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Calcium/calmodulin-dependent protein kinase II regulates hippocampal synaptic transmission

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Extracellular application of protein kinase inhibitors was used to examine the role of calcium/calmodulin-dependent protein kinase II (CaM-KII) in synaptic transmission in the CA1 region of rat hippocampus. Bath application of the broad spectrum, membrane permeable kinase inhibitor H7 (250 μM) decreased excitatory synaptic responses elicited in hippocampal slices. Whereas H7 inhibits several protein kinases and has non-specific effects, several synthetic peptides have been developed as specific inhibitors of CaM-KII. Using in situ phosphorylation in hippocampal slices, we demonstrate that extracellular application of synthetic peptide inhibitors of CaM-KII preferentially suppresses the phosphorylation of synapsin I at the CaM-KII specific site. This suppression was not reversed by the application of a calcium ionophore indicating the decrease in phosphorylation does not result only from blockade of presynaptic calcium influx. Thus, it appears the peptides gain access to intracellular compartments and retain their inhibitory properties. Further, we found that extracellular application of these peptide inhibitors decreased excitatory synaptic responses elicited in the CA1 region of hippocampal slices with relative potencies consistent with their ability to block CaM-KII activity in vitro. Peptide application did not alter the input resistance of postsynaptic cells nor responses elicited by glutamate iontophoresis. These results suggest that CaM-KII activity, possibly through phosphorylation of presynaptic synapsin I, is required for sustained synaptic transmission at mammalian synapses.

INTRODUCTION

Synaptic transmission is the foundation for information processing in the nervous system and modulation of synaptic transmission is believed to mediate learning and memory[2,10]. Although classic studies demonstrated that the release of neurotransmitter from presynaptic terminals requires calcium entry through voltage-sensitive channels[11,12], the precise mechanism by which calcium promotes release is unknown. Calcium is also involved in the modulation of synaptic transmission. For example, the transient facilitation that follows synaptic activation is due, at least in part, to elevated residual free calcium in the presynaptic terminal[32]. Recent studies using the squid giant synapse or the Mauthner neuron have shown that calcium may also influence synaptic transmission presynaptically via activation of calcium/calmodulin-dependent protein kinase II (CaM-KII)[7,15,16]. Presynaptic injection of CaM-KII enhances transmitter release while injection of dephospho-synapsin I, a major presynaptic substrate of CaM-KII, decreases release[15,16]. Furthermore, in vitro studies have shown that phosphorylation of synapsin I by CaM-KII greatly decreases its affinity for actin filaments and synaptic vesicles[28]. These data have led to the hypothesis that synapsin I phosphorylation frees synaptic vesicles from the cytoskeleton and thus prepares them for subsequent release[15,16]. In this view, calcium entry into presynaptic terminals promotes both neurotransmitter release via exocytosis of fusion competent or 'front row' vesicles and the preparation of vesicles for subsequent release through phosphorylation of synapsin I by CaM-KII.

To investigate the role of CaM-KII activity in regulating synaptic transmission in the mammalian brain, we have examined the effects of inhibitors of CaM-KII on synaptic transmission in the CA1 region of rat hippocampal slices. Because previously used kinase

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inhibitors (e.g. H7) are relatively non-specific and have had inconsistent effects on synaptic transmission[23,24], we have used synthetic peptide kinase inhibitors based on pseudosubstrate regions of the target kinases. We have used three peptides that differ in the way they inhibit CaM-KII. One, designated CBP (calmodulin binding peptide), inhibits CaM-KII by blocking both its activation by calcium/calmodulin and also by blocking its activity directly[4,13]. A second peptide, CBP_{-3}, lacks the active-site-directed inhibitory region and thus blocks only the activation of CaM-KII by binding to calcium/calmodulin. A third peptide, MB-II, specifically blocks only the activity of CaM-KII and does not bind calcium/calmodulin. Intracellular injection of these peptides into CA1 pyramidal cells has been used previously to demonstrate that the induction of LTP requires CaM and perhaps CaM-KII activity[18,22], suggesting that the peptides retain inhibitory activity intracellularly. Here we demonstrate that extracellular application of CaM-KII inhibitory peptides to hippocampal slices suppresses the in situ phosphorylation of a CaM-KII-specific site on synapsin I and attenuates synaptic transmission at hippocampal synapses.

