Proc. Natl. Acad. Sci. USA
Vol. 87, pp. 4073–4077, June 1990
Neurobiology

Protein kinase C activity is not responsible for the expression of long-term potentiation in hippocampus

(phorbol esters/H-7/plasticity/synaptic transmission)

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Communicated by James L. McGaugh, March 12, 1990 (received for review December 22, 1989)

ABSTRACT **Long-term potentiation (LTP) in hippocampus has been proposed to result from a tonic activation of protein kinase C. This hypothesis predicts that stimulation of the kinase would produce a smaller change in response size on potentiated versus control pathways and, conversely, that inhibition of the kinase would reduce potentiated inputs to a greater degree than control responses. We tested these predictions using phorbol esters to activate and using the antagonist H-7 to inhibit protein kinase C; we found that the actions of these drugs on synaptic transmission were not affected by prior induction of LTP. Both compounds, however, significantly decreased the contribution of *N*-methyl-D-aspartate receptors to synaptic potentials, a result that accounts for the suppressive effects of these compounds on LTP formation. Thus protein kinase C is probably not involved in the expression of LTP but may play a role in the receptor-mediated events participating in its induction.**

Long-term potentiation (LTP) is a remarkably stable form of synaptic plasticity (1) that may be involved in certain memory processes. While considerable progress has been made in identifying the mechanisms that induce LTP, the changes responsible for its expression and maintenance remain controversial. One much discussed hypothesis holds that expression is due to a tonic activation of protein kinase C (PKC), as suggested by the following results: (*i*) activation of the enzyme by phorbol esters increases the size of synaptic responses and prevents LTP induction, a result that has been interpreted as suggesting an occlusion of LTP by prior activation of PKC and thus a common mechanism for the two effects (2, 3); (*ii*) high concentrations of H-7, an antagonist of PKC, block the generation of LTP and are reported to abolish potentiation produced *before* application of the drug (4); (*iii*) there are indications that translocation of the enzyme to the membrane might occur during the first hour after intense stimulation (5). Other work, however, suggests that phorbol ester-induced synaptic facilitation is qualitatively different than LTP (6) and there are clear indications from the work of Gustafsson *et al.* (7) that the occlusion argument may not be valid. We therefore reexamined several aspects of the persistent activation hypothesis—first, by quantifying the effects of PKC activation or inhibition on control and potentiated synaptic responses. If the potentiation effect were due to chronic activation of the enzyme, prior induction of LTP should significantly reduce the effects of phorbol esters. Similarly, the kinase inhibitor H-7 should affect to a greater degree potentiated versus control pathways. Second, we examined the possibility that pharmacological activation or inhibition of PKC with, respectively, phorbol esters and H-7 prevents LTP induction not because the kinase plays an essential role in the expression of this form of plasticity, but rather because the drugs interfere with receptor-mediated events that normally trigger the potentiation effect. The results obtained do not support the hypothesis that PKC is responsible for LTP expression.

MATERIALS AND METHODS

The experiments were done in area CA1 of hippocampal slices prepared from 5- to 7-week old Sivz rats (Geneva, Switzerland) and maintained in an interface chamber (8). The perfusion medium contained 124 mM NaCl, 1.6 mM KCl, 3 mM $CaCl_2$, 1 mM $MgCl_2$, 26 mM $NaHCO_3$, 1.25 mM KH_2PO_4, 10 mM glucose, and 2 mM ascorbate, at pH 7.4 and was oxygenated with 95% O_2/5% CO_2. Phorbol diacetate (PdAc; Sigma), 1-(5-isoquinolinylsulfonyl)-2-methylpiperazine (H-7) (Sigma), D-2-amino-5-phosphonopentanoic acid (D-AP5; Tocris, London) and 6-cyano-2,3-dihydroxy-7-nitroquinoxaline (CNQX) (Tocris) were all applied in the perfusing medium.

