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Postsynaptic protein kinase C essential to induction and maintenance of long-term potentiation in the hippocampal CA1 region

(rat hippocampal slice/protein kinase inhibitor/synaptic plasticity)

JIN-HUI WANG AND DE-PEI FENG*

Shanghai Institute of Physiology, Chinese Academy of Sciences, Shanghai 200031, China

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ABSTRACT **Previous studies on the effects of protein kinase C (PKC) inhibitors intracellularly introduced into the postsynaptic neuron on long-term potentiation (LTP) in the hippocampal CA1 region showed that given before the tetanic stimulation they only blocked the development of the maintenance phase of LTP and that given after the tetanus they did not affect the continued maintenance of established LTP. We now report different results in such experiments obtained by looking into the dose–effect relationship of the inhibitors given to the postsynaptic neuron and making use of a synergistic effect of two inhibitors given together. We used the following three PKC inhibitors: polymyxin B (PMB), PKC-(19–31), and H7. With the intracellular delivery of the inhibitor(s) beginning 30 min before the tetanus, PMB in adequate dosage or a combination of PMB and PKC-(19–31), each at a low dosage, could block the development of LTP completely including its initial induction phase. With the delivery beginning at the time of the tetanus, PKC-(19–31) or H7 slowly caused the established LTP to decline to the baseline; this decline was greatly accelerated when PMB and PKC-(19–31) or PMB and H7 were given together. PMB and PKC-(19–31) given together 75–90 min or even 3 h after the tetanus caused a decline of the maintained LTP similar to the decline observed when both inhibitors were given at the time of the tetanus. These results show that postsynaptic PKC is essentially involved in both the initial induction and the subsequent maintenance of LTP, contrary to current views on the subject.**

Long-term potentiation (LTP) is a striking phenomenon of use-dependent synaptic plasticity and provides a useful model of a candidate mechanism for learning and memory in the mammalian brain (1, 2). During the last decade, this phenomenon in the hippocampus, especially in the CA1 region, has received increasing attention from investigators, and important progress has been made. Revelation of the properties of the *N*-methyl-D-aspartate receptors (3, 4) has provided a basis for explaining all the three special properties of LTP in the CA1 region (5): input specificity, cooperativity, and associativity. Further, the influx of Ca^{2+} through the activated *N*-methyl-D-aspartate receptor channels into the postsynaptic neuron is now generally conceded to be the trigger for the development of LTP. But the biochemical processes after calcium entry that occur in the postsynaptic neuron and lead to the induction and maintenance of LTP are still little understood. Several candidates for such processes have been under study (1): the activation of protein kinase C (PKC), of Ca^{2+}/calmodulin-dependent protein kinase type II (CaMKII), and of a calcium-sensitive protease, calpain I. So far PKC seems to have received the most attention. But although there is a considerable body of evidence pointing to the involvement of PKC in LTP (6, 7), the role of PKC in LTP is as yet not only far from clear but also has now even become a somewhat controversial issue (8).

Most *in vitro* studies on the role of PKC in LTP employed inhibitors of PKC. The inhibitors were in most cases given in the solution bathing the whole brain slice (9–11). This procedure left the site of action of the substance tested unclear. In a few cases they were given intracellularly to the postsynaptic neuron under observation (12, 13). This is the proper thing to do when one wants to direct one's inquiry to the biochemical processes after calcium entry in the postsynaptic neuron. In these cases it was reported that the intracellular delivery of any of several PKC inhibitors before the high-frequency stimulation blocked the full development of long-lasting stable LTP, leaving a decremental synaptic potentiation lasting about 30 min; that is, inhibition of PKC in the postsynaptic neuron blocked the generation of the maintenance phase but not the induction (i.e., the initial development of LTP). Yet when the PKC inhibitors were given to the postsynaptic neuron after the tetanic stimulation, the maintenance of the established LTP was not affected (13). By looking into the dose–effect relationship of the PKC inhibitors administered to the postsynaptic neuron and making use of a synergistic effect of two inhibitors given together, we have observed results different from those just mentioned. Our results showed that postsynaptic PKC inhibition before the tetanus could block the development of LTP completely, including the initial induction, and after the tetanus could cut short the continued maintenance of the established LTP.