MATERIALS AND METHODS

Synthetic peptides

Synthetic peptides were prepared on an Applied Biosystems 430A solid-phase peptide synthesizer and were analyzed as previously described[13]. CBP has the amino acid sequence MHRQETVDCLKKFNARRKLKGAILTTMLA-NH_2. The sequence of CBP_{-3} is QETVDCLKKFNARRKLKGAILTTMLA-NH_2, MB-II is MHRQEAVDCLKKFNARRKLKGA-NH_2, the control peptide CK-II long with no CaM-KII inhibitory activity in vitro is MHRQETVDCLKKFNA-NH_2, and PKC_i, the peptide that blocks PKC activity in vitro, is RFARKGALRQKNV. PKC_i is identical to that previously described[8]. CBP has two functional domains; calmodulin-binding (IC_{50} = 80 nM) and active-site-directed inhibition (IC_{50} = 2 μM). The active-site-directed inhibitory domain is not present in CBP_{-3}. MB-II contains only the active-site-directed inhibitory domain and only blocks CaM-KII activity (IC_{50} = 2.5 μM). Peptide stocks (10 mM) were made in electrophysiology buffer (see below) and diluted to the indicated final concentrations just before use.

Hippocampal slice preparation and electrophysiology

Hippocampal slices were prepared as described[26]. After rapidly removing the hippocampus from rats (3 to 10 weeks old) deeply anesthetized with sodium pentobarbitol (75 mg/kg), the hippocampus was placed in cold (4°C) buffer solution containing the following (in mM): NaCl 124, KCl 3.0, NaH_2PO_4 2.0, $MgCl_2$ 2.0, $CaCl_2$ 2.0, $NaHCO_3$ 26, dextrose 10, and HEPES (pH 7.35) 10. In some experiments the buffer contained: NaCl 119, KCl 2.5, NaH_2PO_4 1, $MgSO_4$ 1.3, $CaCl_2$ 2.5, $NaHCO_3$ 26.2, and dextrose 11. Both buffers were continuously bubbled with O_2/CO_2 (95/5%), and similar results were obtained with either buffer. Slices (400 μM) were prepared using a tissue chopper and were maintained in normal buffer at 30°C for at least 1 h before experiments were initiated. All experiments were performed in a standard submersion chamber with buffers perfused at a rate of 0.5–2 ml per min at a temperature of 30–34°C. Extracellular field potential recordings were obtained using pipette electrodes containing 3 M NaCl (2–7 MΩ). Responses were obtained by stimulating Schaffer collateral and commissural axons in the stratum radiatum of area CA1 and recording in an adjacent area of CA1. Responses were digitized and stored on computer for subsequent analyses.

Iontophoresis of glutamate was accomplished using a second electrode containing 10 mM monosodium glutamate (pH 7.5) positioned near the recording electrode. Constant current pulses lasting 200 ms and ranging from 1 to 4 μA were used to apply glutamate. The glutamate-evoked responses were recorded from the same electrode used for recording the synaptic responses, however, the amplifier was DC coupled during the glutamate responses.

Blind, whole-cell patch clamp recordings[1] were obtained using electrodes filled with a solution containing (in mM): NaCl 10; CsF 130; EGTA 10; HEPES (pH = 7.2) 10. Cells were voltage clamped between −70 and −75 mV throughout the experiment using the continuous mode of an Axoclamp 2A amplifier. Series resistance was 11–35 MΩ; input resistance was 180–250 MΩ.

In situ phosphorylation of hippocampal slices

The in situ $^{32}P_i$-labeling of hippocampal proteins in Kreb's low phosphate buffer (pH 7.3; 25 μM Na_2PO_4) was carried out as previously described[31]. HEPES (10 mM final concentration) was added to this buffer for better control of pH. Hippocampal slices (400 μm thick) were equilibrated for 30–40 min at 30°C before being labeled for 60 min in $^{32}P_i$ (0.5 mCi/ml buffer). The media bathing slices was gassed constantly with O_2/CO_2 (95/5%) prior to the addition of peptides or ionophore. Peptides were added from 10 mM stocks (in buffer) for 10 min before stopping the reactions. Calcium ionophore (A23187) was solubilized in DMSO and diluted 200-fold when used; this resulted in a final ionophore concentration of 10 μM and 0.5% (v/v) DMSO. Control experiments showed that 0.5% DMSO had no effect on protein phosphorylation (data not shown). Following peptide or ionophore additions, reactions were terminated by the rapid removal of radioactive media, and slices were immediately solubilized in 200 μl of SDS sample buffer pre-warmed to 70–75°C (this took 10–15 s).

