Journal of Physiology (1988), **400**, *pp.* 321–333
With 3 text-figures
Printed in Great Britain

SYNAPSE-SPECIFIC PROTEIN KINASE C ACTIVATION ENHANCES MAINTENANCE OF LONG-TERM POTENTIATION IN RAT HIPPOCAMPUS

BY DAVID M. LOVINGER* AND ARYEH ROUTTENBERG†

From the Cresap Neuroscience Laboratory, Northwestern University, Evanston, IL 60201, U.S.A.

(*Received 4 June 1987*)

SUMMARY

1. Protein kinase C (PKC) stimulators, 12-*O*-tetradecanoyl-phorbol-13-acetate (TPA) or *cis*-unsaturated fatty acid (UFA), have been shown to prolong synaptic enhancement induced by long-term potentiation (LTP). This observation suggests a role for PKC in the biochemical mechanisms underlying maintained enhancement.

2. To determine if PKC stimulators prolong LTP by acting selectively at synapses given high-frequency stimulation or by actions that are not synapse-specific (e.g. increased postsynaptic excitability) we examined the effect of TPA or UFA on input-selective enhancement. Population EPSPs, evoked in the same granule cell population by either the medial (MPP) or lateral (LPP) perforant path, can be selectively enhanced leaving the other perforant path input which receives only low-frequency stimulation as an internal control for PKC stimulator effects not specific to enhanced synapses.

3. Synapse-specific effects were in fact observed, as UFA or TPA selectively prolonged MPP enhancement following two trains of high-frequency MPP stimulation, without affecting responses evoked by the LPP. A similar synapse selectivity of PKC stimulator action was seen following high-frequency LPP stimulation.

4. These findings suggest that PKC stimulators prolong enhancement by acting specifically at high-frequency-stimulated synapses. PKC stimulators do not appear to affect either postsynaptic neurone excitability or synapses given only low-frequency stimulation. This provides further evidence that PKC acts synergistically with the consequences of repetitive synaptic activation to maintain enhancement.

INTRODUCTION

Phosphorylation of synaptic protein kinase C (PKC) substrates has been proposed as a key step in the molecular mechanisms which regulate synaptic plasticity (Routtenberg, 1984, 1985). Indeed, a growing body of evidence (Linden, Murakami

* Present address: NIAAA, 12501 Washington Avenue (DANAC 4), Rockville, MD 20852, U.S.A.

† To whom correspondence should be addressed.

& Routtenberg, 1986; Lynch & Bliss, 1986; Malenka, Madison & Nicoll, 1986; Routtenberg, Colley, Linden, Lovinger, Murakami & Sheu, 1986; Neary, 1987; Shapira, Silberberg, Ginsberg & Rahamimoff, 1987) has implicated PKC activity in plastic changes taking place at a number of synapses (see also Miller, 1986; Routtenberg, 1986; Baraban, 1987; Kaczmerak, 1987). Increases in both membrane PKC activity (Akers, Lovinger, Colley, Linden & Routtenberg, 1986) and phosphorylation of the PKC substrate protein F1 (Lovinger, Akers, Nelson, Barnes, McNaughton & Routtenberg, 1985; Lovinger, Colley, Akers, Nelson & Routtenberg, 1986; Schrama, De Graan, Wadman, Lopes da Silva & Gispen, 1986) have been observed in association with synaptic enhancement induced by high-frequency stimulation (long-term potentiation, LTP, Bliss & Lomo, 1973).

Prolongation of enhancement by intrahippocampal application of the PKC stimulators 12-*O*-tetradecanoyl-phorbol-13-acetate (TPA, Routtenberg *et al.* 1986) or *cis*-unsaturated fatty acid (UFA, Linden *et al.* 1986) has also been observed. The site of action of these compounds is not known. PKC stimulators might prolong synaptic enhancement by increasing postsynaptic cell excitability (e.g. increasing receptor availability or ion channel function). Conversely, these compounds might act specifically at synapses given high-frequency stimulation without altering the efficacy of other inputs to the same neurones. If enhancement were restricted to high-frequency stimulated synapses, as several studies suggest (Andersen, Sundberg, Sveen & Wigstrom, 1977; McNaughton & Barnes, 1977; Barrionuevo, Kelso, Johnston & Brown, 1986), then the latter hypothesis would likely be correct. Consistent with a synaptic site of PKC stimulatory action, we have observed that UFA application to the synaptic region of the dentate gyrus is more potent in prolonging enhancement than UFA application to the granule cell body region (Linden, Sheu, Murakami & Routtenberg, 1987*a*). Furthermore, UFAs do not increase the efficacy of inputs stimulated at low frequencies, suggesting a specificity of PKC stimulators both with respect to location and pattern of synaptic activation.

To test directly the hypothesis that PKC stimulators prolong potentiation by a synapse-specific action we took advantage of the fact that medial (MPP) and lateral (LPP) perforant path inputs to the same dentate gyrus granule cells can be activated and enhanced separately *in vivo* (McNaughton & Barnes, 1977; McNaughton, 1980; Abraham, Bliss & Goddard, 1985; White, Levy & Steward, 1986; Winson & Dahl, 1986). In the present report we determined the synapse selectivity of PKC stimulator action when high-frequency stimulation was given to either the MPP or the LPP while, in the same animal, the other pathway received only low-frequency, non-potentiating stimulation.

METHODS

Surgical preparation

Subjects were forty-four male 180–250 g Sprague–Dawley rats (Harlan Breeding Farms). Animals were anaesthetized by I.P. urethane (600–1000 mg/kg) and placed in the stereotaxic apparatus. Body temperature was maintained at ~ 37 °C using a water jacket heating pad (American Hospital Supply). Each animal received implants of two bipolar, twisted nichrome wire stimulating electrodes (tip diameter = 154 μm) inserted through holes in the skull over the left hemisphere. The first electrode was positioned to stimulate the LPP (co-ordinates = 0·5 mm posterior to lambda, 5·5 mm lateral to the mid-line, 4·0–5·0 mm ventral to the brain surface, skull level) by monitoring responses to single stimuli. This electrode was advanced ventrally until

observation of the sharp increase in population EPSP peak latency and half-width characteristic of the transition from MPP to LPP stimulation (McNaughton & Barnes, 1977). The second stimulating electrode was positioned to stimulate the MPP (0·5 mm anterior, 3·5–4·0 mm lateral, 3·0–3·5 mm ventral) by advancing the electrode until single pulses evoked a hilar response consisting of a negative-going population spike superimposed on a positive-going population EPSP.

