentiated responses (Figs. 4, 5; 2-way

ANOVA: $F = 9.4$; $df = 3,288$; $p < 0.05$). A comparison of the rates of LTP decay following H-7 at different time points showed a significantly slower rate of decay following ejections of 250 pmol H-7 240 min after HFS than after low- and high-dose H-7 ejections at earlier time points (1-way ANOVA: $F = 5.11$; $df = 3,12$; $p < 0.05$; Fig. 6).

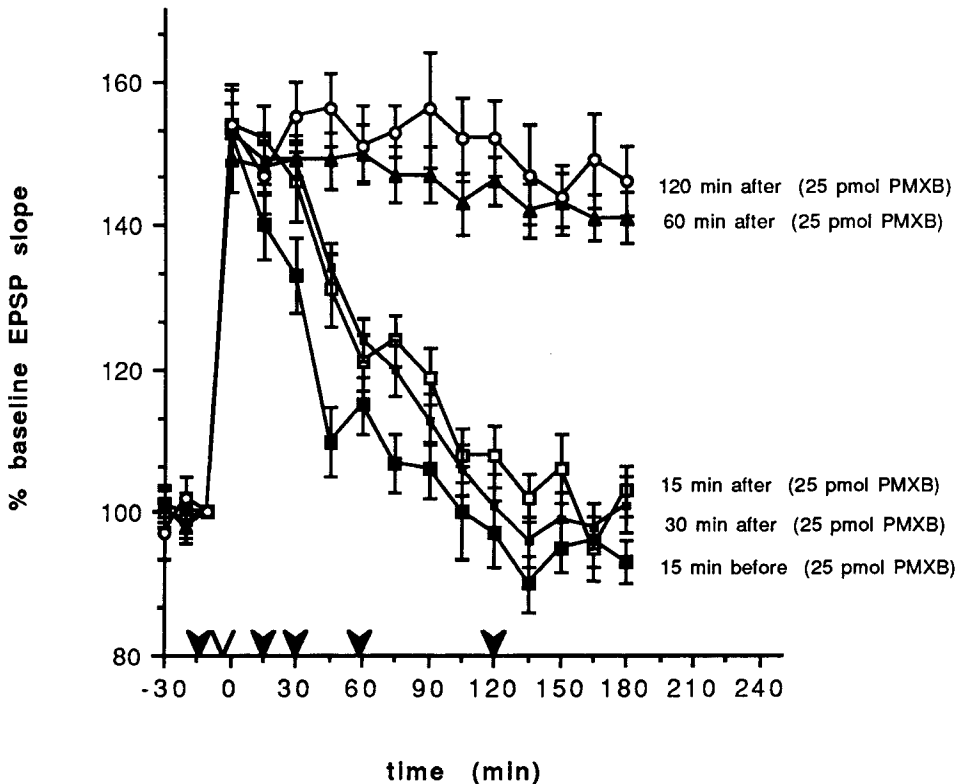


Figure 2. Time course of PMXB inhibition of LTP persistence. Ejections of 25 pmol PMXB (*thick arrowheads*) were effective in producing decay of the LTP response when delivered up to 30 min after LTP HFS (*thin arrowhead*). Ejections delivered 60 and 120 min after HFS, however, had no effect on LTP, as did the Tris controls in Figure 1 ($N = 4$ per group). Error bars represent SEM values.