MATERIALS AND METHODS

We studied LTP in the CA1 region of rat hippocampal slices, using intracellular microelectrode both for recording excitatory postsynaptic potentials (EPSPs) and for delivering PKC inhibitors into the postsynaptic pyramidal neuron. Transverse hippocampal slices (400 μm) from male Sprague–Dawley rats (150–200 g) were cut in ice-cold Earle's solution on a Vibratome. The experimental chamber used was as described in ref. 14. The modified Earle's solution contained 125 mM NaCl, 4.0 mM KCl, 1.3 mM $MgSO_4$, 1.2 mM NaH_2PO_4, 26 mM $NaHCO_3$, 2.4 mM $CaCl_2$, and 10 mM glucose and was gassed with 95% O_2/5% CO_2 to give pH 7.4. The experimental temperature was 33.5 ± 0.5°C. Bipolar tungsten microelectrodes about 50 μm in diameter, insulated except at their tips, were placed in stratum radiatum and stratum lacunosum-moleculare for stimulation of the Schaffer collateral/commissural (S/C) and the perforant pathway

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Abbreviations: PKC, protein kinase C; LTP and STP, long- and short-term potentiation; PMB, polymyxin B; CaMKII, Ca^{2+}/calmodulin-dependent protein kinase type II; EPSP, excitatory postsynaptic potential; PP, perforant pathway; S/C, Schaffer collateral/commissural.

*To whom reprint requests should be addressed.

(PP), respectively; the tetanic stimulation for producing LTP consisted of 10 trains and each train was 10 pulses at 200 Hz, the strength of each pulse being the same as that of control and test stimulus. The intracellular microelectrode (45–70 MΩ) for insertion into the pyramidal neuron was filled with 4 M KOAc. For simultaneous recording of field EPSPs in stratum radiatum in some experiments, a microelectrode containing 2 M NaCl was used. In those experiments in which the intracellular microelectrode was used both for recording EPSPs and for delivering drug and the drug delivery was required to begin at the time of the tetanus, a short series of alternate positive and negative weak pulses (0.1 nA, 100 ms, 1 Hz for about 20 s) was applied to the intracellular microelectrode after the impalement, a manoeuvre empirically found to quicken the attainment of stable control EPSP readings within 2–3 min, and during most of this short pretetanus period a backing current was usually applied to retard the diffusion of drug into the cell, the following three PKC inhibitors used: polymyxin B (PMB), PKC-(19–31), and H7, all carrying a positive charge. Intracellular recording was deemed acceptable, if membrane potentials were 65–75 mV with a change of less than 5 mV during the experiment. PMB and H7 were obtained from Sigma. PKC-(19–31) was synthesized by the Molecular Biology Laboratory of Shanghai Institute of Biochemistry, Chinese Academy of Sciences. These drugs were all dissolved in distilled water and diluted with 4 M KOAc to the desired concentration for filling the intracellular microelectrode.

RESULTS

We first show the effects of PKC inhibitors intracellularly introduced into the postsynaptic neuron before the tetanus on the generation of LTP. Fig. 1 *a* and *b* shows the inhibitory effects on LTP of PMB given before the tetanus with their striking dependence on the dosage. In the experiment shown in Fig. 1*a*, we started giving PMB 30 min before the tetanus to the S/C input and 60 min before the tetanus to the PP input. With the dosage of PMB used, for both the S/C and PP inputs, the maintenance phase of LTP was blocked, leaving an initial decremental phase. The remaining decremental phase was clearly smaller and shorter for the PP input tetanized 30 min later than the S/C input. This provided the first suggestion of a dose–effect relationship, as more PMB