Extracellular recording electrodes were pulled from thick-wall capillaries and had a resistance of 2–7 MΩ when filled with 0.3 M NaCl. Bipolar stimulating electrodes were made by twisting two strands of insulated nichrome wires and were placed on both sides of and ≈1.0 mm from the recording pipette in the stratum radiatum of field CA1. All responses were digitized on line, and their parameters (amplitude, initial slope, decay time, area) were calculated.

The connections between area CA3 and CA1 were cut in all slices to reduce spontaneous activity. In experiments in which *N*-methyl-D-aspartate (NMDA) receptor-mediated components were measured, the magnesium concentration was reduced from 1 mM to 0.2 mM and responses were "primed" (i.e., one of two inputs to a target zone was stimulated 200 msec before the second input) to suppress inhibitory postsynaptic potentials (IPSPs) (see refs. 9–11 for further details on the priming procedure). This technique was also used for the analysis of responses in the presence of phorbol esters because the considerable enhancement of inhibitory postsynaptic potentials (IPSPs) produced by the drug made amplitude measurements less reliable (6). In all cases, LTP was induced using a stimulation pattern consisting of 10 bursts at 5 Hz, each burst being composed of four pulses at 100 Hz (see ref. 12). The duration of the stimulation pulses was increased by 50 μsec during each of the 10 bursts.

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Abbreviations: LTP, long-term potentiation; PdAc, phorbol diacetate; PKC, protein kinase C; EPSP, excitatory postsynaptic potential; IPSP, inhibitory postsynaptic potential; NMDA, *N*-methyl-D-aspartate; D-AP5, D-2-amino-5-phosphonopentanoic acid; CNQX, 6-cyano-2,3-dihydroxy-7-nitroquinoxaline.

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RESULTS

PdAc (15 μM) increased by 146 ± 15% (mean ± SEM; n = 14) the size of excitatory postsynaptic potentials (EPSPs) recorded extracellularly in field CA1 in response to stimulation of Schaffer-commissural fibers. Higher concentrations did not produce larger effects. To test for occlusion between phorbol esters and LTP, we compared the effect of the drug on control and previously potentiated pathways. In a first set of experiments (Fig. 1*A*), the stimulation intensity of two independent collections of Schaffer-commissural axons was adjusted so as to evoke responses of similar size. LTP was then induced in one group of afferents, and PdAc was added to the perfusion medium. If phorbol ester-induced facilitation and LTP are due to a common mechanism, occlusion of the effect of one by the other would be expected and, thus, phorbol esters should produce a much smaller change in EPSP size on potentiated versus control pathways. This effect was not seen in five experiments (Fig. 1*A*), confirming the results of an earlier report (7). As a further test for a possible occlusion effect, we quantitatively compared the effects of phorbol esters on control and potentiated responses set to an equivalent size; experiments of the type illustrated in Fig. 1*B* were done. Stimulation intensity on the two stimulating electrodes was adjusted to produce control responses ≈60–80% larger than those on the second input. LTP was then induced in the group of afferent fibers generating the smaller responses, and the effect of PdAc was compared on control and potentiated EPSPs. In 11 experiments, PdAc increased the size of control responses by 138 ± 18% and of potentiated responses by 125 ± 14%; (P > 0.5) (for data summary, see Fig. 1*C*). These results show that LTP induction and phorbol ester treatment produced a much larger change in synaptic responses than did phorbol ester treatment alone (299 ± 36% versus 138 ± 18%; P < 0.001). In fact, the change in response size seen after combining the two treatments (LTP + PdAc) is even larger than the arithmetic sum of the individual effects (299% versus 216% increase, Fig. 1*C*). It thus seems very unlikely that a single mechanism accounts for the two forms of potentiation. Rather, the results

