# $\gamma$-Aminobutyric acid type A receptors modulate cAMP-mediated long-term potentiation and long-term depression at monosynaptic CA3–CA1 synapses

**Tzu-ping Yu, Sheri McKinney*, Henry A. Lester, and Norman Davidson†**

Division of Biology, 1200 East California Boulevard, California Institute of Technology, Pasadena, CA 91125

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**cAMP induces a protein-synthesis-dependent late phase of long-term potentiation (LTP) at CA3–CA1 synapses in acute hippocampal slices. Herein we report cAMP-mediated LTP and long-term depression (LTD) at monosynaptic CA3–CA1 cell pairs in organotypic hippocampal slice cultures. After bath application of the membrane-permeable cAMP analog adenosine 3′,5′-cyclic monophosphorothioate, Sp isomer (Sp-cAMPS), synaptic transmission was enhanced for at least 2 h. Consistent with previous findings, the late phase of LTP requires activation of cAMP-dependent protein kinase A and protein synthesis. There is also an early phase of LTP induced by cAMP; the early phase depends on protein kinase A but, in contrast to the later phase, does not require protein synthesis. In addition, the cAMP-induced LTP is associated with a reduction of paired-pulse facilitation, suggesting that presynaptic modification may be involved. Furthermore, we found that Sp-cAMPS induced LTD in slices pretreated with picrotoxin, a $\gamma$-aminobutyric acid type A ($GABA_A$) receptor antagonist. This form of LTD depends on protein synthesis and protein phosphatase(s) and is accompanied by an increased ratio of failed synaptic transmission. These results suggest that $GABA_A$ receptors can modulate the effect of cAMP on synaptic transmission and thus determine the direction of synaptic plasticity.**

Long-term potentiation (LTP) and long-term depression (LTD) are persistent and activity-dependent modifications in synaptic efficacy that are thought to underlie some mechanisms for memory (1). Considerable evidence has suggested the involvement of the cAMP-signaling pathway and of cAMP-dependent transcription in both forms of synaptic plasticity (2–6). At least two phases of LTP have been reported in CA1 of the hippocampus: an early phase that occurs immediately after tetanus and a late phase that usually begins 2–3 h after induction. There is general agreement that the early phase of LTP does not require protein synthesis and that the late phase requires both transcription and translation (3, 7). Stimuli that induce LTP can result in a rise in cAMP level (8), activation of cAMP-dependent protein kinase A (PKA) (9, 10), and phosphorylation of some PKA substrates (11). Despite these correlative data and despite the general agreement that the late phase of LTP requires cAMP and activation of PKA (3, 4), there is no general agreement that the early phase of LTP requires the cAMP pathway. Some lines of pharmacological and genetic evidence suggest that the early phase of LTP does not require the cAMP pathway (12–14); however, another pharmacological study suggests that PKA is required (15).

In this study, we investigated the cAMP-induced synaptic plasticity at monosynaptic CA3–CA1 synapses by using organotypic hippocampal slice cultures that retain hippocampal cytoarchitecture and apparently develop additional synaptic connections from CA3 to CA1 cells (16). We used the membrane-permeable analog of cAMP, adenosine 3′,5′-cyclic monophosphorothioate, Sp isomer (Sp-cAMPS), as an agent for inducing cAMP-mediated synaptic plasticity because Sp-cAMPS can selectively induce a protein-synthesis-dependent late phase of field LTP in CA1 in acutely prepared hippocampal slices (3). At monosynaptic CA3–CA1 synapses, in addition to a protein-synthesis-dependent late phase of LTP, we observed an early phase of LTP induced by Sp-cAMPS. This early phase of LTP requires PKA but is independent of protein synthesis. We have also observed a cAMP-mediated LTD in slices pretreated with picrotoxin, a $\gamma$-aminobutyric acid type A ($GABA_A$) receptor antagonist. This form of LTD can be blocked by the protein phosphatase inhibitor calyculin A, suggesting a requirement for activation of protein phosphatases. The protein synthesis inhibitor anisomycin prevents the induction of this form of LTD, indicating a dependence on protein synthesis.