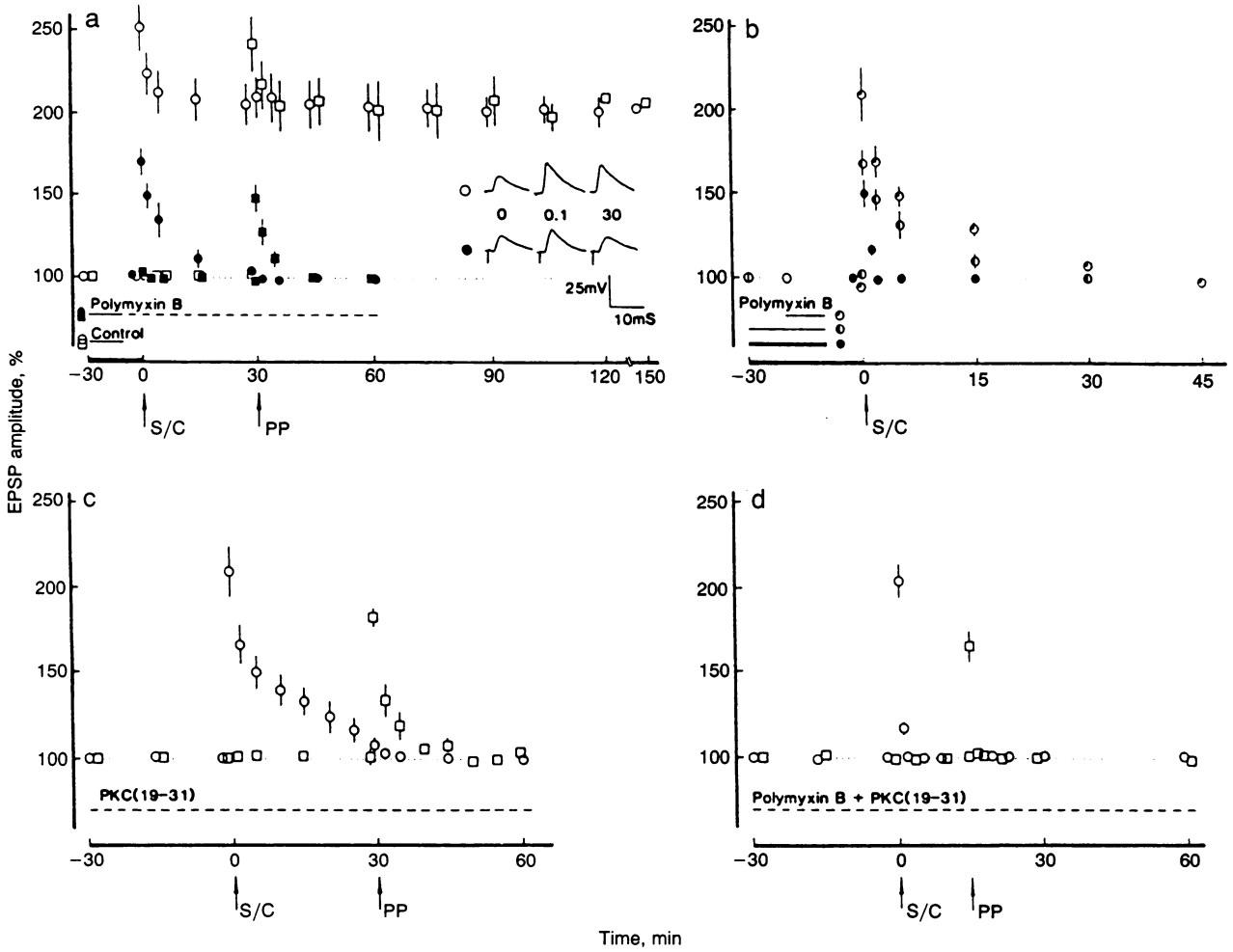

FIG. 1. Inhibitory effects of PMB and PKC-(19–31) given either separately or together before the tetanus on the development of LTP show a striking dependence on the dosage. (*a*) Comparison of control (open symbols; $n = 13$, SEM indicated) and PMB-inhibited (solid symbols; $n = 5$) LTPs elicited by S/C (circles) and by PP (squares) inputs at times marked by arrows. Note that the same 20-min pulsed current (indicated by the continuous horizontal line segment) was applied before the control and the PMB-treated observations, showing that such current by itself did not affect the result. (*Inset*) Sample EPSP records taken at the following times: Zero (just before the tetanus), 0.1 min (just after the tetanus), and 30 min. (*b*) The induction phase of LTP is blocked by increasing the dosage of PMB as determined by varying the duration of the driving current (quarter-solid circle, $n = 6$, 0.5 nA, 100 ms, 0.5 Hz, 10 min; half-solid circle, $n = 5$, 0.5 nA, 100 ms, 0.5 Hz, 20 min; solid circle, $n = 4$, 0.5 nA, 200 ms, 0.5 Hz, 20 min). (*c*) Inhibitory effects of PKC-(19–31) (100 μM in the micropipette, entering the cell by free diffusion; $n = 7$) on LTP in the S/C and the PP pathways are shown. Note that they are comparable to those of PMB in *a* and likewise show the greater inhibition of the LTP in the PP pathway elicited 30 min later. (*d*) With PMB and PKC-(19–31) given together, each at a low dose ($n = 4$), the effect became much more striking, the block of LTP being now practically complete.

must have entered the cell during the 30 min separating the tetanization of S/C and of PP. The experiments in Fig. 1*b* gave full confirmation for this suggestion. With three increasing doses of PMB, the induction phases of the three S/C-elicited LTPs decreased progressively in size, so that with the highest dose used almost nothing more than post-tetanic potentiation was left, which meant practically complete prevention of the generation of LTP.

