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CHAPTER 5

The involvement of PKC and multifunctional CaM kinase II of the postsynaptic neuron in induction and maintenance of long-term potentiation

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Introduction

Regarding long-term potentiation (LTP) — a striking phenomenon of activity-dependent synaptic plasticity now widely and intensively studied by neurobiologists because of its relevance for understanding the cellular mechanisms of learning and memory — it is now generally conceded that its induction in the CA1 region of the hippocampus takes place in the postsynaptic neuron triggered by calcium ion influx. The biochemical processes in the postsynaptic neuron following calcium entry that underlie the initial development and the subsequent maintenance of LTP are still largely unknown. In recent years evidence has begun to accumulate to show the importance of protein kinases of the postsynaptic neuron, especially PKC and CaMKII, in both the induction and the maintenance of LTP, although some inconsistencies among the reports exist. This chapter gives a summary account of our studies on the role of postsynaptic PKC and CaMKII in LTP in rat hippocampal CA1 region. We studied the effects of various inhibitors of PKC and CaMKII given intracellularly to the postsynaptic neuron, either singly or jointly, on the induction and the maintenance of tetanus-induced LTP. In particular it will be shown that the peptide pseudosubstrate inhibitors, PKC(19-31) and CaMKII(273-302), can each block the induction of LTP and the maintenance of established LTP; and when given together there is a strong synergism between the two, greatly accelerating the development of the blocking effects.

It is now generally agreed that the induction of the usual type of LTP produced by brief high-frequency stimulation (HFS) in the CA1 region of the hippocampus takes place in the postsynaptic neuron triggered by calcium ion influx through the NMDA receptor channel. The biochemical processes in the postsynaptic neuron following calcium entry that underlie the development and maintenance of LTP have now logically become more and more the focus of research on the cellular and molecular mechanisms of LTP. Among the candidate biochemical events to be studied, the possible involvement of calcium-related protein kinases, especially PKC and CaMKII, has quite naturally attracted initial attention. Extracellular recording of EPSP and bath application of various inhibitors of these kinases were employed in the early experiments on the effects of PKC and CaMKII inhibitors on LTP in hippocampal