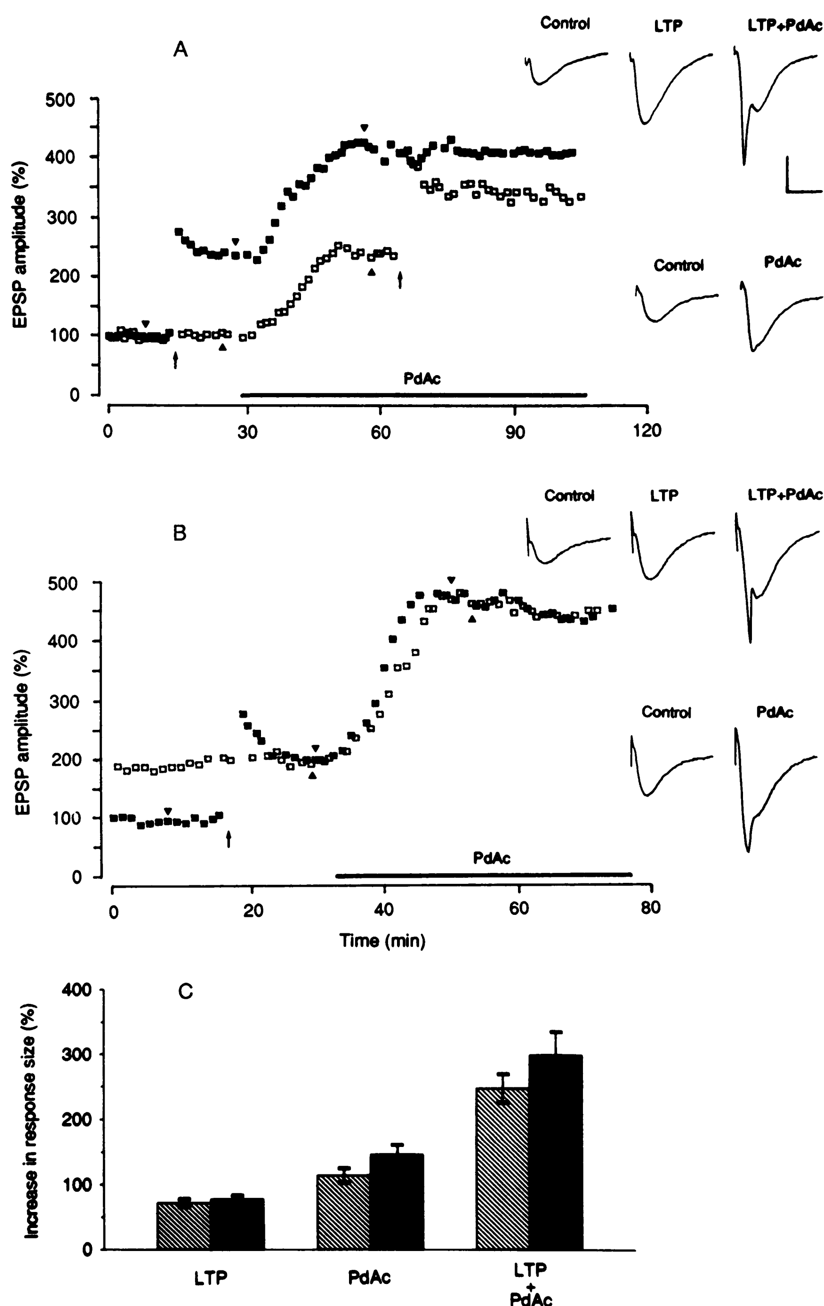

FIG. 1. Effect of PKC activation on independent control and potentiated pathways. (*A*) Amplitude changes measured on responses elicited by two stimulating electrodes placed either side of the recording pipette. At the time indicated by the first arrow, LTP was induced through one input (■) and PdAc (15 μM; black line) was added to the perfusion medium. At end of experiment, high-frequency stimulation was applied through the second input (second arrow, □). ▼ and ▲, The field potential traces illustrated, respectively, above (first input, ■) and below (second input, □). Voltage and time scales for representative EPSP are 1 mV and 10 msec. (*B*) Changes in amplitude measured on responses elicited on two independent inputs, for which responses from one input (control input, □) were 60–80% larger than those from the second input (potentiated input, ■). Symbols (arrow, black line, and triangles) are as in *A*. (*C*) Summary of the amplitude changes (hatched bars) and initial slope (black bars) measured in 11 experiments of the type illustrated in *A* or *B*. The three groups of data refer to the mean ± SEM of the increases in response sizes produced, respectively, by LTP induction, PdAc treatment (15 μM) alone, and LTP induction plus PdAc treatment (black inputs of *A* and *B*).