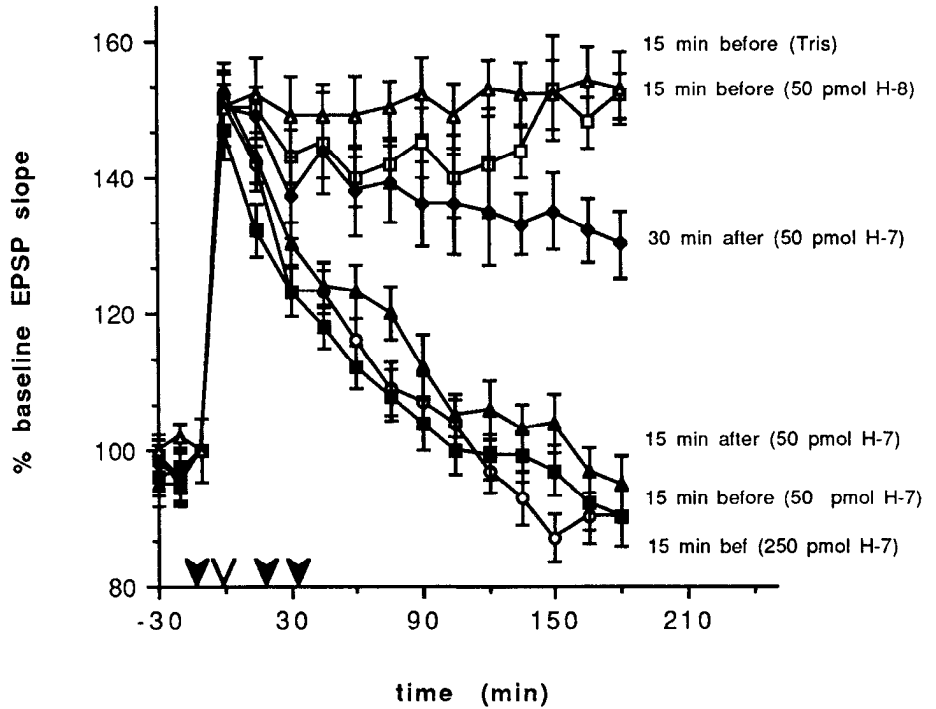


Figure 3. H-7, like PMXB, inhibits LTP persistence without affecting the initial potentiation. Ejections of H-7 (thick arrowheads) 15 min before and up to 30 min after HFS (thin arrowheads) significantly inhibited LTP persistence as compared to H-8 (a cAMP kinase inhibitor) or Tris controls ($N = 4$ per group). Neither 50 nor 250 pmol H-7 ejected before HFS had an effect on initial potentiation. Error bars represent SEM values.

To insure that the lack of inhibitory effect of low-dose ejections delivered 240 min after the induction of LTP was not due to loss of inhibitor potency with time in the micropipette, inhibitor solutions were kept at room temperature for 240 min before testing their effectiveness in inhibiting LTP. These drug solutions showed inhibition of LTP persistence similar to solutions used immediately after thawing (data not shown).

Experiment 4: In vitro phosphorylation of hippocampal protein F1 following inhibition of LTP persistence with PKC inhibitors

To determine whether the inhibition of LTP by PMXB or H-7 was related to an inhibition of PKC activity, we measured PKC substrate phosphorylation 30 min after treatment with PKC

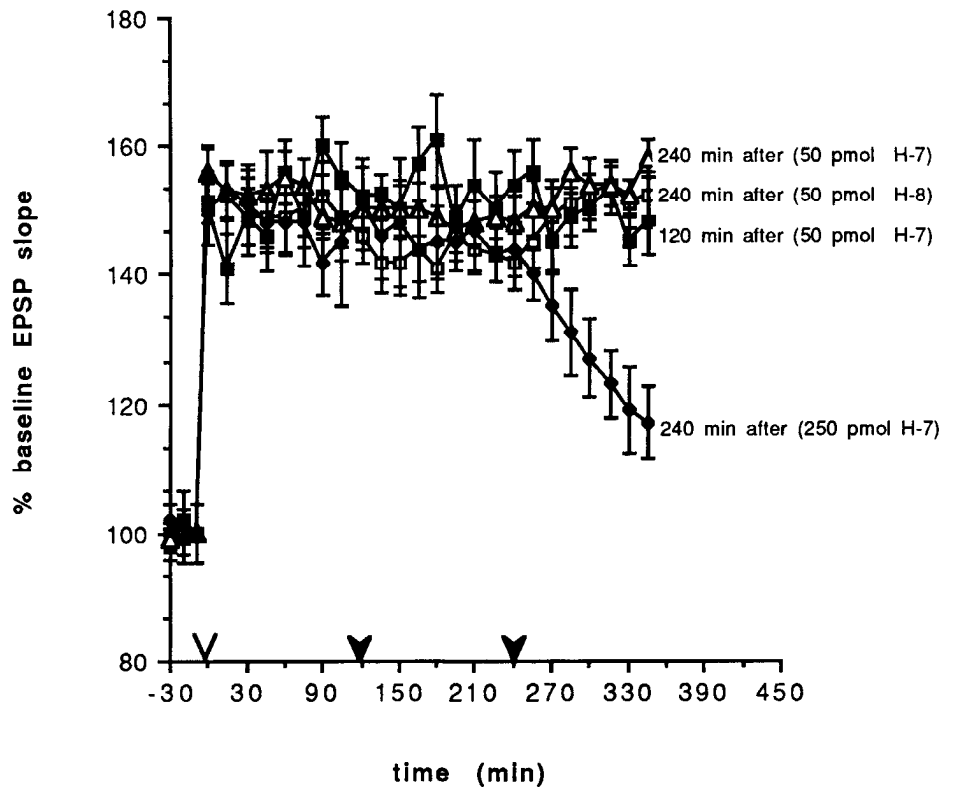


Figure 4. High dose of H-7 blocks late component of LTP. Ejections of 50 pmol (thick arrowheads) fail to inhibit LTP persistence when delivered 120 and 240 min after induction (thin arrowhead). Two-hundred fifty pmol H-7, however, delivered 240 min after HFS produced a significant decay of LTP over 120 min after ejection ($N = 4$ per group). Error bars represent SEM values.