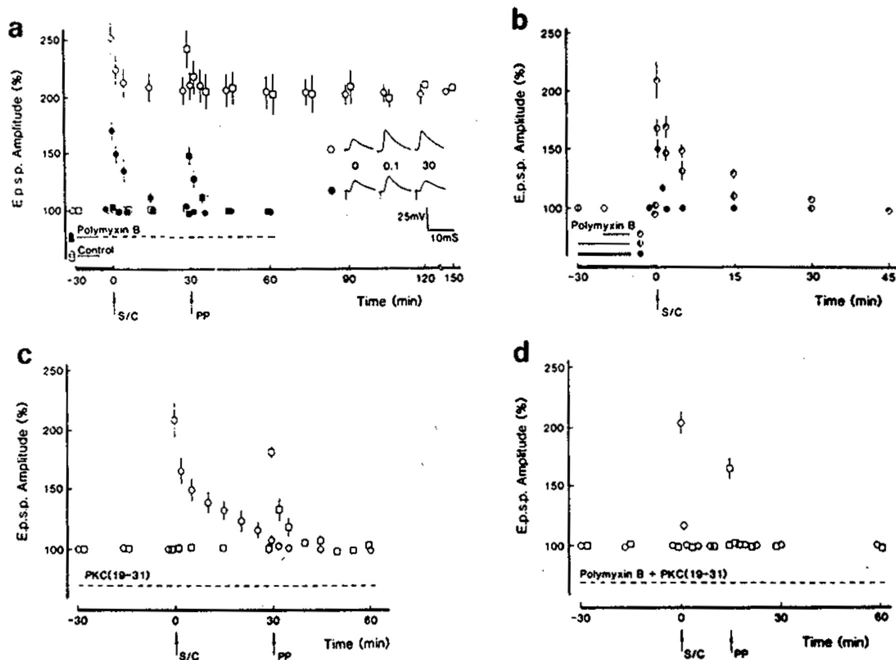


Fig. 1. Inhibitory effects of PMB and PKC(19–31) given either separately or together before the tetanus on the induction of LTP, showing, first, a striking dose–effect relationship and, second, a striking synergistic action of the two PKC inhibitors given together. Tetanic stimulation (in all experiments 10 trains of 10 stimuli each at 200 Hz, interval between trains 5 s) given separately to two afferents: Schaffer-collateral commissural (S/C, circles) and perforant pathway (PP, squares) at times marked by arrows: (a) Comparison of control (open circles) and PMB-inhibited (solid symbols) LTPs, note the greater inhibition in PP tetanized 30 min later; (b) showing increasing block of induction with increasing dosage of PMB regulated by the duration and strength of the driving current (quarter-filled, half-filled and completely filled circles represent three increasing doses); (c) showing the inhibitory effects of PKC(91–31) given alone (100 μM in the microelectrode, entering the cell by free diffusion), again note the greater inhibition in PP tetanized 30 min later; (d) showing that with PMB and PKC(19–31) given together there was much greater inhibition, actually complete inhibition of LTP, leaving only PTP.

slices (Lovinger et al., 1987; Reymann et al., 1988a,b). However, extracellular application of inhibitors does not address questions concerning their sites of action. To specifically study the biochemical events in the postsynaptic neuron, intracellular recording and delivery of the inhibitor has to be used. In recent years, this approach has begun to be employed (Malenka et al., 1989; Malinow et al., 1989). In the limited space of this chapter I will give a brief summary of the main results of our own studies (Wang and Feng, 1992; Feng and Wang, 1992) using intracellular recording and delivery of kinase inhibitors. I will emphasize certain findings that differ from the results of other authors in the hope of stimulating discussion of the data.

Effect of PKC inhibitors on LTP

We will first examine the effect of PKC inhibitors introduced into the postsynaptic neuron before the HFS on the induction of LTP. It is seen in Figs. 1a and 1b that with increasing dosage of polymyxin B (PMB), there is a corre-

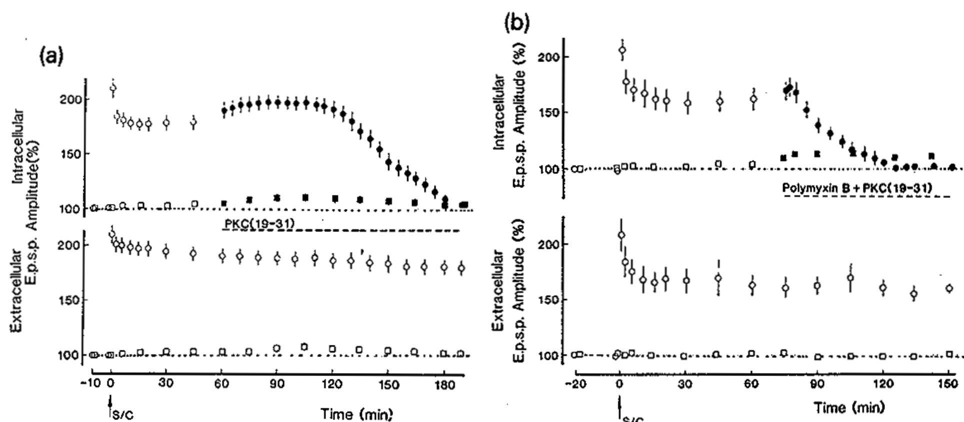


Fig. 2. Comparison of the rate of development of the blocking effect on the maintenance of established LTP: (a) PKC(19–31) given alone, and (b) PMB and PKC(19–31) given together to the postsynaptic neuron 60–75 min after the high-frequency stimulation. Simultaneous extracellular recording of the population EPSPs used as control to make sure that the LTP was well maintained throughout the experimental period. Intracellular recording in this type of experiment required a change of the microelectrode: the first microelectrode (open circles) containing no inhibitors was inserted into one neuron and recorded the well-maintained LTP; 60–75 min later, a second microelectrode (solid circles) containing inhibitors was inserted into another neuron of the same population which at first recorded a similar potentiated EPSP as the first microelectrode, then with very different latencies (in a about 50 min and in b several min) the EPSP began to decline, returning to the pretetanus baseline in about 1 h.