SDS gel electrophoresis and peptide mapping

Equivalent amounts of protein from individual slices were analyzed by one-dimensional electrophoresis in SDS[31]. ^{32}P-labeled proteins were localized by autoradiography and subjected to limited proteolytic peptide mapping using *S. aureus* V8 protease as described[3]. Purified synapsin I was used to produce V8 maps after being phosphorylated in vitro by purified CaM-KII[31] or cAMP-dependent protein kinase[9]. Electrophoretically separated peptides were localized by autoradiography; individual ^{32}P-peptides were excised and CPMs were quantified by liquid scintillation spectroscopy. Each experimental variable was analyzed with pairs of hippocampal slices, and the synapsin I band (synapsin Ia and Ib) from each tissue slice was analyzed by two independent V8-maps.

Statistical analyses

Synaptic responses were normalized to the baseline 10 min prior to inhibitor application. In the in situ experiments the counts for each slice were normalized to the counts seen in control slices run with the same reagents and on the same gels. The in situ phosphorylation and synaptic physiology experiments required comparisons between group means and a baseline. Separate *t*-tests were used to make these comparisons. Where appropriate, a confidence level of 0.01 was used to correct for multiple comparisons, yielding an overall confidence level of less than 0.05. One-way randomized analyses of variance were used to make between-group comparisons. The Tukey test was used for post-hoc individual comparisons ($P < 0.05$). All confidence intervals are for two-tailed tests.

RESULTS

As an initial test as to whether kinase activity may be important for synaptic transmission the effects of the membrane permeable, broad-spectrum protein ki-

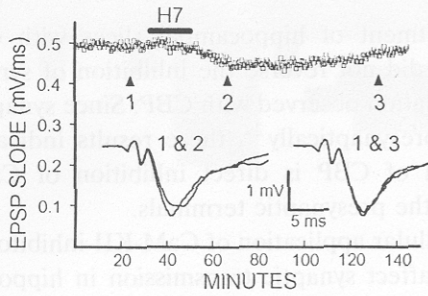

Fig. 1. Application of the broad spectrum protein kinase inhibitor H7 (250 μM) decreases synaptic responses recorded in CA1 region of hippocampus. The initial slope of the EPSPs elicited every 15 seconds are displayed over time. The sample responses were taken from the times indicated by the arrows. Similar results were obtain in a total of 16 experiments.

nase inhibitor H7 were examined. Application of H7 (250 μM, 15 min) produced a slow, but reliable decrease in the initial slope of the EPSPs (Fig. 1). The mean decrease in EPSP slope measured 30 min following the onset of application was $14 \pm 2.8\%$. This decrease was significantly different from baseline; $t_{15} = 4.83$, $P < 0.001$. The time course for reversal of the decrease was slow and in several experiments synaptic responses did not return to baseline within 1 h. These data replicate previous findings that H7 decreases baseline synaptic transmission[24].

Previous studies showed that extracellular application of CaM-binding synthetic peptides blocked intracellular CaM-dependent processes in vitro[6]. To determine whether extracellularly applied CaM-KII inhibitory peptides gain access to intracellular compartments in hippocampal slices we first analyzed the effects of inhibitory peptides on the in situ phosphorylation of synapsin I. If extracellularly applied CBP gains access to intracellular compartments then the in situ phosphorylation of synapsin I on the peptide fragment specifically modified by CaM-KII (i.e., serines 354 and/or 369 of site 2) should be inhibited. Fig. 2 shows that the in situ phosphorylation of synapsin I (site 2, 30 K peptide) was inhibited $75 \pm 4.5\%$ in the presence of 200 μM CBP compared to control slices. In contrast, the application of a pseudosubstrate inhibitor of PKC (PKC_i, 200 μM) inhibited synapsin I phosphorylation by only $14 \pm 13.3\%$. The decrease in phosphorylation produced by CBP was statistically reliable ($t_{13} = 61.29$) whereas the effect of PKC_i was not significant ($t_7 = 2.97$). Fig. 2A shows that two additional phosphopeptides (10 and 13 kDa) are generated by V8 proteolysis of synapsin I. The 10 kDa phosphopeptide appears to correspond to site 1 of synapsin I phosphorylated by PKA[9], and the 13 kDa phosphopeptide is most likely derived from the 87 kDa MARCKs protein that is a substrate for PKC[30]. The 30 kDa and 10 kDa phosphopeptides generated by V8 digestion of synapsin I phosphorylated in situ co-migrated with those obtained from pure synapsin I phosphorylated in vitro by purified CaM-KII or PKA, respectively (lanes 7 and 8, Fig. 2A). The phosphorylation of the 10 kDa fragment of synapsin I (site 1) was also inhibited by CBP; its phosphorylation decreased $30 \pm 10\%$ ($n = 12$). This re-