Stimuli consisted of constant-voltage, monophasic, square-wave pulses of 0·1 ms duration. Stimulus pulses were delivered at 0·1 Hz at all times except during high-frequency stimulation used to induce LTP. All potentials were recorded using the micropipettes described below with reference to a stainless-steel screw implanted in the frontal skull. Potentials were amplified 460–680 × with half-amplitude filters at 10 Hz and 25 kHz.

In experiments examining TPA effects on enhancement a multibarrelled micropipette was placed in the dentate hilus (co-ordinates = 4·0 mm posterior to bregma, 2·0 mm lateral, 3·1–3·3 mm ventral). The pipette barrel used for ionophoresis contained 1·6 mM-TPA (LC Services) in 20% DMSO–20 mM-Tris (pH = 7·5), and the remaining barrels, one of which was used for recording field potentials, contained physiological saline. Figure 1*A* schematically illustrates the placement of stimulating and recording–ionophoresis electrodes. The micropipette barrel used for ionophoresis in control animals contained either physiological saline or a 2·4 mM solution of the inactive phorbol analogue 4-α-phorbol (LC Services) in DMSO–Tris vehicle.

In experiments examining enhancement following application of the UFA oleate both a multibarrelled micropipette for ionophoresis of oleate and a second micropipette for recording evoked field potentials were implanted. The multibarrelled pipette, with one barrel containing 5·2 mM-oleate in 20 mM-Tris (pH = 7·5) and the rest containing saline, was first placed in the dentate hilus. In control animals the pipette barrel used for ionophoresis contained 20 mM-Tris (pH = 7·5). Individual barrels of pipettes used in all experiments had tip diameters of ~ 1 μM.

After positioning the multibarrelled pipette and the stimulating electrodes the second micropipette was moved into the hilus at a site near the tip of the multibarrelled pipette. The proximity of the pipette tips was optimized such that MPP- or LPP-evoked population EPSPs recorded using either micropipette displayed a similar amplitude and latency to onset. Such placement of the single-barrelled pipette was achieved by advancing it posteroventrally at a 20 deg angle relative to the frontal plane with the pipette tip entering the brain 1·5–2·0 mm anterior to the entry point of the multibarrelled pipette. After all electrodes were in position the multibarrelled pipette was moved dorsally into the dentate molecular layer at a point midway between the maximum current sinks evoked by MPP and LPP stimulation.

Characterization of responses to medial and lateral perforant path stimulation

In each animal we ensured that the stimulating electrodes were correctly placed in the MPP and LPP, and that the synaptic populations activated by the two electrodes showed minimal overlap, using the criteria of McNaughton & Barnes (1977): first, laminar profile analysis, performed by moving the multibarrelled electrode dorsally over a 350 μm range through the dentate molecular layer, revealed a difference in the localization of the current sinks evoked by stimulation using the two different electrodes. Figure 1*B* illustrates typical responses to LPP and MPP stimulation recorded at different lamina in the molecular layer and hilus of the dentate gyrus dorsal blade. When potentials were analysed using the current source density technique two distinct current peaks were observed with the MPP peak falling 75–125 μm ventral to the LPP peak. Current source density analysis was performed using the Freeman & Nicholson (1975) procedure with current localized along an axis parallel to the granule cell dendrites at a lattice spacing of 25 μm. Their formula D_2 was used to minimize high-frequency spatial noise. Figure 1*C* shows a current source density profile which represents averaged data from ten subjects. To generate this averaged profile we first designated the depth at which the MPP peak was observed as the 0 point for each subject, and the other points were designated by their distance from 0. Current density at the 0 point and at given points dorsal and ventral to 0 were then averaged across animals for both MPP- and LPP-evoked responses.

Second, we observed that LPP-evoked population EPSPs exhibited a greater latency to peak and a greater half-width than population EPSPs evoked by MPP stimulation (see McNaughton & Barnes (1977) for further discussion of these measures).

Finally, following the delivery of high-frequency stimulation to one pathway no response