Fig. 1*c* shows the effect of PKC-(19–31) (100 μM in the micropipette) entering the postsynaptic neuron by free diffusion starting 30 min and 60 min before tetanizing S/C and PP, respectively. For both inputs the maintenance phase of LTP was abolished. Comparison of the size of the remaining decremental phases for the two inputs again shows the dose–effect relationship: the remaining decremental phase for the PP input tetanized 60 min after the start of the delivery of PKC-(19–31) was clearly smaller than that for the S/C input tetanized 30 min earlier.

In the experiment shown in Fig. 1*d*, the micropipette contained 100 μM PMB and 100 μM PKC-(19–31) and the two inhibitors were allowed to enter the postsynaptic neuron together by free diffusion, starting 30 min and 45 min before tetanizing the S/C and the PP inputs, respectively. It is seen that the combined inhibitory effect of PMB and PKC-(19–31) was much greater than that of either given alone (compare Fig. 1 *d* with *a* and *c*). The use of a combination of two PKC inhibitors at low doses to achieve a high-dose effect helped ensure that the blocking effect on the generation of LTP described above was specifically due to inhibition of postsynaptic PKC.

We now turn to the effects of PKC inhibitors on the maintenance of established LTP with their delivery from the microelectrode into the postsynaptic neuron beginning at the time of the tetanus (Fig. 2). Because of the slowness of such delivery, it may be assumed that the inhibitors actually only took effect after the establishment of LTP. Fig. 2*a* shows the effect of PMB given at the time of the tetanus for the S/C pathway but 45 min before the tetanus for the PP. In this experiment PMB (100 μM in the micropipette) was driven into the cell by pulsed current (0.5 nA, 100 ms, and 0.5 Hz) for the first 30 min and then allowed to continue to freely diffuse into the cell. Judging from the nearly complete suppression of the PP-induced LTP (which served as a sort of internal control for adequate delivery of the drug), the dose of PMB used in this experiment appeared to be a rather large one, but it only lowered the maintained level of the estab-