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obtained are consistent with the idea that the effects are produced by two different processes acting in cascade.

We next compared the effect of the protein kinase C antagonist H-7 on control and potentiated responses set to an equivalent size with the results of Fig. 2*A*. Two independent inputs were used in which responses from one were 60–80% larger than from the other. LTP was then generated in the weaker input, resulting in equivalent responses to the two input pathways. H-7 at a concentration of 400 μM reduced the size of both EPSPs. The effect of the drug could be monitored by the progressive increase in the decay time of field potentials it produced, an effect probably related to IPSP blockade (6, 21). The reductions in amplitude and slope measured on control and potentiated responses were indistinguishable (respectively, 32 ± 6% and 37 ± 7% versus 31 ± 4% and 37 ± 4%, $P > 0.8$). Thus H-7 does not appear to have a selective effect on potentiated pathways.

An additional indication that LTP was indeed expressed in the presence of H-7 is the fact that high-frequency stimulation applied at the end of the experiment produced a strong short-term potentiation effect on the control pathway but not on the previously potentiated one (see Fig. 2*A*). The mean degree of short-term potentiation measured as the increase in slope seen 1–2 min after high-frequency stimulation averaged 101 ± 11% on the control versus 43 ± 4% on the previously potentiated pathway ($n = 5$; $P < 0.01$). This difference can be accounted for by the fact that LTP partially occludes the short-term potentiation effect (9).

Another point illustrated in Fig. 1*A* is that LTP induction in the presence of phorbol ester is not equivalent to LTP induction before drug application. In nine slices perfused with a low magnesium medium (0.2 mM) to facilitate induction mechanisms, the degree of potentiation measured as the increase in amplitude and slope of field potentials averaged, respectively, 58 ± 7% and 64 ± 8% in the absence of PdAc and 30 ± 6% and 34 ± 6% in its presence ($P < 0.01$). This difference was seen even when comparing responses set to an equivalent amplitude before application of high-frequency stimulation. One possible explanation, other than an occlusion effect, was that phorbol esters interacted with the mechanisms of LTP induction. We therefore analyzed the effect of PdAc on the components of synaptic responses mediated by NMDA receptors. To reveal those components, we proceeded, as described (9–11), using a low-magnesium medium and primed responses. Two conditions were analyzed, the responses to single-pulse stimulation and to the short bursts of high-frequency stimulation (four pulses at 100 Hz) used to induce LTP (12). NMDA-dependent components were determined as the reduction in response area produced by application of 50 μM D-2-amino-5-phosphonopentanoic acid (D-AP5). Results are illustrated in Fig. 3. In control conditions, D-AP5 caused 23 ± 2% and 28 ± 2% reductions in the response areas to single-pulse and burst stimulation, respectively. In contrast, the reduction in response area in phorbol ester-treated slices was only 15 ± 3% and 14 ± 2% ($P < 0.05$; Fig. 3*B*). Note that the facilitation that occurs within the burst of high-frequency stimulation in control conditions is reduced with PdAc.

Fig. 3 also illustrates the effect of H-7, an antagonist of protein kinase C, on NMDA receptor-mediated aspects of synaptic responses. As with phorbol esters, we found that H-7 (400 μM) substantially blocks the D-AP5-sensitive portions of field potentials. In fact, D-AP5 reduced the area of responses to, respectively, single-pulse and burst stimulation by only 6 ± 2% and 7 ± 2% ($P < 0.001$). In some cases, D-AP5 seemed even to enlarge some aspects of the complex responses to burst stimulation elicited with H-7. Corresponding to this blockade of NMDA components and, in agreement with earlier reports, we were unable to induce robust LTP with these high concentrations of H-7 (see Fig. 2). Thus, inhibition as well as activation of PKC appeared to interact with the mechanisms responsible for LTP induction.