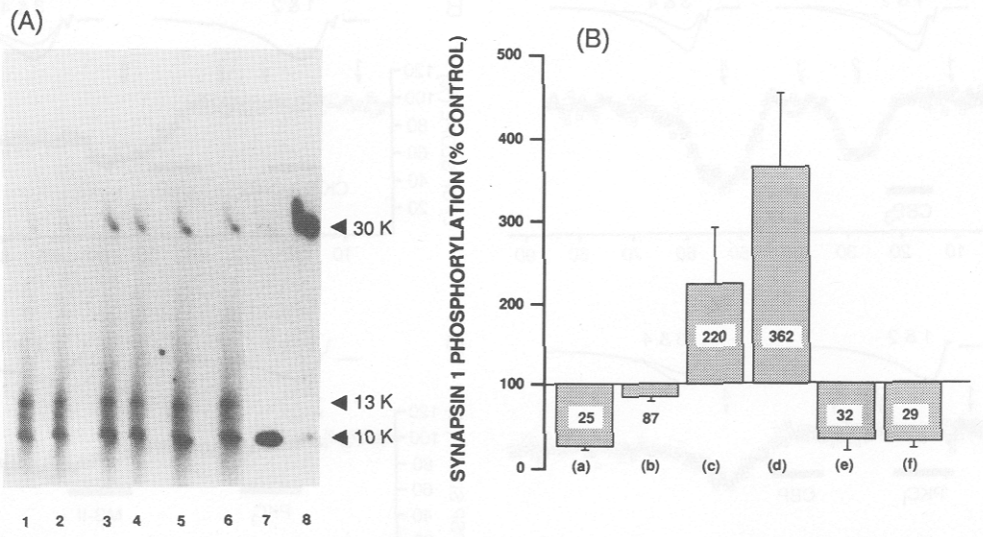

Fig. 2. Analysis of in situ phosphorylated synapsin I by limited digestion with V-8 protease. Pairs of hippocampal slices were phosphorylated under control conditions, or in 200 μM PKC_i for 10 min, or in 200 μM CBP for 10 min. Gel bands corresponding to synapsin I were subjected to V-8 proteolysis (2.5 μg/band). A: lanes 1&2 are from treatment with CBP; lanes 3&4 from treatment with PKC_i; lanes 5&6 from control reactions; lanes 7&8 are from purified synapsin I phosphorylated in vitro by purified PKA or CaM-KII, respectively. B: summary of effects of inhibitory peptides and/or calcium ionophore A23187 on the in situ phosphorylation of the 30 kDa site of synapsin I as determined by V8 protease mapping. Hippocampal slices were labeled with $^{32}P_i$ followed by incubations in: (a) CBP (200 μM) for 10 min ($n = 14$), (b) PKC_i (200 μM) for 10 min ($n = 8$), (c) A23187 (10 μM) for 2 min ($n = 4$), (d) A23187 (10 μM) for 5 min ($n = 4$), (e) CBP (200 μM) for 8 min followed by CBP (200 μM) plus A23187 (10 μM) for 2 min ($n = 4$), or (f) CBP (200 μM) for 5 min followed by CBP (200 μM) plus A23187 (10 μM) for 5 min ($n = 6$); values represent averages $\pm$ S.D.

sult is consistent with CBP's known bifunctional properties of being a potent calmodulin antagonist in addition to an active-site-directed inhibitor of CaM-KII. If extracellularly applied CBP gains access to intracellular compartments it should also decrease PKA activity by inhibiting the calcium/calmodulin-dependent adenylate cyclase which is present in high concentrations in brain[5], and reduce phosphorylation of site 1 as shown.