sponding increasing block of the induction of LTP until it becomes complete, i.e. the maintained LTP is first changed into a decremental STP lasting 30–40 min, which then becomes briefer and briefer until nothing but PTP remains. A similar result was obtained using PKC(19–31) (Fig. 1c). When PMB and PKC(19–31) were delivered together to the postsynaptic neuron, a much greater inhibitory effect on the induction was obtained than with either PMB or PKC(19–31) alone (Fig. 1d). Thus, a striking synergistic action between the two inhibitors of PKC was brought to light. We next examine the effect of giving the PKC inhibitor(s) to the postsynaptic neuron after the HFS on the maintenance of LTP. We began the delivery of the inhibitor(s) at times varying from immediately after to 3 h after the HFS and obtained similar results. The blocking effect on the maintenance of LTP with PKC(19–31) given alone or with PMB and PKC(19–31) given together 60–75 min after the establishment of LTP is shown in Figs. 2a and 2b. These two figures provide a very interesting comparison: when PKC(19-31) was given alone, the blocking effect on the maintenance developed very slowly, only beginning to be noticeable after about 1 h so that if the observation were terminated in 1 h or less, the blocking effect would have been missed; when PMB and PKC(19-31) were given together, the blocking effect developed very rapidly, becoming noticeable within minutes. Here again there is a striking synergism between PMB and PKC(19-31).

Effect of CaMKII inhibitor on LTP

We now look at the blocking effects on the induction of LTP and on the maintenance of established LTP by the specific peptide inhibitor of CaMKII, CaMKII(273–302), delivered to the post-synaptic neuron before and after the

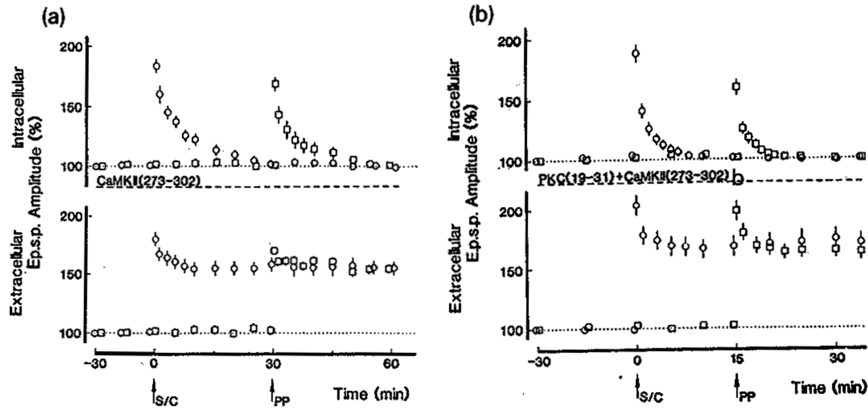


Fig. 3. The blocking effect on the induction of LTP of CaMKII(273–302) given alone (a) is much less than that of PKC(19–31) and CaMKII(273–302) given together (b). Note the larger time scale in (b) than in (a).