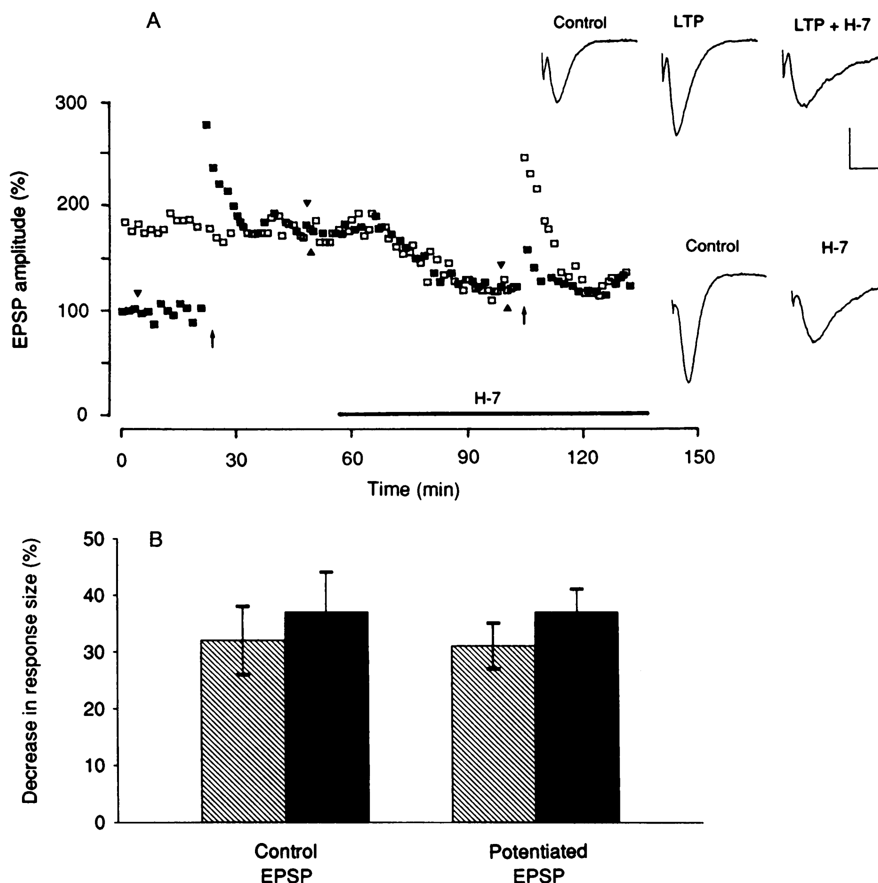

FIG. 2. Effect of PKC inhibition on control and potentiated pathways. (*A*) Changes in amplitude of responses elicited on two independent groups of afferents. At time indicated by the arrow, LTP was induced through the input generating the smaller responses (■). H-7 (400 μM, black line) was then added to the perfusion medium, reducing the sizes of field potentials from both inputs in a parallel fashion. At end of the experiment, high-frequency stimulation (arrow) was again applied to both inputs, generating a large short-term potentiation effect but no LTP on the control pathway (□) and only a small short-term effect on the potentiated pathway (■). ▼ and ▲, Upper and lower trace, respectively, at right. Note the characteristic prolongation of decay time produced by H-7 treatment. Voltage and time scales are 1 mV and 10 msec. (*B*) Summary of the reduction in response amplitudes (hatched bars) and initial slope (black bars) produced by 400 μM H-7 on control and potentiated pathways. Results are presented as the mean ± SEM of nine experiments.