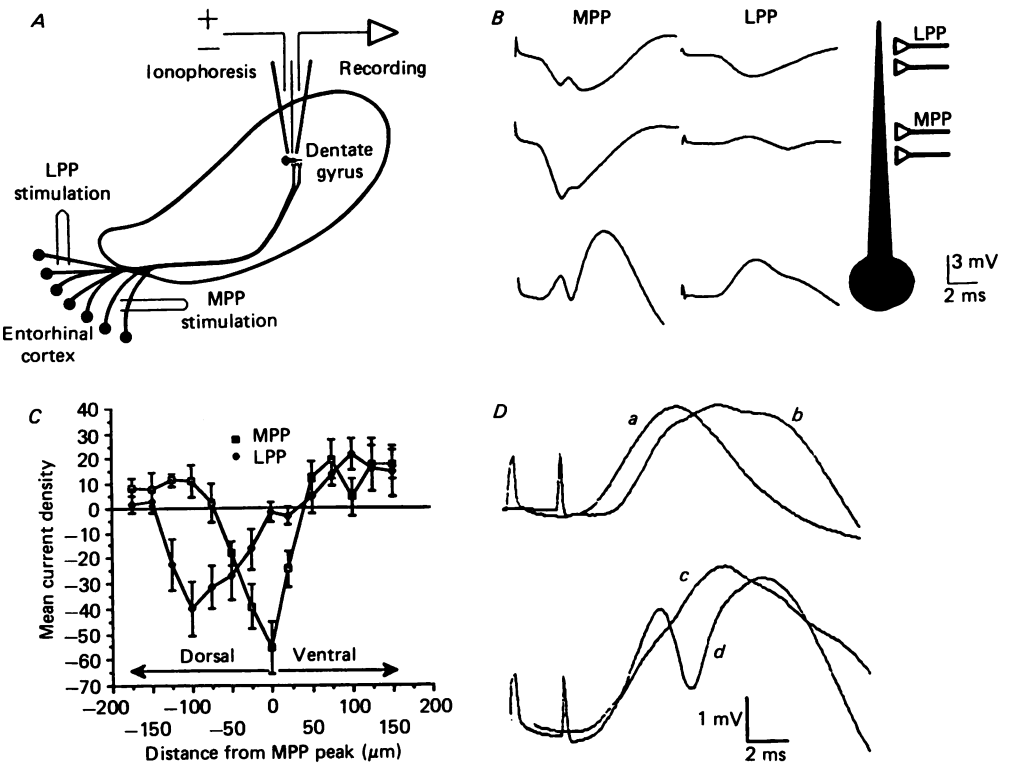

Fig. 1. Characterization of LPP- and MPP-evoked responses: lack of overlap of stimulated synapses, and convergence of inputs onto the same granule cell population. *A*, schematic illustration showing placement of electrodes for stimulation of the medial and lateral perforant path fibres. This Figure also illustrates the placement of the multibarrelled micropipette for ionophoresis and field potential recording in the dentate gyrus. In experiments examining oleate effects on potentiation a second recording pipette was placed near the cell bodies in the dentate gyrus. *B*, highly schematic representation of a dentate gyrus granule cell showing the termination site of LPP and MPP fibres on the outer and middle third of the dendritic arbor respectively. Representative averaged field potentials evoked by LPP and MPP stimulation and recorded in stratum moleculare at the level of the LPP or MPP terminal fields or in the dentate hilus near to the granule cell somata are also shown (positive-going potentials have an upward slope). *C*, this averaged current source density profile generated from potentials evoked by LPP and MPP stimulation in ten subjects demonstrates separation of influence of the stimulated pathways. Each point represents the mean ± S.E.M. current density observed at a given distance dorsal or ventral to the depth at which the peak of current density to MPP stimulation was observed (calculated as described in Methods). Note that the peak of current density evoked by LPP stimulation falls on average 100 μm dorsal to the MPP peak. Current source density analysis was performed as described in Methods. MPP and LPP stimuli performed in the same animals were alternated in a counterbalanced pattern which ensured that differences in current peak localization could not be attributed to effects of stimulus order. *D*, spatio-temporal summation of responses to LPP and MPP stimulation indicates convergence of inputs onto the same granule cell population. The two averaged field potentials shown at the top of this Figure are representative responses to LPP (*a*) and MPP (*b*) stimulation. The field potentials shown at the bottom are: (*c*) The computed linear sum of *a* and *b*; and (*d*) the actual response observed when the two pathways were activated with the LPP stimulus preceding the MPP stimulus by 2 ms. Note that the population spike amplitude with combined stimulation is much greater than the linear sum of the spikes produced by *a* and *b*, suggesting that these two pathways are acting in concert to activate the same set of postsynaptic cells.

enhancement was observed in the pathway which received only low-frequency stimulation. In some cases the low-frequency-stimulated pathway exhibited 'heterosynaptic depression' of responding (Dunwiddie & Lynch, 1978; Levy & Steward, 1979; Abraham *et al.* 1985). A response was considered to be depressed if the EPSP slope observed following high-frequency stimulation decreased to < 90% of its baseline level.

In each animal we ensured that the stimulated pathways converged to activate the same population of dentate gyrus granule cells by demonstrating spatio-temporal summation of responses when the LPP received a single stimulus pulse a few milliseconds prior to MPP stimulation. Figure 1*D* shows an example of such summation. Stimuli which produced a response below spike threshold when given to either the MPP or LPP alone summed to produce a large granule cell discharge when the two pathways were stimulated with LPP activation preceding MPP stimulation by 2 ms.

Experimental protocol

Over the first 20–30 min following electrode placement we often observed increases in responses to single, low-frequency (0·1 Hz) stimuli. Therefore all preparations were allowed to recover for at least 45 min until responses reached a stable level. We then initiated the experiment, recording baseline potentials evoked by single LPP and MPP stimuli. PKC stimulators or control compounds were then delivered by ionophoresis. In experiments examining TPA effects on enhancement, phorbol compounds were ejected into the dentate hilus using 38 nA of cathodal current for 15 min. It has previously been observed that ~ 32 pmol of [^{3}H]TPA is ejected *in vitro* using these parameters (Routtenberg *et al.* 1986). Responses were recorded for an additional 5 min after which enhancement was induced by the delivery of two high-frequency stimulus trains, 1 train/30 s consisting of eight, 0·1 ms duration, 10 V amplitude pulses (within-train frequency = 400 Hz) to either the LPP or the MPP. Responses to 0·1 Hz, single stimuli were recorded for 120 min following high-frequency stimulation. Each animal received only one ejection and high-frequency stimulation of only one perforant pathway.

The protocol for oleate experiments was similar to that just described with the following exceptions: first, oleate or Tris was ejected by 25 nA of cathodal current for 5 min. These parameters have been shown to result in ejection of ~ 193 pmol of [^{14}C]oleate (Linden *et al.* 1986*a*). Second, the interval between ejection and the onset of high-frequency stimulation was < 2·5 min. A 10 nA anodal backing current, which has been demonstrated to prevent leakage of TPA (Routtenberg *et al.* 1986) or oleate (Linden *et al.* 1986) was applied to all ionophoretic solutions except during the ejection periods described above.

Data analysis

Evoked potential waveforms were collected by an Isaac 2000 A/D conversion system (Cyborg Inc.) interfaced with an IBM PC-XT microcomputer (A/D conversion rate = 132 kHz). All waveforms used for analysis or presentation in Fig. 1 were averaged on-line using the responses to five individual stimulus pulses. Waveforms were stored on disc for analysis off-line. Synaptic efficacy was measured using the maximal slope of the population EPSP (Abraham *et al.* 1985).

Data were expressed as the percentage of baseline EPSP slope calculated using the formula:

$$(\text{Population EPSP slope at } t_x/\text{EPSP slope at } t_0) \times 100,$$

where t_0 equals a time point just before, and t_x is a given time point after potentiation. Two-way ANOVAs (analysis of variance) were used to compare the effect of compounds ejected and their interaction with time following potentiation in each experiment. In addition planned comparisons among treatment groups were run using the EPSP slope data at individual time points following LTP. The Fisher exact-probability test was used to compare the incidence of heterosynaptic depression across treatment groups. Paired *t* tests were run to compare EPSP slope before and after PKC stimulator ejection in individual animals. The criterion for H_0 rejection was $P < 0.05$ for all comparisons.

RESULTS

TPA selectively prolongs medial or lateral perforant path synaptic enhancement

Application of TPA to the dentate hilus prolonged enhancement induced by two trains of high-frequency MPP stimulation. All of the TPA-treated animals ($n = 4$)

showed prolonged increases in the MPP-evoked population EPSP slope (Fig. 2*A*, $F = 20{\cdot}83$, d.f. = 2·9, $P < 0{\cdot}001$) over a 120 min period following high-frequency stimulation relative to 4-α-phorbol ($n = 4$) or no ejection controls ($n = 4$).