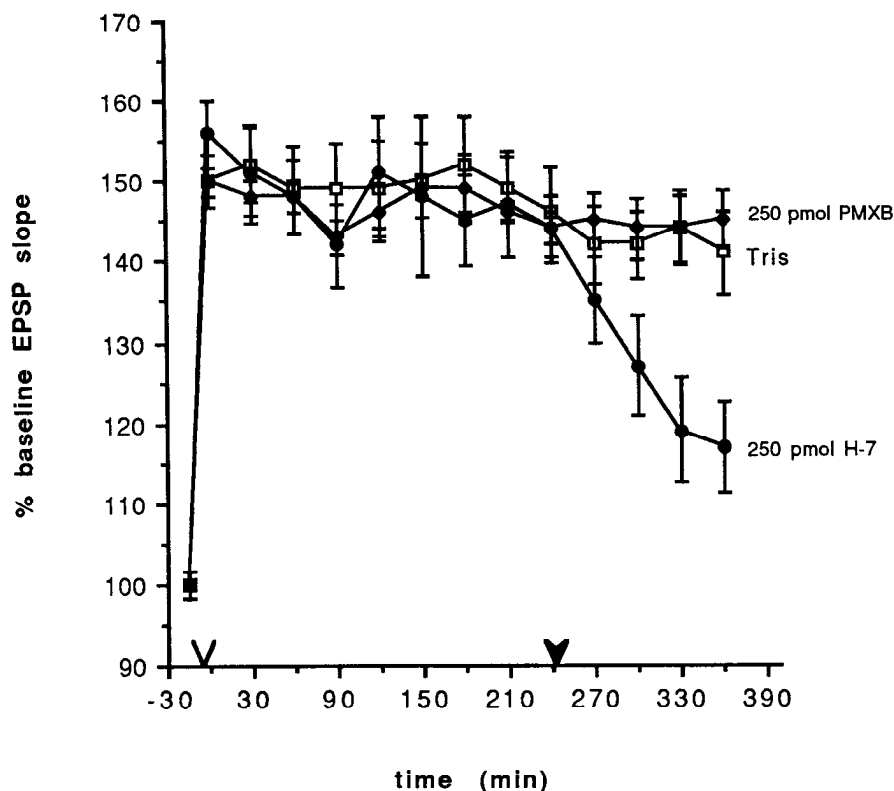


Figure 5. Unlike high dose (250 pmol) of H-7 (Fig. 4), PMXB (250 pmol) delivered 240 min (thick arrowhead) after HFS (thin arrowhead) failed to produce decay of LTP ($N = 4$ per group). Error bars represent SEM values.

inhibitors and HFS. To determine whether inhibitors ejected *in vivo* might be having their effect directly on the *in vitro* reaction rather than *in vivo*, PMXB was delivered in LTP animals immediately before death (*in vitro* phosphorylation controls).

Animals that received ejections of either H-7 or PMXB immediately before HFS showed a significant decrease in the *in vitro* phosphorylation of the PKC substrate protein F1 as com-

pared to LTP controls (1-way ANOVA: $F = 7.81$; $df = 1,10$; $p < 0.025$). Both LTP animals and *in vitro* phosphorylation controls showed greater levels of protein F1 phosphorylation as compared to animals that received only low-frequency stimulation (1-way ANOVA: $F = 8.71$ and 11.83 , $df = 1,6$; $p < 0.05$ and 0.025 , respectively), suggesting that the PKC inhibitors PMXB and H-7 are affecting an *in vivo* process rather than the