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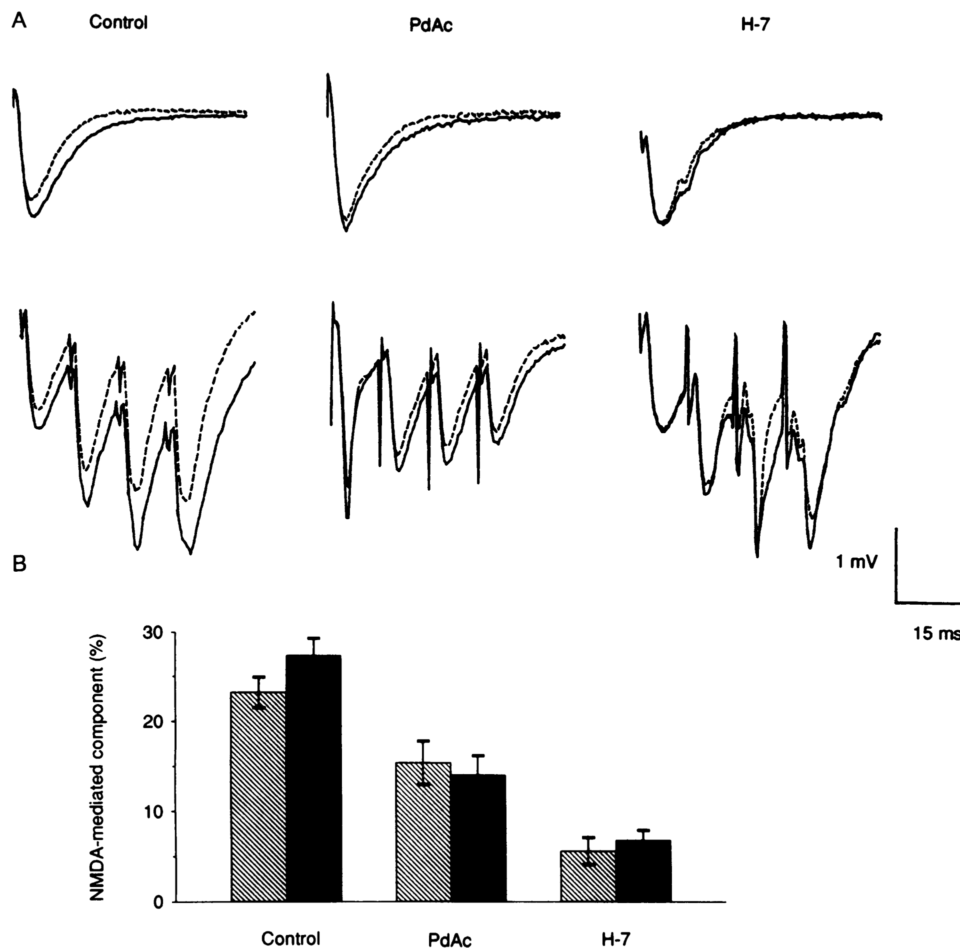

FIG. 3. NMDA receptor-mediated components of responses to single-pulse (upper traces) and burst (lower traces) stimulation. (*A*) Representative field EPSPs recorded before (—) and after (- - -) application of 50 μM D-AP5 in control conditions (*Left*), with 15 μM PdAc (*Middle*), or with 400 μM H-7 (*Right*). The medium contained 0.2 mM magnesium, and all responses were primed (see text). Bursts of high-frequency stimulation were composed of four pulses delivered at 100 Hz. (*B*) Summary representing the size of NMDA receptor-mediated components of responses to single-pulse (hatched bars) and burst (black bars) stimulation. NMDA components were measured as the reduction in response areas produced by applying 50 μM D-AP5. Results are presented as the mean ± SEM of 6–10 values.