In the unpotentiated LPP (Fig. 2*B*) no difference among treatment groups was observed in the slope of the population EPSP in the same animals exhibiting MPP enhancement ($F = 0{\cdot}003$, d.f. = 2·9, $P > 0{\cdot}25$). Thus TPA was without effect at low-frequency-stimulated synapses even when adjacent synaptic inputs were expressing prolonged enhancement. These data also reveal that the heterosynaptic depression of LPP-evoked population EPSPs sometimes observed following high-frequency stimulation of the MPP was not influenced by TPA. One animal in each treatment group exhibited depression. Thus the probability of occurrence of depression did not differ among treatment groups (Fisher test $P > 0{\cdot}10$). The population EPSP slope observed immediately after high-frequency stimulation in animals exhibiting depression ranged from 72 to 83% of baseline.

If TPA applied as in the above experiment were also accessing LPP synapses then this PKC stimulator should increase the maintenance of enhancement when high-frequency stimulation is restricted to the LPP. To test this prediction, a second group of animals ($n = 12$) was employed. We found that TPA increased the maintenance of enhancement following two trains of high-frequency LPP stimulation relative to 4-α-phorbol or no-ejection controls (Fig. 2*C*, $F = 4{\cdot}39$, d.f. = 2·9, $P < 0{\cdot}05$, $n = 4$ for all groups). In the non-potentiated MPP we observed no difference among treatment groups (Fig. 2*D*, $F = 1{\cdot}07$, d.f. = 2·9, $P > 0{\cdot}25$) in the population EPSP slope observed following high-frequency LPP stimulation. The incidence of heterosynaptic depression at MPP synapses did not differ among treatment groups (Fisher test $P > 0{\cdot}10$). Three of the four TPA-treated animals, and two of the four animals in each of the control groups showed depression. EPSP slopes immediately after potentiation ranged from 57 to 89% of baseline in animals showing depression. Thus, TPA produced a selective prolongation of LPP enhancement similar to that described above for the MPP.

Oleate selectively prolongs medial or lateral perforant path synaptic enhancement

We have recently demonstrated that UFAs, PKC stimulators (Murakami & Routtenberg, 1985; Murakami, Chan & Routtenberg, 1986), prolong enhancement in a manner similar to TPA (Linden *et al.* 1986, 1987*a*). We thus examined the synapse specificity of UFA effects using stimulation of the MPP and LPP. The UFA oleate was shown to be more potent when applied to the dentate molecular layer than when applied to the hilus (Linden *et al.* 1986). We ejected oleate in the molecular layer midway between the maximal current sinks evoked by MPP and LPP stimulation, as determined by laminar profile analysis, to allow the compound to access both LPP and MPP synapses. Since recording from another barrel at such an intermediate site would not allow us to obtain accurate measures of population EPSPs evoked by either pathway, we recorded responses using a second micropipette placed in the dentate hilus just ventral to the ejection site.

Oleate ejection in the molecular layer produced a selective prolongation of enhancement similar to that observed following hilar TPA ejection. Increased maintenance of MPP enhancement was observed in all six animals receiving oleate