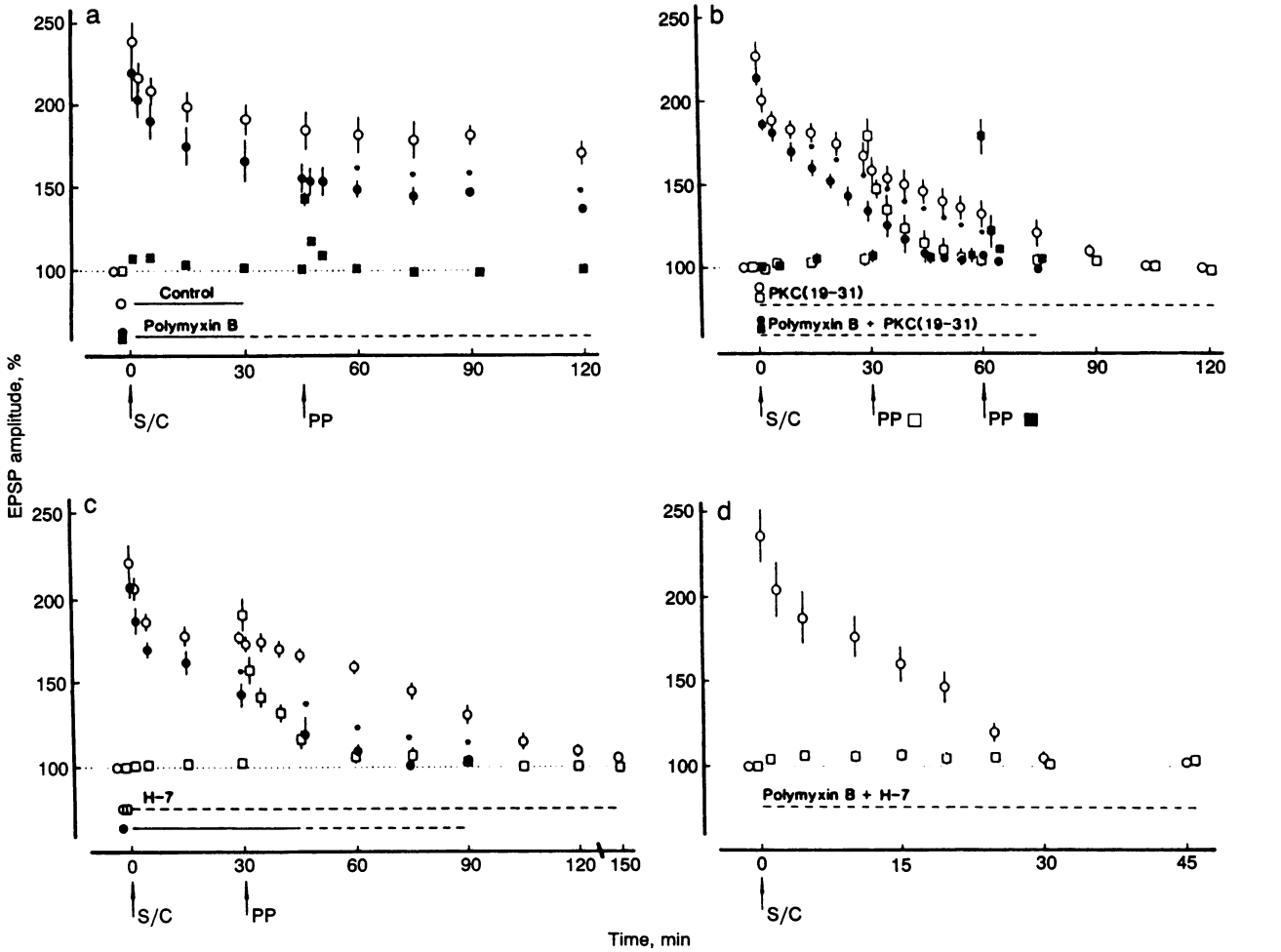

FIG. 2. Effects of PKC inhibitors given separately or in pairs at the time of the tetanus on the maintenance of LTP. (*a*) PMB given alone at the time of tetanizing S/C allowed the LTP in this pathway (solid circles, $n = 5$) to be maintained but at a lower level than the control (open circles, $n = 10$). The small remaining decremental phase of the LTP in the PP (solid squares) tetanized 45 min after beginning the drug delivery served as an internal control for the effective entry of the drug. (*b*) PKC-(19–31) given alone (open circles) beginning at the time of the tetanus caused the established LTP to decline to the baseline ($n = 7$) in about 100 min, and, with PMB and PKC-(19–31) given together (solid circles), this decline was greatly accelerated ($n = 5$). (*c*) H7 given alone at the time of the tetanus also caused the LTP to decline; note the different rates of the decline with two doses of H7 (smaller dose, open circles, $n = 6$; larger dose, solid circles, $n = 5$). The small asterisks in *a*–*c* indicate $P < 0.05$. (*d*) PMB and H7 given together at the time of the tetanus caused a much more rapid decline of the LTP ($n = 4$) than the larger dose of H7 given alone (compare with *c*, note the difference in the time scale).

lished LTP in the S/C pathway. Fig. 2*b* shows the effect of PKC-(19–31) and of PMB plus PKC-(19–31) given beginning at the time of the tetanus. In these experiments the drugs (both 100 μM in micropipette) entered the cell by free diffusion, so the dosage of each drug may be assumed to be relatively small. (*i*) Note that PKC-(19–31) alone slowly but clearly caused the established LTP to decline, falling to the baseline in about 100 min. (*ii*) Note that PMB plus PKC-(19–31) made the LTP fall much more rapidly, reaching the baseline in about 50 min.

Fig. 2*c* shows the effects of H7 in two doses given at the time of tetanizing the S/C pathway on the LTP in this pathway. The smaller dose very slowly caused the LTP to decline, falling to the base line in about 150 min, and the larger dose caused the LTP to fall more rapidly, reaching the baseline in about 70 min. For the smaller dose, the change in the LTP in the PP elicited 30 min after beginning the drug delivery is also shown, indicating that H7 given before the tetanus at the dosage used in this experiment was effective in blocking the generation of the maintenance phase of LTP.