To examine the possibility that CBP inhibits synapsin I phosphorylation by simply blocking calcium entry into presynaptic terminals, we tested the effects of CBP in the presence of the calcium ionophore A23187. Treatment of slices with A23187 alone resulted in a large, time-dependent stimulation of synapsin I phosphorylation (site 2) following 2 or 5 min treatments (Fig. 2B). The increase in phosphorylation following 2 min application of A23187 was not significant ($t_3 = 3.75$), whereas the increase following 5 min application was reliable ($t_3 = 6.48$). When slices were pre-incubated in CBP for 10 min prior to the addition of A23187 for 2 or 5 min (Fig. 2B), CBP still inhibited the phosphorylation of synapsin I by $68 \pm 10\%$ and $70 \pm 7\%$, respectively. An analysis of variance revealed a significant difference between the six treatments shown in Fig. 2B ($F_{5,34} = 69.27$, $P < 0.001$). Pair-wise comparisons using the Tukey test revealed that (i) the three groups with CBP were reliably different from PKC_i and from the two A23187 groups ($P < 0.01$), and (ii) the three CBP groups were not significantly different from each other. Thus, treatment of hippocampal slices with calcium ionophore did not reverse the inhibition of synapsin I phosphorylation observed with CBP. Since synapsin I is localized presynaptically[29], these results indicate that one action of CBP is direct inhibition of CaM-KII activity in the presynaptic terminals.

Extracellular application of CaM-KII inhibitory peptides also affect synaptic transmission in hippocampal slices. Fig. 3A shows that the extracellular application of either CBP or CBP_{-3} reversibly attenuated excitatory postsynaptic potentials (EPSPs) evoked in CA1 pyramidal cells. Application of CK-II LONG, a peptide structurally similar to CBP, but ineffective in blocking CaM-KII activity *in vitro,* had no detectable effect on EPSPs (Fig. 3B). Fig. 3C compares the effects of CBP and a selective peptide inhibitor of PKC (PKC_i). Whereas CBP attenuated EPSPs at a concentration of 30 μM, PKC_i had no detectable effect at 100 μM. Fig. 3D contrasts the absence of a detectable effect with 100 μM PKC_i with the reversible attenuation of EPSPs produced by 50 μM MB-II, a selective inhibitor of CaM-KII *which does not bind calmodulin*[25]. Thus, all three peptides that inhibit CaM-KII reversibly attenuated synaptic transmission whereas a control peptide similar in structure but lacking inhibitory activity had no effect. Both CBP and CBP_{-3} more potently attenuated synaptic transmission than did MB-II; at 50 μM the average attenuation was $43 \pm 12\%$ ($n = 22$), $38 \pm 15\%$ ($n = 5$) and $31 \pm 5\%$ ($n = 11$), respectively. In