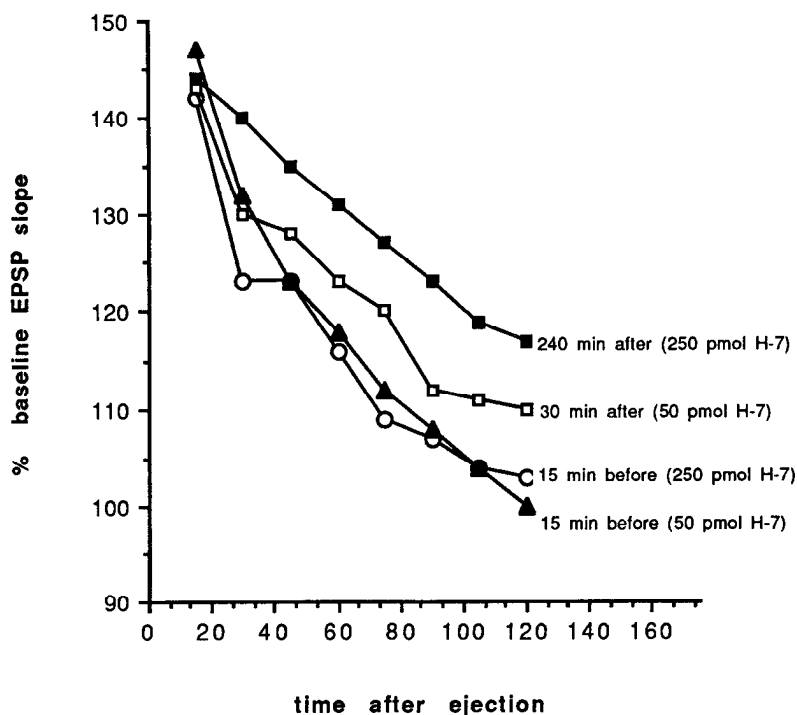


Figure 6. Comparison of decay rates following ejections of H-7 at different time points. The rate of decay following 250 pmol H-7 ejected 240 min after HFS is significantly slower than LTP decay following ejections 15 min before (50 and 250 pmol) or 30 min after HFS (1-way ANOVA comparing all groups: $F = 5.11$; $df = 3,12$; $p < 0.05$). Decay rate was calculated as the mean change in percent of baseline EPSP slope over each 15 min interval.

Table 1. Effect of PKC inhibitor ejections and LTP on *in vitro* phosphorylation of PKC substrate protein F1

Group	Number of hippocampi sampled	Protein F1 ^a (³² P incorporation cpm)	Percent baseline EPSP slope (after 30 min) ^b
Low-frequency control (0.1 Hz)	4	634.3 ± 17.4	101 ± 5.42
LTP alone	4	753.0 ± 36.4	149 ± 6.59
LTP plus PMXB	5	509.3 ± 97.4	104 ± 7.67
LTP plus H-7	4	480.0 ± 89.9	107 ± 4.76
<i>In vitro</i> phosphorylation control ^c	4	713.7 ± 15.2	137 ± 5.03

^a The percentage of ³²P incorporated into protein F1 was significantly greater in LTP and *in vitro* phosphorylation controls (see ^c below) than in animals that received PKC inhibitors 15 min prior to HFS or low-frequency controls (1-way ANOVA: $F = 15.82$ and 18.31 , respectively; $df = 3,12$; $p < 0.001$).

^b The degree of potentiation at 30 min after LTP induction was significantly greater in animals that received HFS alone than in animals that received either PMXB or H-7 prior to HFS (1-way ANOVA: $F = 10.82$ and 27.58 , respectively; $df = 1,7$; $p < 0.01$) and low-frequency controls (1-way ANOVA: $F = 31.99$; $df = 1,7$; $p < 0.001$).

^c *In vitro* phosphorylation control: LTP animals received ejections of PMXB immediately before death and, because this had no electrophysiological effect, were physiologically identical to LTP controls.

in vitro phosphorylation assay directly. This *in vivo* process is likely to be the PKC activation induced by LTP (Akers et al., 1986) because the alternative, that the *in vitro* phosphorylation assay is measuring an increase in vacant sites after LTP, would predict an increase in protein F1 phosphorylation after ejection of PKC inhibitors.

We tested whether PKC activity, as measured by F1 substrate phosphorylation, is related to the persistence of LTP 240 min after its induction. Animals receiving only low-frequency stimulation (0.1 Hz) or 400 Hz stimulation to produce LTP and monitored for 5 hr were used as controls. Experimental groups consisted of animals that received either 50 or 250 pmol H-7 180 min after HFS. Only the 250-pmol group showed significant decay of LTP over an additional 2 hr (Table 2).