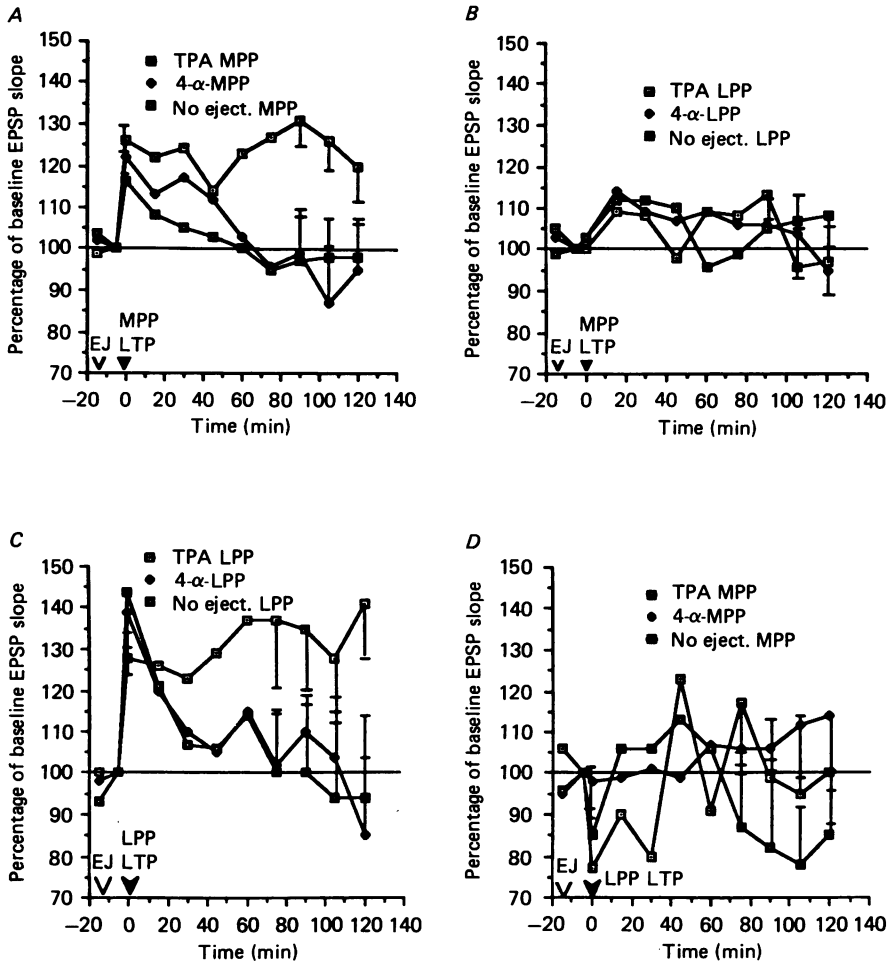

Fig 2. TPA selectively prolongs potentiation of either MPP or LPP synapses. *A*, time course of increases in MPP-evoked population EPSP slope induced by two trains of high-frequency stimulation in animals treated with TPA, 4-α-phorbol or given no ejection. Note that the initial increase does not differ among treatment groups, whereas significantly greater ($P < 0.05$ one-way ANOVA) potentiation is seen in the TPA-treated animals 90–120 min after high-frequency stimulation. $N = 4$ for all groups. Representative half-standard error of the mean values are shown by the error bars at 0 and 90–120 min after LTP. *B*, responses to LPP stimulation in the same animals shown in *A*. Responses did not differ from baseline in any of the treatment groups at any time point, and no significant difference among treatment groups in the percentage of baseline EPSP slope was observed at any time point. Error bars at 90–120 min after LTP show representative half-standard of the mean values. *C*, time course of EPSP slope potentiation of LPP synapses in TPA, 4-α-phorbol or no-ejection animals separate from the animals used for the experiment shown in *A*. The initial potentiation levels are not significantly different among treatment groups, but the TPA-treated animals show significantly greater potentiation ($P < 0.05$, one-way ANOVA *vs.* both control groups) 75, 90 and 120 min after high-frequency stimulation. $N = 4$ for all groups. Representative half-standard error of the mean values are given by the error bars at 0 and 75–120 min after LTP. *D*, responses to MPP stimulation in the same animals shown in *C*. As in *B*, no significant alterations in EPSP slope were observed, either among groups, or relative to baseline responding. Error bars at 0 and 75–120 min after LTP are representative half-standard error values.

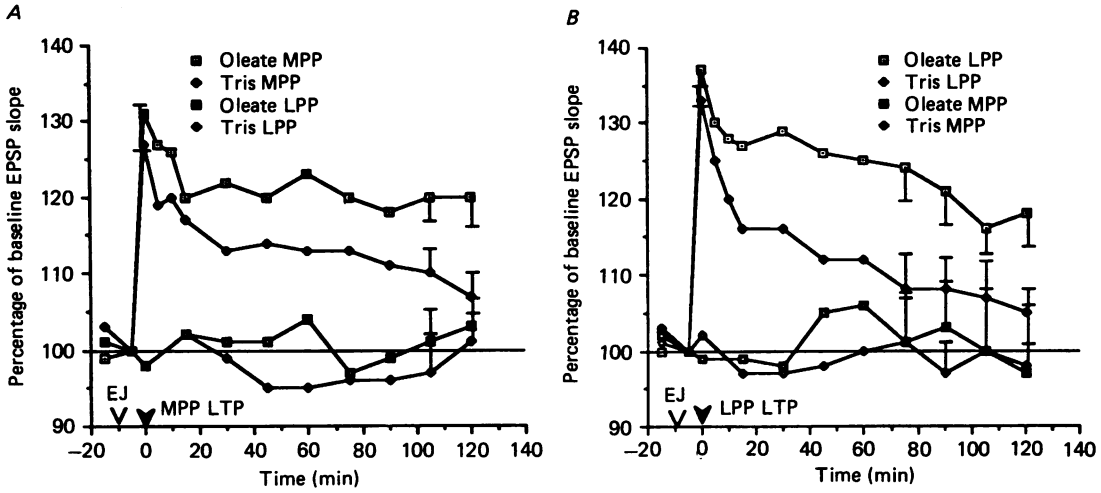

Fig. 3. Oleate selectively prolongs either MPP or LPP potentiation. *A*, time course of responses evoked by MPP or LPP stimulation following two trains of MPP stimulation in oleate- or Tris-treated animals. As observed with TPA, oleate treatment did not alter initial MPP potentiation. MPP-evoked responses in both treatment groups then showed a fast decay phase over the first 10 min after high-frequency stimulation. The oleate-treated animals showed greater maintenance of potentiation over the remainder of the 120 min observation period with significant differences ($P < 0{\cdot}05$, one-way ANOVA *vs.* Tris) at the 105 and 120 min time points. Consistent with the observations following TPA treatment (Fig. 1*B*) LPP-evoked responses showed no alterations following MPP potentiation. $N = 6$ for both oleate and Tris groups. Representative half-standard error of the mean values are shown by the error bars at 0, 105 and 120 min after LTP. *B*, responses evoked by LPP and MPP stimulation following two-train high-frequency stimulation given to the LPP in a second group of oleate- or Tris-treated animals. Consistent with the results observed using TPA, oleate treatment led to significantly greater LPP potentiation at later time points ($P < 0{\cdot}05$, one-way ANOVA *vs.* Tris 75–120 min after LTP), but did not alter initiation of potentiation. The unpotentiated MPP showed no changes in EPSP slope over the time course of the experiment. $N = 4$ for both treatment groups. Half-standard error of the mean values are shown by the error bars at 0 and 75–120 min after LTP.

ejection prior to two trains of high-frequency MPP stimulation relative to six Tris-vehicle-treated controls (Fig. 3*A*, $F = 22{\cdot}46$, d.f. = 1·10, $P < 0{\cdot}001$). Oleate treatment did not induce increases, or prolong depression of non-potentiated LPP-evoked population EPSPs (Fig. 3*A*, $F = 1{\cdot}392$, d.f. = 1·10, $P > 0{\cdot}25$). The incidence of heterosynaptic depression in the non-potentiated LPP was also unaffected by oleate. Immediately after high-frequency stimulation, two of the six animals in each treatment group exhibited depression (Fisher test $P > 0{\cdot}10$), with population EPSP slopes ranging from 79 to 82% of baseline. Thus oleate applied to the molecular layer produced a selective prolongation of enhancement when MPP synapses were given high-frequency stimulation.

To determine if selective prolongation of LPP enhancement could be obtained after UFA treatment (and to ensure that UFA was accessing LPP synapses) a separate experiment was carried out. Oleate application to the synaptic region prior to two-train high-frequency LPP activation also induced a selective prolongation of

enhancement. Four animals given oleate treatment showed increased maintenance of LPP enhancement over the 120 min observation period following LTP relative to four Tris-treated controls (Fig. 3*B*, $F = 31{\cdot}87$, d.f. = 1·6, $P < 0{\cdot}005$). Population EPSPs evoked by low-frequency MPP stimulation were not altered by oleate treatment (Fig. 3*B*, $F = 0{\cdot}806$, d.f. = 1·6, $P > 0{\cdot}25$). Oleate treatment also did not increase the incidence of MPP heterosynaptic depression (Fisher test $P > 0{\cdot}10$). Depression to 88% of baseline was observed in one of the oleate-treated animals and none of the Tris-treated animals.