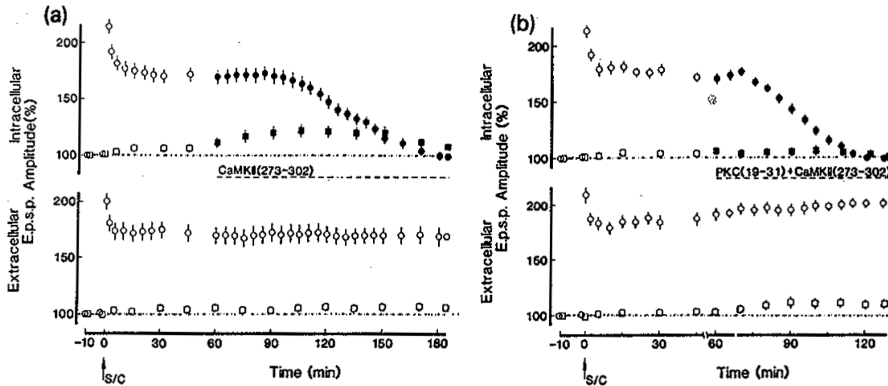


Fig. 4. The faster development of the blocking effect on the maintenance of established LTP of PKC(19–31) and CaMKII(273–302) given together (b) than that of either CaMKII(273–302) (a) or PKC(19–31) (Fig. 2a) given alone.

HFS respectively. Figures 3a and 3b show the blocking effects on the LTP induction of CaMKII(273–302) given alone and of PKC(19–31) and CaMKII(273–302) given together respectively. It is seen that the two inhibitors of the two different kinases given together produced a more complete inhibition of LTP induction than either of them given alone. Figures 4a and 4b show the blocking effect on the maintenance of established LTP of CaMKII(273–302) given alone and PKC(19–31) and CaMKII(273–302) given together 1 h after the HFS respectively. It is seen that the blocking effect on the maintenance developed much more rapidly with PKC(19–31) and CaMKII(273–302) given together than with either of them given alone.

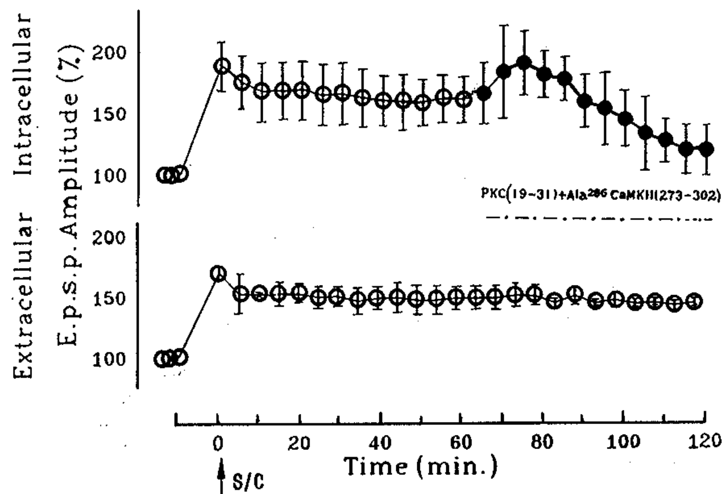


Fig. 5. A comparison of this figure with Fig. 4b shows that Ala^{286}CaMKII(273–302) is equally effective as an inhibitor of CaMKII as CaMKII(273–302) with threonine at the 286 position used in the earlier experiments. Curves shown represent averages of 3 experiments.

These comparisons bring out a new kind of synergism, i.e. synergism between two inhibitors of two different protein kinases. This result was first obtained nearly two years ago by one collaborator (J.H. Wang) and confirmed more recently by another (H.X. Chen). The CaMKII(273–302) we first used was obtained from Dr. Howard Schulman through the kindness of Dr. Richard Tsien at Stanford University; the peptide used in our more recent experiments, kindly supplied by Dr. Czernik of Rockefeller University, is Ala^{286}CaMKII(273–302) with threonine at the 286 position replaced by alanine. We have found that Ala^{286}CaMKII (273–302) has a similar inhibitory effect on the maintenance of established LTP as the CaMKII(273–302) used earlier (Fig. 5). In the above experiments the concentration of PKC(19–31) used was 100 μM and that of CaMKII(273–302) 200 μM. We have tested whether the greatly accelerated blocking effect on the maintenance of established LTP produced by the combination of PKC(19–31) and CaMKII(273–302) could be limited by simply increasing the concentration of PKC(19–31) alone. We found that 300 μM PKC(19–31) did not produce any more rapid blocking effect than 100 μM. This provides some extra assurance that the increased effect due to the addition of Ala^{286}CaMKII(273–302) must result from the additional inhibition of CaMKII.