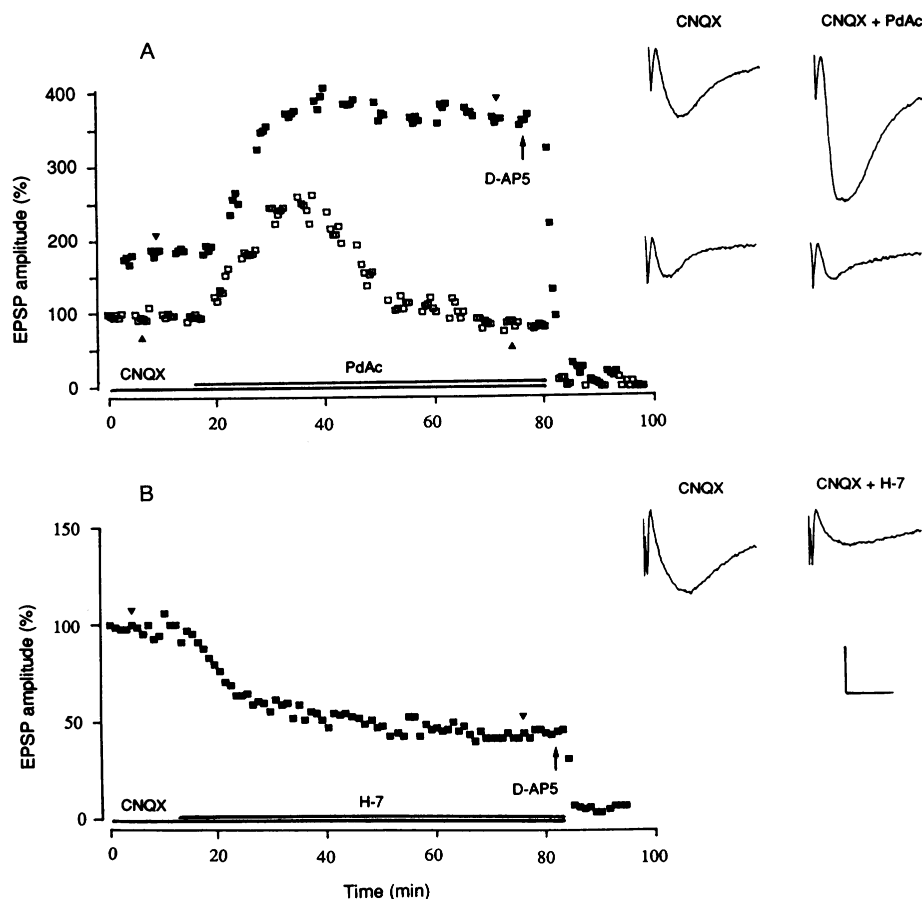

FIG. 4. Effect of PdAc and H-7 on NMDA receptor-mediated responses recorded in 10 μM CNQX/0.2 mM magnesium. (*A*) Changes in amplitude of primed (■, upper traces) and unprimed (□, lower traces) EPSPs produced by application of 15 μM PdAc (black line; CNQX present throughout the experiment). Note the large increase in the difference observed between primed and unprimed responses. ▼ and ▲, Primed and unprimed EPSPs, respectively, illustrated at right. At end of the experiment, D-AP5 (50 μM) was applied to the medium, completely blockading all responses. (*B*) Reduction in amplitude of NMDA receptor-mediated responses to primed single-pulse stimulation produced by applying 400 μM H-7 (CNQX present throughout the experiment). Application of D-AP5 at end of the experiment abolished the NMDA-mediated response. Scales are 1 mV and 10 msec for all EPSPs.

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In an effort to further clarify this point, we also measured the effects of PdAc and H-7 on synaptic responses recorded with CNQX (100 μM), an antagonist of non-NMDA receptors, and in a low-magnesium medium (0.2 mM $MgCl_2$). The field potentials recorded in these conditions are mediated exclusively by activation of NMDA receptors (10, 13). As illustrated in Fig. 4*A*, PdAc (15 μM) produced a dual action on NMDA-mediated responses in field CA1. When EPSPs were primed [i.e., inhibitory components were suppressed (9, 11)] PdAc acted as on quisqualate-mediated potentials—i.e., the drug increased considerably the size of NMDA responses. In five experiments, the mean change in amplitude of primed NMDA responses averaged 96 ± 6%. However, if the NMDA-mediated synaptic potentials were not primed (i.e., IPSPs were still active) the effect of PdAc was biphasic. During a first transitory period, the drug increased the size of the unprimed response, but with further time the facilitatory effect was reversed and NMDA-mediated EPSPs became smaller than before drug application (mean reduction in response amplitude in five experiments: 18 ± 2%). Thus, the difference between a primed and unprimed response increased considerably with PdAc (237 ± 5%; n = 5), thereby indicating that the influence of inhibitory processes on NMDA-mediated components was amplified by the drug.