Application of TPA or oleate did not alter baseline responses to stimulation of either pathway prior to LTP in any of the experiments described (assessed by paired *t* tests on the differences between pre- and post-ejection responses, $P > 0{\cdot}20$ for all tests). This observation is consistent with our previous demonstration that TPA (Colley & Routtenberg, 1986) or oleate (Linden *et al.* 1987*a*) applied as in the present study did not induce potentiation in the absence of high-frequency stimulation even when responses were observed for 120 min after PKC stimulator treatment. Initial levels of enhancement ($t = 0$, Figs 2 and 3) were also not affected by TPA and oleate application in the above experiments. This observation agrees with earlier reports (Routtenberg *et al.* 1986; Linden *et al.* 1986), which indicated that PKC stimulators applied as in the present study do not alter the initial magnitude of enhancement.

DISCUSSION

The present data suggest that PKC stimulators enhance the durability of potentiation by acting at perforant path synapses, and that these actions are specific to synapses undergoing LTP. Neither PKC stimulators alone or two trains of high-frequency stimulation in the absence of PKC activation was sufficient to produce enhancement of 2 h duration. However, prolonged enhancement resulted from combined PKC stimulator treatment and high-frequency stimulation. These findings support the hypothesis that PKC activation in synergism with molecular events which are brought about by high-frequency stimulation prolongs synaptic enhancement (Routtenberg, 1986). That the prolonged synaptic enhancement induced by the synergistic action of PKC activators and brief high-frequency stimulation may occur via the same mechanism as produced with a greater number of high-frequency stimuli is supported by the recent observation that phorbol ester-induced prolongation of enhancement occludes response increases produced by subsequent high-frequency stimulation (Colley & Routtenberg, 1987).

It has previously been suggested that LTP consists of both fast- and slow-decaying components (Racine, Milgram & Hafner, 1983). In this view, the two trains of high-frequency stimulation given in the present study might induce only the fast-decaying component, while the function of PKC activation may be critical for the longer-lasting phase of enhancement.

PKC stimulators might act at the presynaptic terminal since PKC is localized presynaptically in the dentate gyrus (Girard, Mazzei, Word & Kuo, 1985; Worley, Baraban, De Souza & Snyder, 1986). One mechanism of PKC action that we have previously discussed involves presynaptic terminal growth following LTP (Routtenberg, 1985). The recent reports that protein F1 (B-50; Gispen, De Graan,

Chan & Routtenberg, 1986), a PKC substrate related to enhancement (Routtenberg, Lovinger & Steward, 1985), is present in presynaptic terminals (Gispen, Leunissen, Oestreicher, Verkliej & Zwiers, 1985), and is probably identical to the growth-related axonal proteins GAP-43 (Snipes, Freeman, Costello, Chan & Routtenberg, 1987), pp46 (Nelson, Routtenberg, Hyman & Pfenninger, 1985), and GAP-48 (Benowitz & Routtenberg, 1987) suggest a role for this PKC substrate in presynaptic terminal growth. PKC-mediated phosphorylation may also regulate changes in transmitter release following LTP (Lynch & Bliss, 1986; see also Shapira *et al.* 1987; Malenka, Ayoub & Nicoll, 1987).

The lack of PKC stimulator action at unpotentiated synapses could result from selective access of PKC stimulators to LPP or MPP synapses, or selective localization of target molecules for stimulator action at one set of synapses. If either of these explanations were correct then PKC stimulators should prolong enhancement following high-frequency stimulation at one set of synapses, but should not affect enhancement following high-frequency stimulation of the other pathway. This appears not to be the case as PKC stimulators selectively prolonged enhancement at high-frequency-stimulated synapses, whether LPP or MPP.

The recent demonstration that phorbol ester bath-applied to the hippocampal slice in the absence of high-frequency stimulation can induce lasting increases in responses at Schaffer collateral–CA1 synapses (Malenka *et al.* 1986) is at variance with the hypothesis that PKC activation alone is not sufficient to initiate enhancement. However, the difference in the findings might result from differences in PKC distribution in the dentate gyrus relative to field CA1 (Worley *et al.* 1986). Alternatively this apparent discrepancy might reflect non-specific effects of PKC stimulators bath-applied at high concentrations (10 μM) to the hippocampal slice compared to the effects of local application of small amounts (32 pmol, Routtenberg *et al.* 1986) of phorbol ester. High concentrations of phorbol ester can activate PKC-independent processes (see Nishizuka, 1986), suggesting that the initiation of response enhancement in the slice might result from non-specific effects of these compounds.

The effects of phorbol ester applied to the molecular layer of the dentate gyrus has been the subject of a study recently completed in our laboratory (Colley & Routtenberg, 1987). We demonstrated that phorbol ester ejected into the molecular layer synergistically prolongs enhancement at doses considerably lower (6 pmol for molecular layer application *vs.* 32 pmol for hilar application) than the doses needed for such action with hilar ejection. We anticipate that prolongation following molecular layer ejection will show a synapse specificity similar to that observed in the present study.

While PKC may not be involved in the initiation of enhancement, activation of the NMDA class of amino acid receptor appears to be necessary for this process at Schaffer collateral–CA1 (cf. Harris, Ganong & Cotman, 1984; Collingridge, 1985), and perforant path–dentate gyrus (Errington, Lynch & Bliss, 1987) synapses. A link between NMDA receptors and PKC is suggested by the observation that NMDA receptor blockade does not prevent enhancement at mossy fibre–CA3 synapses (Harris & Cotman, 1986), and that the mossy fibres contain little if any PKC (Worley *et al.* 1986). The recent observation (Linden, Sheu & Routtenberg, 1987*b*) that NMDA

receptor blockade prevents the LTP-induced increase in protein F1 phosphorylation, previously related to maintenance of enhancement, provides further evidence for this link. It will be of interest to determine if PKC (which is present in pyramidal cell bodies, Worley *et al.* 1986) plays a role in the maintenance of potentiation at these synapses.

We also observed that PKC stimulators did not affect the incidence of heterosynaptic depression of non-potentiated synapses, or the duration of depression in those cases in which it occurred. This finding indicates that PKC activation does not play a role in the onset or maintenance of such depression. This observation further suggests that the increases in membrane PKC activity (Akers *et al.* 1986) and protein F1 phosphorylation (Routtenberg *et al.* 1985) following high-frequency stimulation are more likely to be related to homosynaptic enhancement than to heterosynaptic depression. While PKC is not related to heterosynaptic depression in the perforant path–dentate gyrus system it has been implicated in homosynaptic depression observed in the dorsal root ganglion (Shapira *et al.* 1987).