LTP controls showed higher protein F1 phosphorylation 5 hr after HFS as compared to low-frequency controls (1-way ANOVA: $F = 9.97$; $df = 1,9$; $p < 0.05$; Table 2). In addition, protein F1 phosphorylation was lower in animals that received 250 pmol H-7 3 hr after HFS and showed decay of the LTP than in animals that received HFS alone (1-way ANOVA: $F = 8.05$; $df = 1,10$; $p < 0.05$; Table 2). Animals that received 50 pmol H-7 3 hr after HFS showed protein F1 phosphorylation levels intermediate to those seen in LTP controls and animals receiving 250 pmol H-7 3 hr after HFS.

Discussion

We conclude from these data that PKC activation is important for the persistence of LTP, but not for initial potentiation. As summarized in Table 3, it has previously been demonstrated that extracellular PKC inhibitors have no effect on initial potentiation, yet produce decay to baseline of LTP both in the intact hippocampus (Lovinger et al., 1987) and in the hippocampal slice preparation (Malinow et al., 1988; Reymann et al., 1988a,b). In the present study, a low dose of PMXB or a range of H-7 doses ejected prior to HFS also had no effect on initial

Table 2. *In vitro* phosphorylation of protein F1 5 hr after the induction of LTP: does H-7 delivered 3 hr after HFS inhibit PKC substrate phosphorylation?

Group	Number of hippocampi sampled	Protein F1 ^a (% total P ³² incorporation)	Percent baseline EPSP slope (after 5 hr)
0.1 Hz control	5	4 ± 0.41	98 ± 3.21
LTP alone	5	6 ± 0.47	140 ± 4.43
LTP plus 50 pmol H-7	4	6 ± 0.63	145 ± 3.80
LTP plus 250 pmol H-7	4	4 ± 0.25	127 ± 5.54

^a Animals that received 250 pmol H-7 3 hr after LTP induction to produce decay of LTP showed similar levels of protein F1 phosphorylation to those seen in low-frequency controls and significantly lower levels of protein F1 phosphorylation as compared to LTP controls (1-way ANOVA: $F = 8.05$; $df = 1,10$; $p < 0.05$).

potentiation, but produced a decay to baseline of the potentiated response.

It has been reported that intracellular injections of H-7 into CA1 pyramidal cells blocked LTP in hippocampal slices (Malinow et al., 1989). Although not discussed, their Figure 1 shows that intracellular injection of H-7 reduced initial potentiation of the intracellular response. Such an effect of H-7 was not observed in the present study.

Three possible explanations for the discrepancy may be suggested: (1) high doses of H-7 (200 nM) used by Malinow et al. (1989) may have nonselective effects, (2) the perforant path-granule cell synapses express a different set of PKC subtypes than CA1 pyramidal synapses, or (3) procedural differences such as *in vivo* versus *in vitro* preparation or intracellular versus extracellular drug application. The first explanation should be considered in view of a related study that showed that lower doses (20 nM) of H-7 intracellularly injected into CA1 cells *in vitro* had no effect on initial potentiation, but resulted in the total decay of LTP 30 min after HFS (Malenka et al., 1989).

Another conclusion we draw from the present data is that the persistence of LTP consists of 2 separable components, both of which may require PKC activation. Malinow et al. (1988), perfusing a 300- μ M solution of H-7 in a hippocampal slice preparation, showed that LTP expression could be inhibited with applications as late as 3 hr after induction. This late inhibition of LTP by H-7 is now proposed by Malinow et al. (1989) to be mediated by effects of H-7 at the presynaptic terminal because intracellular postsynaptic injection of H-7 into CA1 pyramidal cells blocks the induction of LTP but has no effect on established LTP. In the present study, a high dose of H-7 is effective in inhibiting LTP persistence when applied 240 min after HFS. Because the same dose does not affect responses elicited with low-frequency stimulation for 90 min after ejection, this dose is not nonspecifically altering synaptic transmission. Interestingly, the rate of LTP decay following 250 pmol H-7 240 min after LTP induction is significantly slower than decay following earlier H-7 ejections. These observations suggest that, at this later time point, H-7 is having an effect on a process that appears to be either more resistant to PKC inhibitors or at a distance from the site of inhibitor ejection.