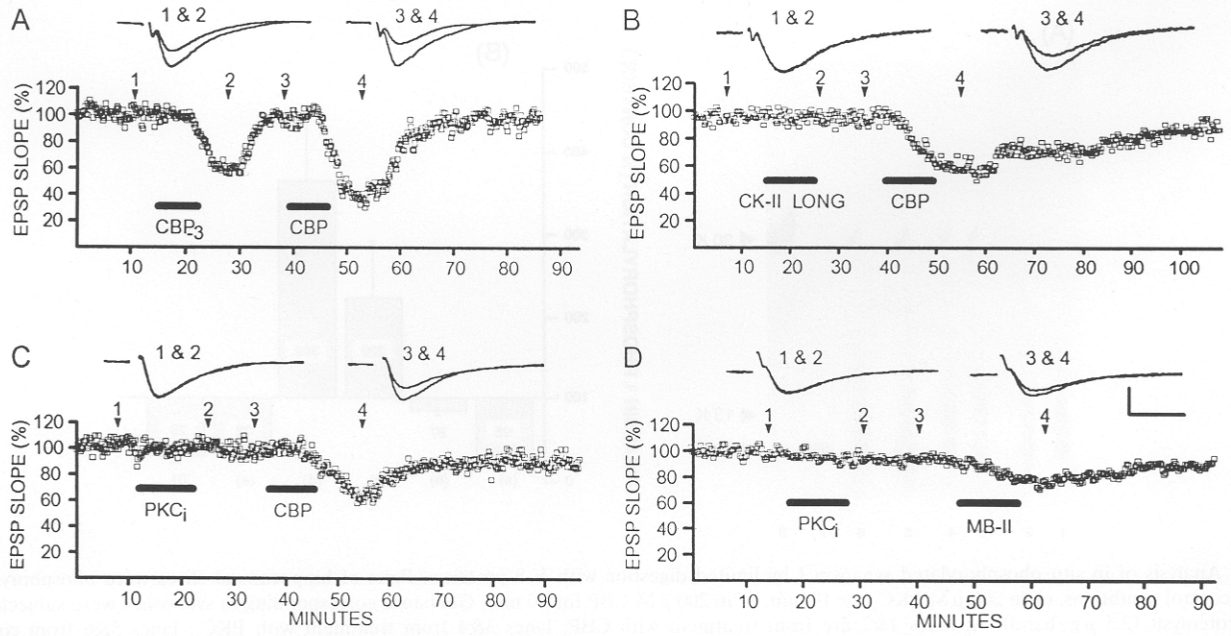

Fig. 3. The effects of extracellular application of peptides on the magnitude (initial slope) of EPSPs elicited in the CA1 region of hippocampal slices. In each experiment, two different peptides were applied sequentially to the same slice. A: application of CBP_{-3} or CBP (30 μM) reversibly attenuated synaptic transmission. B: application of CK-II long (100 μM) had no detectable effect on synaptic transmission, whereas CBP (30 μM) reversibly attenuated synaptic transmission. C: application of PKC_i (50 μM) had no detectable effect whereas CBP (30 μM) produced reversible attenuation. D: PKC_i (100 μM) had no detectable effect whereas MB-II (50 μM) reversibly attenuated synaptic transmission. Representative EPSPs were taken at the times indicated by arrows. Calibration bars represent 0.5 mV and 10 ms.

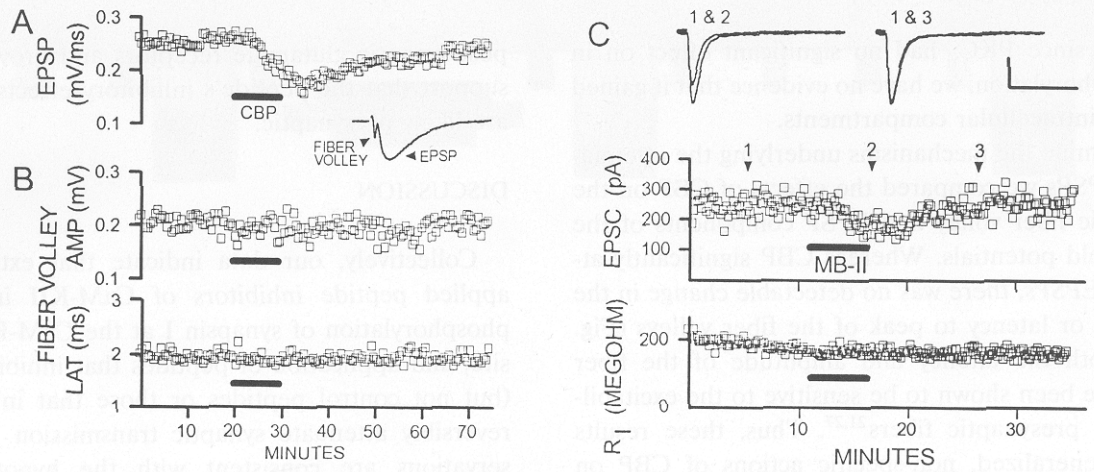

Fig. 4. Differential effects of CBP application on presynaptic fiber volleys and synaptic responses. A,B: an experiment comparing the attenuation of EPSPs produced by CBP (30 μM) and the absence of detectable effects on the presynaptic fiber component of the response. The inset shows a sample recording with fiber volley and EPSP components identified. Similar data were obtained in four additional experiments. C: whole cell patch recording demonstrating the effects of MB-II (50 μM) on excitatory postsynaptic currents (EPSCs) and on input resistance calculated from a 10 mV hyperpolarizing step. MB-II reversibly attenuated synaptic transmission without detectable effects on the holding current(not shown) or input resistance. Similar data were obtained in four additional experiments. Calibration bars represent 0.5 mV and 10 ms (A) and 100 pA and 20 ms (B).

general, this parallels the relative potencies of these peptides in inhibiting CaM-KII activity in vitro. The reductions for CBP, CBP_{-3} and MB-II were statistically significant ($P < 0.01$), whereas the effects of CK-II long and PKC_i were not. An analysis of variance revealed a significant difference among the five peptides ($F_{4,48} = 31.33$, $P < 0.001$) and pair-wise comparisons using the Tukey test revealed that (i) CBP, CBP_{-3} and MB-II were significantly different from CK-II long and PKC_i, and (ii) CBP, CBP_{-3} and MB-II were not significantly different from each other. Although previous studies have shown that activation of PKC by phorbol esters enhances both synaptic transmission[19] and transmitter release in hippocampal slices[20], we find that PKC_i, which selectively inhibits PKC activity in vitro, had no detectable effect on synaptic transmission.