Conclusion and discussion

In summary, by more fully exploring the dose–effect relationship of kinase inhibitors, by taking advantage of the newly discovered synergistic action of two inhibitors delivered together into the postsynaptic neuron, and by more patiently following the development of the effect of the inhibitors on established LTP for a longer time, we have been able to obtain some new experimental results, which call for a revision of certain conclusions by previous authors:

1. "PKC activation is necessary for maintenance but not for induction of LTP" (Reymann et al., 1988; Linden and Routtenberg, 1989).

2. "Intracellular postsynaptic delivery of either PKC(19–31) alone or CaMKII(273–302) alone or even a combination of PKC(19–31) and CaMKII(273–302) together were all ineffective with respect to established LTP" (Malinow et al., 1989; Malgaroli et al., 1992).

Our conclusions are:

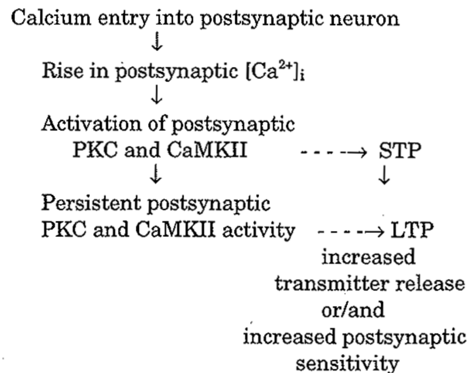
1. Polymyxin B given to the postsynaptic neuron in adequate dosage before the HFS can block completely the induction of LTP, leaving only the brief PTP. The two PKC inhibitors, polymyxin B and PKC(19–31), each at a relatively low dosage, when given together, can summate to give a high-dosage effect.

2. Either PKC(19–31) alone or CaMKII(273–302) alone given to the postsynaptic neuron before the HFS can block the induction of LTP; PKC(19–31) and CaMKII(273–302) given together act synergistically and block induction even more effectively.

3. Either PKC(19–31) alone or CaMKII(273–302) alone given to the postsynaptic neuron after the establishment of LTP can slowly arrest its continued maintenance; PKC(19–31) and CaMKII(273–302) given together to the postsynaptic neuron act synergistically, greatly accelerating the development of the blocking effect on the maintenance of LTP.

From the above contrasting conclusions the question naturally arises as to how to explain the difference between our results and those of others. The first point is easiest to explain: simply because we consciously paid attention to the dose–effect relationship and made a special effort to show that with adequate inhibition of the postsynaptic PKC the STP taken as an index of induction can be completely blocked. The second point of disagreement with Dr. Tsien's group concerning the inhibitory effect of PKC(19–31) and CaMKII(273–302) given separately to the postsynaptic neuron on the maintenance of established LTP at first also appeared to have a ready explanation: the blocking effect of these peptide inhibitors given separately to the postsynaptic neuron on the maintenance of established LTP takes about one hour to be detectable. Dr. Tsien's group usually terminated their observation about one hour after starting the intracellular delivery of the inhibitor, so they might well have missed the effect. But the real puzzle arises in connection with the inhibitory effect of the combined intracellular delivery of PKC(19–31) and CaMKII(273–302) on the maintenance of established LTP which in our hands began to develop in 10–20 min. Dr. Tsien's group report that expression of LTP is not inhibited by the combined postsynaptic delivery of PKC(19–31) and CaMKII(273–302) (Malgaroli et al., 1992). Furthermore they used microelectrodes containing 3 mM PKC(19–31) and 1 mM CaMKII(273–302) while we used 100 μM PKC(19–31) and 200 μM CaMKII(273–302).