Based on these later effects of H-7 on LTP persistence, we suggest that there are at least 3 mechanistically separable events. First, HFS triggers the voltage-dependent release of Mg²⁺ blockade of postsynaptic NMDA receptor-associated Ca²⁺ channels

Table 3. Summary of PKC inhibitor studies

Study	Preparation	Inhibitor(s)	Extracellular application ^a	Effect on LTP persistence ^b
Colley et al., this study	Dentate <i>in vivo</i>	PMXB, H-7	25–250-pmol ejections	inhibition up to 240 min
Lovinger et al., 1987	Dentate <i>in vivo</i>	PMXB, mellitin, H-7	50-pmol ejections	inhibition, but not at 240 min
Malinow et al., 1988, 1989	CA1 slice	H-7	300- μ M, 3–8-hr perfusion	inhibition up to 3 hr
Muller et al., 1988	CA1 slice	H-7	100- μ M, 20-min static bath	no inhibition
Reymann et al., 1988a	CA1 slice	PMXB	20- μ M, 60-min perfusion	inhibition

^a The parameters of inhibitor application include the dose of inhibitors used, the duration of application, and the method of application. Only studies investigating the effect of extracellular inhibitor application are included in the table for reasons of comparison.

^b References to time indicate the time after initiation of LTP at which inhibitors are still effective in producing decay of LTP.

allowing for a transient influx of Ca²⁺ (Collingridge, 1985). The resulting initial potentiation is mediated, at least in part, through activation of a CaM-dependent kinase (Popov et al., 1988; Reymann et al., 1988b). These events may regulate an increased sensitivity of quisqualate/kainate-type receptors after LTP (Collingridge and Lester, 1988; Davies et al., 1989).

Second, presynaptic PKC is activated, and subsequently, protein F1 is phosphorylated, possibly via a retrograde mechanism (Linden and Routtenberg, 1989). This event may regulate the persistence of potentiation for 5–60 min after HFS (Lovinger et al., 1987; Matthies, 1989).

Third, protein synthesis is initiated (>60 min after HFS) to provide a long-lasting synaptic enhancement (Krug et al., 1984; Stanton and Sarvey, 1984; Frey et al., 1988; Cole et al., 1989; Otani and Abraham, 1989). This protein-synthetic event mediating the long-term persistence of LTP may be regulated by PKC (Imagawa et al., 1987; Lee et al., 1987; Reymann et al., 1988a).

It is known that protein-synthetic events can be regulated by PKC- as well as cAMP-dependent processes. cAMP-dependent kinases have been shown to regulate the induction of RNA and protein synthesis of at least some proteins via nuclear protein phosphorylation (Imagawa et al., 1987; Yamamoto et al., 1988). A possible role of cAMP in the regulation of a later persistence phase of LTP is interesting in view of evidence that cAMP regulation of protein synthesis has been proposed in invertebrates to underlie other forms of long-term synaptic change (Goelet et al., 1986). However, our results show that the cyclic nucleotide inhibitor H-8 failed to have an effect on LTP persistence at a time point when a high dose of H-7 was effective in producing decay of LTP. This observation argues against a role of cAMP in the regulation of long-term persistence of LTP.

If the late component of LTP persistence is not regulated by cAMP, PKC activation may be required for protein synthetic events associated with LTP. This suggestion is consistent with the observed decrease in PKC substrate phosphorylation in animals that received 250 pmol H-7, a dose effective in producing decay of LTP 3 and 4 hr after its induction (Table 2).

The present data therefore suggest that the 2 components of LTP persistence may both be regulated by PKC activation, but by different mechanisms. We propose that the early component of LTP persistence, which in the present report is first measured 15 min after HFS, is mediated by translocation of PKC activity to the synaptic membrane and subsequent synaptic protein phosphorylation. The later component of LTP persistence (>60 min) may be mediated by PKC regulation of protein synthesis.

By what mechanism is persistent PKC maintained? Malinow et al. (1988) suggested that, at later time points, PKC activation may be maintained in the absence of activators, that is, cleavage of the catalytic from the regulatory subunit, or by long-lasting translocation of the kinase to the surface membrane. If the cleaved catalytic subunit of PKC is responsible for persistent PKC activity after LTP, only PKC inhibitors such as H-7 acting at the catalytic site should be effective. This prediction was confirmed in the present report.

PKC has been identified as a class of several closely related enzymes (Coussens et al., 1986; Knopf et al., 1986; Ono et al., 1987; Nishizuka, 1988) that can be differentiated on the basis of sensitivity to activators (Nishizuka, 1988; Sekiguchi et al., 1987), as well as regional (Brandt et al., 1987; Huang et al., 1987, 1988) and subcellular localization in the brain (Leach et al., 1989; for review, see Huang, 1989). The possibility exists, then, that these 2 phases of LTP persistence could be regulated by different PKC subtypes.