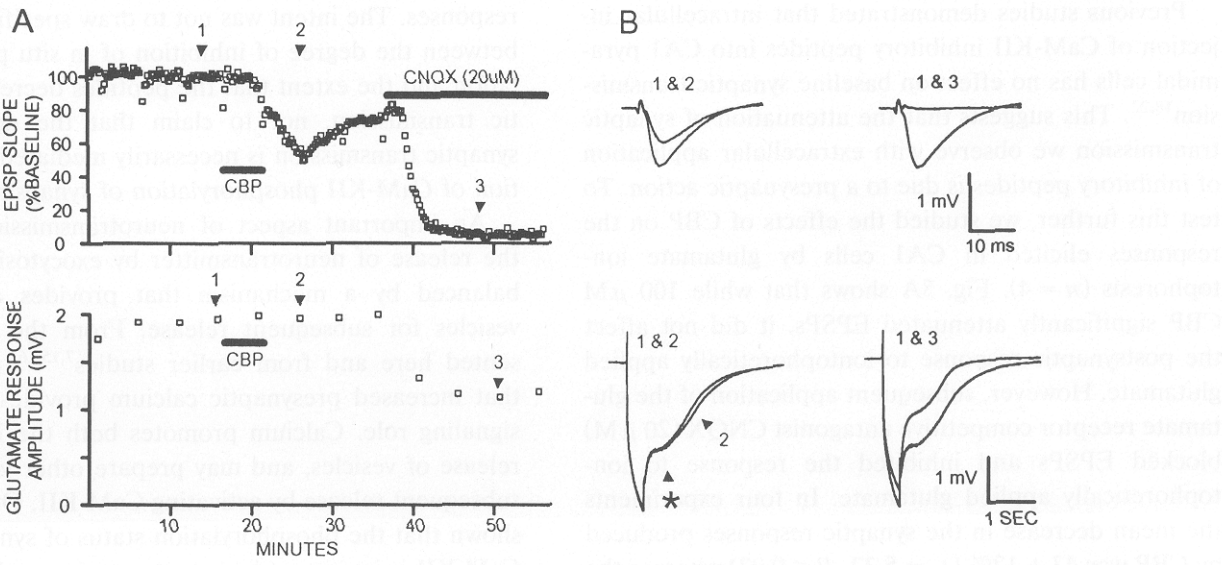

Fig. 5. A comparson of the actions of CBP on synaptic responses and responses elicited by glutamate iontophoresis in the same slice. The bathing solution contained the NMDA receptor antagonist APV (50 μM) to prevent the potentiation of responses. Synaptic responses were elicited every 15 s and the iontophoretic responses once every 5 min. A: applicaion of CBP (100 μM) produced robust attenuation of synaptic responses but had no detectable effect on iontophoresis responses. Subsequent application of CNQX (20 μM) decreased both responses. B: sample synaptic and iontophoretic responses taken from the times indicated in A. The amplitude of the iontophoretic responses was calculated at the time point marked by the asterisk in the sample sweeps. The early (first 200 ms) portion of the iontophoretic responses is made up of the glutamate-induced depolarization and the current artifact.

However, since PKC_i had no significant effect on in situ phosphorylation, we have no evidence that it gained access to intracellular compartments.

To examine the mechanisms underlying the attenuation of EPSPs we compared the effects of CBP on the presynaptic fiber volley and EPSP components of the evoked field potentials. Whereas CBP significantly attenuated EPSPs, there was no detectable change in the amplitude or latency to peak of the fiber volleys (Fig. 4A,B). Both the latency and amplitude of the fiber volley have been shown to be sensitive to the excitability of the presynaptic fibers[21,27]. Thus, these results exclude generalized, non-specific actions of CBP on the action potential-generating properties and membrane potential of the presynaptic fibers.

Whole-cell patch clamp recordings were used to identify possible postsynaptic changes in membrane conductance or input resistance produced by peptide application. In these experiments, each synaptic response was preceded by a 10 mV hyperpolarizing step to determine input resistance. These recordings ($n = 5$) revealed that MB-II produced no consistent effects on input resistance or holding current (not shown) but produced a robust and reversible suppression of EPSCs (Fig. 4C). Similar results were obtained with CBP ($n = 5$, data not shown). These experiments demonstrate that extracellular application of peptide inhibitors of CaM-KII does not produce detectable changes in the excitability of the presynaptic fibers or in the general membrane properties of the postsynaptic neurons.