We gratefully acknowledge the valuable suggestions of Dr Bruce L. McNaughton on an earlier version of this manuscript. We also thank David Linden, Dr Robert Nelson, Dr Oswald Steward and Dr Geoff White for helpful advice. Invaluable technical assistance was provided by Laura Maurer, Peter Naumann and Hope Robinson. This work was supported by MH 25281-13 and AFOSR87-0042 to A.R.

REFERENCES

ABRAHAM, W. C., BLISS, T. V. P. & GODDARD, G. V. (1985). Heterosynaptic changes accompany long-term but not short-term potentiation of the perforant path in the anaesthetized rat. *Journal of Physiology* **363**, 335–349.

AKERS, R. F., LOVINGER, D. M., COLLEY, P. A., LINDEN, D. J. & ROUTTENBERG, A. (1986). Translocation of protein kinase C activity may mediate hippocampal long term potentiation. *Science* **231**, 587–589.

ANDERSEN, P., SUNDBERG, S. H., SVEEN, O. & WIGSTROM, H. (1977). Specific long-lasting potentiation of synaptic transmission in hippocampal slices. *Nature* **266**, 736–737.

BARABAN, J. M. (1987). Phorbol esters: probes of protein kinase C activity in the brain. *Trends in Neurosciences* **10**, 57–59.

BARRIONUEVO, G., KELSO, S. R., JOHNSTON, D. & BROWN T. H. (1986). Conductance mechanism responsible for long-term potentiation in monosynaptic and isolated excitatory synaptic inputs to hippocampus. *Journal of Neurophysiology* **55** (5), 540–550.

BENOWITZ, L. I. & ROUTTENBERG, A. (1987). A membrane phosphoprotein associated with neural development axonal regeneration, phospholipid metabolism, and synaptic plasticity. *Trends in Neurosciences* **10** (12), 527–531.

BLISS, T. V. P. & LOMO, T. (1973). Long-lasting potentiation of synaptic transmission in the dentate area of the anesthetized rabbit following stimulation of the perforant path. *Journal of Physiology* **232**, 357–374.

COLLEY, P. A. & ROUTTENBERG, A. (1986). Hypothesis: Protein kinase C activation acts synergistically with a calcium-mediated event to induce long-lasting synaptic changes in the hippocampus. *Society for Neuroscience Abstracts* **12**, 1168.

COLLEY, P. A. & ROUTTENBERG, A. (1987). Induction, enhancement and blockade of hippocampal synaptic potentiation: dose-dependent effects of phorbol 12,13-dibutyrate (PDBu). *Society for Neuroscience Abstracts* **13**, 1232.

COLLINGRIDGE, G. L. (1985). Long term potentiation in the hippocampus: mechanisms of initiation and modulation by neurotransmitters. *Trends in Pharmacological Science* **6**, 407–411.

DUNWIDDIE, T. & LYNCH, G. (1978). Long-term potentiation and depression of synaptic responses in the rat hippocampus: Localization and frequency dependency. *Journal of Physiology* **276**, 353–367.

ERRINGTON, M. L., LYNCH, M. A. & BLISS, T. V. P. (1987). Long-term potentiation in the dentate gyrus: induction and increased glutamate release are blocked by D(–) aminophosphonovalerate. *Neuroscience* **20** (1), 279–284.

FREEMAN, J. A. & NICHOLSON, C. (1975). Experimental optimization of current source-density technique for anuran cerebellum. *Journal of Neurophysiology* **38**, 369–382.

GIRARD, P. R., MAZZEI, G. J., WORD, J. G. & KUO, J. F. (1985). Polyclonal antibodies to phospholipid/Ca^{2+}-dependent protein kinase and immunocytochemical localization of the enzyme in rat brain. *Proceedings of the National Academy of Sciences of the U.S.A.* **82**, 3030–3034.

GISPEN, W. H., DE GRAAN, P. N. E., CHAN, S. Y. & ROUTTENBERG, A. (1986). Comparison between the neural acidic proteins B-50 and F1. In *Phosphoproteins in Neuronal Function, Progress Brain Research*, vol. 69, ed. GISPEN, W. H. & ROUTTENBERG, A., pp. 383–386. Amsterdam: Elsevier.

GISPEN, W. H., LEUNISSEN, J. L. M., OESTREICHER, A. B., VERKLIEJ, A. J. & ZWIERS, H. (1985). Presynaptic localization of B-50 phosphoprotein: the ACTH-sensitive protein kinase substrate involved in rat brain polyphosphoinositide metabolism. *Brain Research* **328**, 199–413.

HARRIS, E. C. & COTMAN, C. W. (1986). Long-term potentiation of guinea pig mossy fiber responses is not blocked by *N*-methyl-D-aspartate antagonists. *Neuroscience Letters* **70** (1), 132–137.

HARRIS, E. C., GANONG, A. H. & COTMAN, C. W. (1984). Long-term potentiation in the hippocampus involves activation of *N*-methyl-D-aspartate receptors. *Brain Research* **323**, 132–137.

KACZMERAK, L. K. (1987). The role of protein kinase C in the regulation of ion channels and neurotransmitter release. *Trends in Neurosciences* **10** (1), 30–33.

LEVY, W. B. & STEWARD, O. (1979). Synapses as associative memory elements in the hippocampal formation. *Brain Research* **175**, 233–245.

LINDEN, D. J., MURAKAMI, K. & ROUTTENBERG, A. (1986). A newly discovered protein kinase C activator (oleic acid) enhances long-term potentiation in the intact hippocampus. *Brain Research* **379**, 358–363.

LINDEN, D. J., SHEU, F.-S., MURAKAMI, K. & ROUTTENBERG, A. (1987*a*). Enhancement of long-term potentiation by cis-unsaturated fatty acid: relation to protein kinase C and phospholipase A_2. *Journal of Neuroscience* **7**, 3783–3792.

LINDEN, D. J., SHEU, F.-S. & ROUTTENBERG, A. (1987*b*). DL-Aminophosphonovalerate (APV) blockade of hippocampal long-term potentiation (LTP) prevents an LTP-associated increase in protein F1 phosphorylation. *Society for Neuroscience Abstracts* **13**, 1232.