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* 231:374–378.
- Bliss TVP, Lomo T (1973) Long-lasting potentiation of synaptic transmission in the dentate area of the anesthetized rabbit following stimulation of the perforant path. *J Physiol (Lond)* 233:334–356.
- Brandt SJ, Niedel JE, Bell RM, Young WS III (1987) Distinct patterns of expression of different protein kinase C mRNAs in rat tissue. *Cell* 49:57–63.
- Cole AJ, Saffen DW, Baraban JM, Worley PF (1989) Rapid increase of an immediate early gene messenger RNA in hippocampal neurons by synaptic NMDA receptor activation. *Nature* 340:474–476.
- Colley PA, Sheu F-S, Routtenberg A (1989) Dose-dependent phorbol ester facilitation or blockade of hippocampal long-term potentiation: relation to membrane/cytosol distribution of protein kinase C activity. *Brain Res* 495:205–216.
- Collingridge GL (1985) Long-term potentiation in the hippocampus: mechanisms of initiation and modulation by neurotransmitters. *Trends Pharmacol Sci* 6:407–411.
- Collingridge GL, Lester RAJ (1988) The sensitivity of CA1 neurones to quisqualate following high-frequency stimulation in rat hippocampus *in vitro*. *J Physiol (Lond)* 398:22P.
- Coussens L, Parker PJ, Rhee L, Yang-Feng TL, Chen E, Waterfield MD, Francke U, Ullrich A (1986) Multiple distinct forms of bovine and human protein kinase C suggest diversity in cellular signalling pathways. *Science* 233:859–866.
- Davies SN, Lester RAJ, Reymann KG, Collingridge GL (1989) Temporally distinct pre- and post-synaptic mechanisms maintain long-term potentiation. *Nature* 338:500–503.
- Frey U, Krug M, Reymann K, Matthies H (1988) Anisomycin, a