The effects of PMB plus H7, each at a low dose given at the time of the tetanus, on the maintenance of established LTP are shown in Fig. 2*d*. This combination caused the LTP to fall to the baseline in 30 min; that is, the effect was much stronger than that of the larger dose of H7 given alone, as shown in Fig. 2*c*.

The results shown in Fig. 2 demonstrate that PKC inhibition in the postsynaptic neuron occurring shortly after the establishment of LTP could interfere with its maintenance. Would such inhibition occurring much later in the course of the maintained LTP also be able to block its continued maintenance? Additional experiments shown in Fig. 3 provide the answer. PMB and PKC-(19–31) given together to the postsynaptic neuron 75 min (Fig. 3*a*) and 3 h (Fig. 3*b*) after the tetanus could still inhibit the maintenance of established LTP as effectively as when given at the time of the tetanus.

DISCUSSION

The three PKC inhibitors used have different modes of action. PMB inhibits PKC by blocking its activation (15). PKC-(19–31) is a selective pseudosubstrate blocker of PKC activity (16). H7 inhibits activated protein kinases by competing with ATP (17). The synergistic inhibitory action on LTP of PMB and PKC-(19–31) or of PMB and H7 given together to the postsynaptic neuron might have its basis in the fact that PMB acts on the regulatory subunit of PKC, whereas PKC-(19–31) or H7 acts on its catalytic subunit; there may be cooperativity between the changes in the two subunits. The precise molecular mechanism involved requires further study, but empirically, we have now a useful method for achieving very strong PKC inhibition with a combination of two inhibitors each at a low dose. The observation that, while PMB alone only lowered the maintained level of the established LTP, either PKC-(19–31) alone or H7 alone could cause the established LTP to disappear might also be related to the difference in their modes of action on PKC just referred to.

The above results necessitate a revision of earlier conclusions such as "PKC activation is necessary for maintenance, but not for induction of LTP" (6, 9) or "Intracellular postsynaptic delivery of H7 or of PKC-(19–31) was ineffective with respect to established LTP" (13). The present conclusion is that postsynaptic PKC is essentially involved in the generation of LTP including its two phases of induction and maintenance and the long-lasting maintenance of the established LTP, at least during the first 3 h. As additional support for this conclusion, we mention our unpublished observation that prior enhancement of EPSP by phorbol ester intracellularly delivered to the postsynaptic neuron occludes the subsequent generation of LTP and that, conversely, prior establishment of LTP occludes the EPSP-enhancing effect of phorbol ester given subsequently.

The facts that the development of LTP can be completely blocked by PKC inhibition confined to the postsynaptic neuron and that also the maintenance of established LTP can be completely arrested by such PKC inhibition anytime during a period of several hours after its induction indicate that postsynaptic events involving PKC activation and related protein phosphorylation play a determinative role in both the initiation and the subsequent long-lasting mainte-