LOVINGER, D. M., AKERS, R. F., NELSON, R. B., BARNES, C. A., MCNAUGHTON, B. L. & ROUTTENBERG, A. (1985). A selective increase in the phosphorylation of protein F1, a protein kinase C substrate, directly related to three day growth of long term synaptic enhancement. *Brain Research* **343**, 137–143.

LOVINGER, D. M., COLLEY, P. A., AKERS, R. F., NELSON, R. B. & ROUTTENBERG, A. (1986). Direct relation of long-term synaptic potentiation to phosphorylation of membrane protein F1: A substrate for membrane protein kinase C. *Brain Research* **399**, 205–211.

LYNCH, M. A. & BLISS, T. V. P. (1986). Long-term potentiation of synaptic transmission in the hippocampus of the rat; effect of calmodulin and oleoyl-acetyl-glycerol on release of [^{3}H] glutamate. *Neuroscience Letters* **65**, 171–176.

MALENKA, R. C., AYOUB, G. S. & NICOLL, R. A. (1987). Phorbol esters enhance transmitter release in rat hippocampal slices. *Brain Research* **403**, 198–203.

MALENKA, R. C., MADISON, D. V. & NICOLL, R. A. (1986). Potentiation of synaptic transmission in the hippocampus by phorbol esters. *Nature* **321**, 175–177.

MCNAUGHTON, B. L. (1980). Evidence for two physiologically distinct perforant pathways to the fascia dentata. *Brain Research* **199**, 1–19.

MCNAUGHTON, B. L. & BARNES, C. A. (1977). Physiological identification and analysis of dentate granule cell responses to stimulation of the medial and lateral perforant pathways in the rat. *Journal of Comparative Neurology* **175** (4), 439–453.

MILLER, R. J. (1986). Protein kinase C: a key regulator of neuronal excitability. *Trends in Neurosciences* **9**, 537–538.

MURAKAMI, K., CHAN, S. Y. & ROUTTENBERG, A. (1986). Protein kinase C activation by *cis*-fatty acid in the absence of Ca^{2+} and phospholipids. *Journal of Biological Chemistry* **231** (33), 15424–15429.

MURAKAMI, K. & ROUTTENBERG, A. (1985). Direct activation of purified protein kinase C by unsaturated fatty acid in the absence of phospholipids and Ca^{2+}. *FEBS Letters* **192**, 189–193.

NEARY, J. T. (1987). Modulation of ion channels by Ca^{2+}-activated protein phosphorylation: a biochemical mechanism of associative learning. In *Phosphoproteins in Neuronal Function, Progress in Brain Research*, vol. 69, ed. GISPEN, W. H. & ROUTTENBERG, A., pp. 91–106. Amsterdam: Elsevier.

NELSON, R. B. & ROUTTENBERG, A. (1985). Characterization of the 47 kD protein F1 (pI 4·5), a kinase C substrate directly related to neural plasticity. *Experimental Neurology* **89**, 213–224.

NELSON, R. B., ROUTTENBERG, A., HYMAN, C. & PFENNINGER, K. H. (1985). A phosphoprotein, F1, directly related to neuronal plasticity in adult rat brain may be identical to a major growth cone membrane protein. *Society for Neuroscience Abstracts* **11**, 927.

NISHIZUKA, Y. (1986). Studies and perspectives of protein kinase C. *Science* **233**, 305–312.

RACINE, R. J., MILGRAM, N. W. & HAFNER, S. (1983). Long-term potentiation phenomema in the rat limbic forebrain. *Brain Research* **260**, 217–245.

ROUTTENBERG, A. (1984). Brain phosphoproteins, kinase C, and protein F1: Protagonists of plasticity in particular pathways. In *Neurobiology of Learning and Memory*, ed. LYNCH, G., MCGAUGH, J. & WEINBERGER, N., pp. 479–490. New York: Guilford Press.

ROUTTENBERG, A. (1985). Protein kinase C activation leading to protein F1 phosphorylation may regulate synaptic plasticity by presynaptic terminal growth. *Behavioral and Neural Biology* **44**, 186–200.

ROUTTENBERG, A. (1986). Synaptic plasticity and protein kinase C. In *Phosphoproteins in Neuronal Function, Progress in Brain Research*, vol. 69, ed. GISPEN, W. H. & ROUTTENBERG, A., pp. 411–435. Amsterdam: Elsevier.

ROUTTENBERG, A., COLLEY, P. A., LINDEN, D. J., LOVINGER, D., MURAKAMI, K. & SHEU, F.-S. (1986). Phorbol ester promotes growth of synaptic plasticity. *Brain Research* **378**, 374–378.

ROUTTENBERG, A., LOVINGER, D. & STEWARD, O. (1985). Selective increase in the phosphorylation of a 47kD protein (F1) directly related to long-term potentiation. *Behavioral and Neural Biology* **43**, 3–11.

SCHRAMA, L., DE GRAAN, P. N. E., WADMAN, W. J., LOPES DA SILVA, F. H. & GISPEN W. H. (1986). Long-term potentiation and 4-aminopyridine-induced changes in protein and lipid phosphorylation in the hippocampal slice. In *Phosphoproteins in Neuronal Function, Progress in Brain Research*, vol. 69, ed. GISPEN, W. H. & ROUTTENBERG, A., pp. 245–258. Amsterdam: Elsevier.

SHAPIRA, R., SILBERBERG, S. D., GINSBERG, S. & RAHAMIMOFF, R. (1987). Activation of protein kinase C augments evoked transmitter release. *Nature* **325**, 58–60.

SNIPES, J., FREEMAN, J. A., COSTELLO, B., CHAN, S. Y. & ROUTTENBERG, A. (1987). A growth associated protein, GAP43, is immunologically and structurally related to the plasticity associated protein, protein F1. *Journal of Neuroscience* **7** (12), 4066–4075.

WHITE, G., LEVY, W. B. & STEWARD, O. (1986). Associative interactions between afferents during the induction of long-term potentiation occur within limited dendritic domains. *Society for Neuroscience Abstracts* **12**, 505.

WINSON, J. & DAHL, D. (1986). Long-term potentiation in dentate gyrus: Induction by asynchronous volleys in separate afferents. *Science* **234**, 985–988.

WORLEY, P. F., BARABAN, J. M., DE SOUZA, E. B. & SNYDER, S. H. (1986). Mapping second messenger systems in the brain: differential localizations of adenylate cyclase and protein kinase C. *Proceedings of the National Academy of Sciences of the U.S.A.* **83**, 4053–4057.