Previous studies demonstrated that intracellular injection of CaM-KII inhibitory peptides into CA1 pyramidal cells has no effect on baseline synaptic transmission[18,22]. This suggests that the attenuation of synaptic transmission we observe with extracellular application of inhibitory peptides is due to a presynaptic action. To test this further, we studied the effects of CBP on the responses elicited in CA1 cells by glutamate iontophoresis ($n = 4$). Fig. 5A shows that while 100 μM CBP significantly attenuated EPSPs, it did not affect the postsynaptic response to iontophoretically applied glutamate. However, subsequent application of the glutamate receptor competitive antagonist CNQX (20 μM) blocked EPSPs and inhibited the response to iontophoretically applied glutamate. In four experiments the mean decrease in the synaptic responses produced by CBP was $43 \pm 12\%$ ($t_3 = 5.32$, $P < 0.02$) whereas the iontophoretic responses increased $15 \pm 11\%$ ($t_3 = 1.89$, $P > 0.05$). CNQX significantly reduced both the synaptic and iontophoretic responses (91%, $t_3 = 63.27$, $P < 0.001$ and 34%, $t_3 = 6.93$, $P < 0.01$ respectively). These results suggest that CBP is not simply an antagonist of post-synaptic glutamate receptors and provide further support that the peptide's inhibitory effects on EPSPs are likely presynaptic.

DISCUSSION

Collectively, our data indicate that extracellularly applied peptide inhibitors of CaM-KII inhibits the phosphorylation of synapsin I at the CaM-KII specific site, and application of peptides that inhibit CaM-KII (but not control peptides or those that inhibit PKC) reversibly attenuate synaptic transmission. These observations are consistent with the hypothesis that synapsin I phosphorylation by CaM-KII releases vesicles from the cytoskeleton making them available for subsequent release[15,16]. An additional possibility is that CaM-KII-dependent phosphorylation is directly involved in the actual exocytosis of synaptic vesicles. However, to fulfill this role CaM-KII activation and substrate phosphorylation would have to occur within the temporal constraints of synaptic delay (< 1 ms), which seems unlikely[14].

It is important to point out that the purpose of the in situ experiments was to test whether peptides that are applied extracellularly can gain access to intracellular compartments and retain their specific inhibitory activity. The sensitivity of in situ phosphorylation/V8 digest analyses (site 1 of synapsin I) is modest and we did not expect that subtle changes could be detected. Thus, the concentrations of peptide used (200 μM) were higher than those required to attenuate synaptic responses. The intent was not to draw specific parallels between the degree of inhibition of in situ phosphorylation and the extent that the peptides decrease synaptic transmission, nor to claim that the decrease in synaptic transmission is necessarily mediated by inhibition of CaM-KII phosphorylation of synapsin I.

An important aspect of neurotransmission is that the release of neurotransmitter by exocytosis must be balanced by a mechanism that provides a pool of vesicles for subsequent release. From the data presented here and from earlier studies[7,15,16] it appears that increased presynaptic calcium provides this dual signaling role. Calcium promotes both the immediate release of vesicles, and may prepare other vesicles for subsequent release by activating CaM-KII. Others have shown that the phosphorylation status of synapsin I by CaM-KII is important in neurotransmitter release from vertebrate and invertebrate synapses[7,15,16]. In addition, a peptide inhibitor of CaM-KII identical to MB-II used in our studies, attenuates neurotransmitter release from cortical synaptosomes[25]. Variations in the phosphorylation status of the total pool of synapsin I, as deter-

mined by CaM-KII activity, represents one possible mechanism for mediating activity-dependent changes in the magnitude of synaptic transmission[17]. These variations could alter the number of vesicles available for release or the probability of release for individual vesicles. Whereas transient changes in synapsin I phosphorylation might in part mediate paired-pulse facilitation and/or post-tetanic potentiation[32], it is also possible in principle that a longer-lasting change in CaM-KII activity and/or synapsin I phosphorylation could mediate the persistent expression of long-term potentiation[22].

Although extracellular application of CaM-KII inhibitory peptides can reduce the in situ phosphorylation of synapsin I, there is no direct evidence that the observed attenuation of synaptic transmission is mediated via decreased synapsin I phosphorylation. Indeed, the in situ phosphorylation experiments demonstrated only that extracellular application of the peptides inhibits intracellular kinase activity. Despite other evidence for the involvement of synapsin I in synaptic transmission[15,16], the attenuation of synaptic transmission we observed may be mediated by inhibiting the phosphorylation of substrates other than, or in addition to synapsin I. The elucidation of additional presynaptic and postsynaptic substrates for CaM-KII may provide important insights on the molecular mechanisms that regulate synaptic transmission.

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