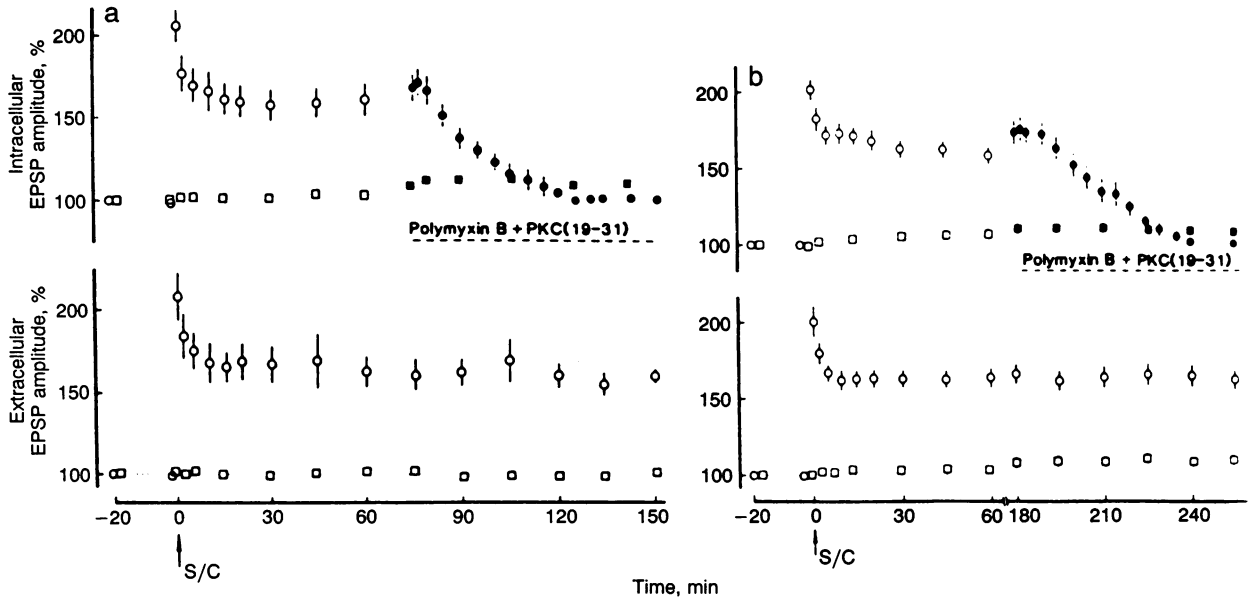

FIG. 3. PMB and PKC-(19–31) given together late in the course of a maintained LTP can arrest its continued maintenance. (*a* and *b*) Delivery of PMB plus PKC-(19–31) began 75 min ($n = 5$) and 180 min ($n = 4$), respectively. In these two groups of experiments the EPSPs of the tetanized S/C and untetanized PP pathways were recorded both extracellularly (lower section) and intracellularly (upper section) first from one pyramidal neuron with a microelectrode containing no drugs (open circles) and then (75 min in *a* and 180 min in *b*) later from another neuron with a microelectrode containing 100 μM PMB and 100 μM PKC-(19–31). Note that the fall of EPSP recorded by the drug-containing microelectrode in the tetanized S/C pathway (solid circles) to the baseline in about 50 min with EPSP in the untetanized PP (solid squares) remained practically unchanged and also that the extracellularly recorded field EPSPs remained unchanged.

nance of LTP. However, it does not follow from this that the final link in the biochemical chain that expresses itself as maintained LTP must also be postsynaptic; it could be presynaptic mediated by a retrograde signal (2, 18). So our results as they stand are neutral to the long-standing debate about whether a postsynaptic mechanism, such as changes in receptor number or sensitivity (19, 20), or a presynaptic sustained increase in transmitter release (21, 22) is responsible for the maintenance of LTP. They only make it necessary to suppose that the final biochemical change responsible for the expression of LTP, brought about within minutes after the tetanus, whether postsynaptic or presynaptic, is not a stable state by itself but is a kind of dynamic state constantly depending for its maintenance on postsynaptic PKC-related events. This supposition seems to be susceptible to specific experimental tests for each candidate postsynaptic or presynaptic mechanism underlying the maintenance of LTP.

Recent reports have provided evidence also for an essential role of postsynaptic CaMKII in the generation of LTP (12). The role of postsynaptic CaMKII in LTP requires a more detailed study. Then the problem of unravelling the working relationship in LTP between PKC and CaMKII in the postsynaptic neuron should be tackled. Current results indicate that, in relation to LTP, PKC and CaMKII cannot function entirely independently of each other—i.e., simply in parallel.

The generation of a full-fledged well-maintained LTP depends first of course on an adequate trigger—i.e., on an adequate calcium influx into the postsynaptic neuron. Next, there must be an intracellular biochemical process ready to respond to the calcium influx. In the light of our experimental results, we suppose the activation of postsynaptic PKC to be at least one of the essential processes triggered by the calcium influx. The degree of PKC activation (including translocation from cytosol to cell membrane) and EPSP enhancement vary with the intensity of the calcium influx. Up to a point all these changes are probably more or less directly dependent on the increase in intracellular Ca^{2+} concentration and the external manifestation is short-term potentiation (STP) of varying duration (23). When the PKC activation and the ensuing protein phosphorylation reach a certain critical level, the changes somehow become self-sustaining, thus bringing about the transition from STP to LTP. With a given calcium influx, the degree of PKC activation will depend on the amount of postsynaptic PKC available for activation. With an increasing degree of inhibition of PKC, less and less of PKC will be available for activation, stably maintained LTP will be first changed into STP, and STP will continue to decrease and eventually disappear, exactly as our experimental results have shown. This hypothetical picture is very tentative and incomplete, waiting for revision and further study.

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