Fig. 4*B* also shows that H-7 significantly reduced the size of NMDA responses to primed single-pulse stimulation recorded in the presence of CNQX and low-magnesium medium. In four experiments, we measured a 44 ± 6% reduction in amplitude of NMDA-mediated EPSPs. These results are thus consistent with and confirm the observations made in Fig. 3.

DISCUSSION

If PKC activity were responsible for the increase in synaptic efficacy produced by high-frequency stimulation, one would expect (*i*) activation of the enzyme by phorbol esters to eliminate the difference between a control and potentiated pathway or, at least, to produce a smaller change in response size on a potentiated versus control input and (*ii*) inhibition of the catalytic subunit of the kinase by H-7 to reverse LTP. These two conditions were not met in the present experiments.

The observation that LTP and phorbol esters interact (i.e., are expressed together and potentiate the effects of each other) suggests that the two treatments act on different steps in a cascade of events controlling the size of synaptic responses. Therefore it is tempting to propose that the main effect of PKC activation on transmission is presynaptic, a possibility supported by several lines of evidence (2, 14, 15), and that the modifications responsible for LTP are postsynaptic, as suggested by recent pharmacological experiments (11, 16, 17).

The results obtained with H-7, an antagonist of the catalytic subunit of the kinase, also do not support the idea that PKC is involved in LTP expression. Previous data indicated that H-7 selectively reversed LTP (4, 18), but we have been unable to reproduce this result. In conditions where control and potentiated inputs were set to a reasonably large and equivalent size, we did, indeed, measure a reduction in amplitude and initial slope of the potentiated pathway, but the same effect was also present on the control input. No statistical difference could be detected between the two pathways.

Finally, the experiments carried out in the presence of CNQX provide an additional indication that PKC activation and LTP involve different mechanisms. Whereas LTP was shown to be expressed almost exclusively by non-NMDA receptors (16, 17), the data obtained by analyzing primed NMDA-mediated responses indicate that the facilitation produced by phorbol esters can be expressed by both types of postsynaptic receptors.

Although the results presented here do not support the hypothesis that persistent stimulation of PKC is responsible for the expression of LTP, they do provide further evidence that activation or inhibition of the enzyme may interact with the mechanisms responsible for its induction. In both cases, we found a significant reduction in the contribution of NMDA receptors to the responses to high-frequency stimulation. It may be surprising that both activation and inhibition of PKC produce the same effect—i.e., blockade of NMDA receptor-mediated currents. Several explanations might be considered to account for this result, including nonspecific drug-related effects. However, for phorbol esters, factors known to be affected by the drugs could also modulate NMDA-mediated responses—e.g., reduced summation of responses within high-frequency bursts, enhancement by phorbol esters of γ-aminobutyric acid (GABA)-ergic transmissions (6), and inactivation of dendritic chloride conductances (19). The dual action of PdAc produced on NMDA responses recorded in the presence of CNQX supports this interpretation. The increase in size of the primed NMDA-mediated EPSP is consistent with the presynaptic action of the drug, whereas the inhibitory effect on the unprimed response reveals an increased influence of inhibitory processes on NMDA-dependent components. A possible explanation for H-7 is that a phosphorylation activity is necessary for the functioning of NMDA receptors; a recent study found an inactivation of NMDA responses in the absence of energy-rich compounds (20). Other mechanisms, however, such as increased participation of potassium conductances (19) or nonspecific effects related to the high concentrations used, cannot be excluded. These results thus emphasize the importance of modulatory influences on the induction of LTP but argue strongly against the possibility that PKC plays an essential role in its expression.

We thank Jackie Porter for her secretarial help. This work was supported by Fonds National Suisse de la Recherche Scientifique 3.173.0.88 and the Sandoz Foundation to D.M. and a grant from the Air Force Office of Scientific Research (AFOSR #89-0383) to G.L.

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