In conclusion, on the basis of our own experimental results I submit a scheme for discussion and further experiments on LTP in the CA1 region:



It may be noted first that in the above scheme we do not limit calcium entry through the NMDA receptor channels, as the scheme is intended to be used also for discussing and planning experiments on other types of LTP that do not depend on NMDA receptors, e.g.

calcium-induced LTP. A comparison of the inhibitor sensitivity of different types of LTP has proven to be instructive (see Cheng et al., 1994), but this will not be dealt with in this chapter. It may also be pointed out that we place dashed arrows in the scheme above to indicate that multiple steps are likely involved.

The experimental results presented in this chapter do not concern directly the current debate about the locus of expression of LTP, that is, whether it is presynaptic due to increased release of neurotransmitter, or postsynaptic due to increased responsiveness of the postsynaptic structure. There are experimental supports for each of these possibilities and there is also evidence that both kinds of changes may occur during LTP with their relative importance varying with experimental conditions (Davies et al., 1989; Bekkers and Stevens, 1990; Malinow and Tsien, 1990; Reymann et al., 1990; Foster and McNaughton, 1991; Kullmann and Nicoll, 1992; Larkman et al., 1992; Liao et al., 1992; Malgaroli and Tsien, 1992; Manabe et al., 1992; Voronin et al., 1992; see also Bliss and Collingridge, 1993, for a review). Our experiments focus on the postsynaptic neuron, and what our results show and emphasize is that if the expression of LTP is presynaptic (whether wholly or partly) the sustaining of the presynaptic change started by some retrograde signal from the postsynaptic neuron must continue to depend on such signaling, at least for the initial few hours of LTP maintenance, and that the biochemical events in the postsynaptic neuron underlying this retrograde signalling must involve in an essential way some ongoing PKC and CaMKII activity. Regarding the possible nature of the retrograde signal, all attention has so far been given to diffusible messengers such as arachidonic acid (Williams and Bliss, 1989), NO (Zhuo et al., 1993) or CO (Stevens and Wang, 1993). But as pointed out by Lisman and Harris (1993), there are intimate structural links between the pre- and postsynaptic components in hippocampal synapses, and there might well be also some more direct way of retrograde communication across the synapse. This is as yet a speculation, but it is good to keep one's mind open.

The biochemical steps in which protein kinases participate in LTP are poorly understood. Only PKC and CaMKII are mentioned, simply because we have so far studied only these two kinases. There is now evidence also for the involvement of tyrosine kinase (O'Dell et al., 1991) and PKA (Roberson and Sweatt, 1993) in LTP. To achieve an understanding of the coordinated functioning of all the protein kinases in the postsynaptic neuron involved in LTP will be a real challenge for future work.

It would be nice to get direct biochemical evidence of persistent postsynaptic PKC or CaMKII activity during LTP. A number of biochemical studies have reported an increase in PKC activity during LTP. So far, most studies (Lovinger et al., 1987; Reymann et al., 1988a,b; Klann et al., 1993; Sacktor et al., 1993) have not addressed the question whether the increase is postsynaptic or presynaptic. When our inquiry relating to LTP becomes specifically concerned with the biochemical events in the postsynaptic neuron triggered by calcium entry, this question is critical. Fortunately, this issue is now beginning to receive attention. The PKC substrate protein RC3 (also called neurogranin) has been shown to be localized post-synaptically. Sweatt and his collaborators (Chen et al., 1993) have now reported that the maintenance phase of LTP in hippocampal CA1 area is associated with an increase in the phosphorylation of RC3 protein *in situ*, thus providing a first piece of biochemical evidence for persistent postsynaptic PKC activity during the maintenance of LTP.

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