- inhibitor of protein synthesis, blocks late phases of LTP phenomena in the hippocampal CA1 region *in vitro*. *Brain Res* 452:57–65.
- Goelet P, Castellucci VF, Schacter S, Kandel ER (1986) The long and the short of long-term memory—a molecular framework. *Nature* 322:419–422.
- Gustafsson B, Huang Y-Y, Wigstrom H (1988) Phorbol ester-induced synaptic potentiation differs from long-term potentiation in the guinea pig hippocampus *in vitro*. *Neurosci Lett* 85:77–81.
- Hannun YA, Loomis CR, Merrill AH Jr, Bell RM (1986) Sphingosine inhibition of protein kinase C activity and of phorbol dibutyrate binding *in vitro* and in human platelets. *J Biol Chem* 261:12604–12606.
- Hidaka H, Inagaki M, Kawamoto S, Sasaki Y (1984) Isoquinoline-sulfonamides, novel and potent inhibitors of cyclic nucleotide-dependent protein kinase and protein kinase C. *Biochemistry* 23:5036–5041.
- Huang FL, Yoshida Y, Nakabayashi H, Huang K-P (1987) Differential distribution of protein kinase C isozymes in the various regions of brain. *J Biol Chem* 262:15714–15720.
- Huang FL, Yoshida Y, Nakabayashi H, Young WS, Huang K-P (1988) Immunocytochemical localization of protein kinase C isozymes in rat brain. *J Neurosci* 8:4734–4744.
- Huang K-P (1989) The mechanism of protein kinase C activation. *TINS* 12:425–432.
- Imagawa M, Chiu R, Karin M (1987) Transcriptional factor AP-2 mediates induction by two different signal transduction pathways: protein kinase C and cAMP. *Cell* 51:251–260.
- Knopf JL, Lee M-H, Sultzman LA, Kriz RW, Loomis CR, Hewick RM, Bell RM (1986) Cloning and expression of multiple protein kinase C cDNAs. *Cell* 46:491–502.
- 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* 13:39–42.
- Leach KL, Powers EA, Ruff VA, Jaken S, Kaufmann S (1989) Type 3 protein kinase C localization to the nuclear envelope of phorbol ester-treated NIH 3T3 cells. *J Cell Biol* 109:685–695.
- Lee W, Mitchell P, Tijan R (1987) Purified transcription factor AP-1 interacts with TPA-inducible enhancer elements. *Cell* 49:741–752.
- Linden DJ, Routtenberg A (1989) The role of protein kinase C in long-term potentiation: a testable model. *Brain Res Rev* 14:279–296.
- Lovinger D, Akers F, Nelson R, Barnes C, McNaughton B, Routtenberg A (1985) Selective increase in hippocampal protein F1 phosphorylation directly related to three day growth of long-term enhancement. *Brain Res* 343:137–143.
- Lovinger DM, Colley PA, Akers RF, Nelson RB, Routtenberg A (1986) Direct relation of long-term synaptic potentiation to phosphorylation membrane protein F1, a substrate for membrane protein kinase C. *Brain Res* 399:205–211.
- Lovinger DM, Wong KL, Murakami K, Routtenberg A (1987) Protein kinase C inhibitors eliminate hippocampal long-term potentiation. *Brain Res* 436:177–183.
- Malenka RC, Madison DV, Nicoll RA (1986) Potentiation of synaptic transmission in the hippocampus by phorbol esters. *Nature* 321:175–177.
- Malenka RC, Kauer JA, Perkel DJ, Mauk MD, Kelly PT, Nicoll RA, Waxham MN (1989) An essential role for postsynaptic calmodulin and protein kinase activity in long-term potentiation. *Nature* 340:554–557.
- Malinow R, Madison DV, Tsien RW (1988) Persistent protein kinase activity underlying long-term potentiation. *Nature* 335:820–824.
- Malinow R, Schulman H, Tsien RW (1989) Inhibition of postsynaptic PKC or CaMKII blocks induction but not expression of LTP. *Science* 245:862–866.
- Matthies H (1989) In search of cellular mechanisms of memory. *Prog Neurobiol* 32:277–349.
- Mazzei GJ, Katch N, Kuo JF (1982) Polymyxin B is a more selective inhibitor for phospholipid-sensitive Ca²⁺-dependent protein kinase than for calmodulin-sensitive Ca²⁺-dependent protein kinase. *Biochem Biophys Res Comm* 109:1129–1133.
- McCrimmon DR, Feldman JL, Speck DF (1986) Respiratory motoneuronal activity is altered by injections of picomoles of glutamate into cat brainstem. *J Neurosci* 6:2384–2392.
- McNaughton BL (1982) Long-term synaptic enhancement and short-term potentiation in rat fascia dentata act through different mechanisms. *J Physiol* 324:249–262.
- Muller D, Turnbull J, Baudry M, Lynch G (1988) Phorbol ester-induced synaptic facilitation is different than long-term potentiation. *Proc Natl Acad Sci USA* 85:6997–7000.
- Nishizuka Y (1986) The role of protein kinase C in cell surface transduction and tumour promotion. *Nature* 308:693–697.
- Nishizuka Y (1988) The molecular heterogeneity of protein kinase C and its implication for cellular regulation. *Nature* 334:661–665.
- Ono Y, Fujii T, Ogita K, Kikkawa U, Igarashi K, Nishizuka Y (1987) Identification of three additional members of rat protein kinase C family: *delta*-, *epsilon*- and *zeta*-subspecies. *FEBS Lett* 266:125–128.
- Otani S, Abraham WC (1989) Inhibition of synthesis in the dentate gyrus, but not the entorhinal cortex, blocks maintenance of long-term potentiation in rats. *Neurosci Lett* 106:175–180.
- Popov NS, Reymann KG, Schulzeck K, Schulzeck S, and Matthies H (1988) Alterations in calmodulin content in fractions of rat hippocampal slices during tetanic- and calcium-induced long-term potentiation. *Brain Res Bull* 21:201–206.
- Reymann KG, Frey U, Jork R, Matthies H (1988a) 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.
- Reymann KG, Brodemann R, Kase H, Matthies H (1988b) Inhibitors of calmodulin and protein kinase C block different phases of hippocampal long-term potentiation. *Brain Res* 461:388–392.
- Routtenberg A (1985) Protein kinase C activation leading to protein F1 phosphorylation may regulate synaptic plasticity by presynaptic terminal growth. *Behav Neurol Biol* 44:186–200.
- Routtenberg A, Lovinger D, Steward O (1985) Selective increase in the phosphorylation of a 47kD protein (F1) directly related to long-term potentiation. *Behav Neurol Biol* 43:3–11.
- Routtenberg A, Colley P, Linden D, Lovinger D, Murakami K, Sheu F-S (1986) Phorbol ester promotes growth of synaptic plasticity. *Brain Res* 378:374–378.
- Sekiguchi K, Tsukuda M, Ogita K, Kikkawa U, Nishizuka Y (1987) Three distinct forms of rat brain protein kinase C: differential response to unsaturated fatty acids. *Biochem Biophys Res Comm* 145:797–802.
- 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.
- Yamamoto KK, Gonzalez GA, Biggs WH III, Montminy MR (1988) Phosphorylation-induced binding and transcriptional efficacy of nuclear factor CREB. *Nature* 334:494–498.