## Materials and Methods

Hippocampal slices were obtained from P7–P9 rats and plated on membrane inserts (MilliCell-CM, from Millipore), as described (17). After at least 14 days in culture, slices were transferred to the recording chamber, which was mounted on an upright Olympus BX50WI microscope. Slices in the recording chamber were continuously perfused with oxygenated artificial cerebrospinal fluid (ACSF) at 32°C for at least 30 min before recording. ACSF contained 149 mM NaCl, 2.7 mM KCl, 2.8 mM $CaCl_2$, 2.0 mM $MgCl_2$, 11.6 mM $NaHCO_3$, 0.4 mM $NaH_2PO_4$, and 5.6 mM glucose, adjusted to pH 7.4 with NaOH; the osmolarity was adjusted to 320 milliosmolar with sucrose.

Two sharp electrodes filled with 1 M potassium methyl sulfate (with a resistance of 80–100 MΩ) were placed in CA3 and CA1 regions, under a ×10 water immersion lens. The electrode tips were then inserted into CA3 and CA1 pyramidal cells by using electrophysiological cues without visualization of individual cells. Recordings of both pre- and postsynaptic responses were made with an Axoclamp 2A (Axon Instruments, Foster City, CA). Single action potentials were elicited in CA3 cells by injection of depolarizing current pulses (10–15 ms, 0.4–0.6 nA). Excitatory postsynaptic potentials (EPSPs) of a single CA1 cell were evoked by single action potentials elicited in a synaptically connected CA3 cell. The criteria for monosynaptic connections between cell pairs included relatively brief (i.e., less than 3 ms) and constant latencies, as described elsewhere (16, 18). The membrane resistances of both pre- and postsynaptic cells were

Abbreviations: LTP, long-term potentiation; LTD, long-term depression; EPSP, excitatory postsynaptic potential; Sp-cAMPS, adenosine 3′,5′-cyclic monophosphorothioate, Sp isomer; Rp-cAMPS, adenosine 3′,5′-cyclic monophosphothioate, Rp isomer; PKA, protein kinase A; $GABA_A$, $\gamma$-aminobutyric acid type A; ACSF, artificial cerebrospinal fluid; sEPSP, spontaneous EPSP.

*Present address: Cortex Pharmaceuticals Inc., 15231 Barranca Parkway, Irvine, CA 92618.

†To whom reprint requests should be addressed. E-mail: normand@caltech.edu.

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monitored during the experiments by injection of hyperpolarizing current pulses (100 ms, 0.2 nA). Baseline responses were recorded for at least 20 min before drug application. Paired-pulse facilitation was measured by application of paired current pulses (0.4 nA, 10 ms in duration at 50-ms intervals) to presynaptic CA3 cells. The amount of paired-pulse facilitation was expressed as the percent increase in the amplitude of the second response relative to the first. Recordings were filtered at 3 kHz, digitized at 10 kHz (TL-1, DMA Interface, Axon Instruments), acquired with PCLAMP 6 (Axon Instruments), and analyzed off-line with CLAMPFIT 8.1 (Axon Instruments) and Microcal ORIGIN 6.0 (Microcal Software, Northampton, MA). Data were presented as mean ± SEM. An unpaired Student's $t$ test was used to compare EPSP amplitudes measured in the control group with those in drug-treated groups, and a paired Student's $t$ test was used to compare responses obtained before and after drug treatments in the same group. Differences were considered significant at the level of $P < 0.05$ or $P < 0.01$, as indicated.

Sp-cAMPS (Calbiochem) and adenosine 3′,5′-cyclic monophosphothioate, Rp isomer (Rp-cAMPS, Calbiochem) were prepared as 10 mM stock solutions and stored at −20°C. Anisomycin (Sigma) and calyculin A (Calbiochem) were prepared as concentrated stock solutions in DMSO and then diluted to a final concentrations of 20 $\mu$M and 1 $\mu$M, respectively (for both, final concentration of DMSO was 0.1%), before application. Picrotoxin was obtained from Sigma.

## Results

**Sp-cAMPS Induces LTP at Monosynaptic CA3–CA1 Cell Pairs.** CA3–CA1 cell pairs in stratum pyramidale of organotypic hippocampal slice cultures were recorded simultaneously (Fig. 1*A*). We tested these cell pairs for synaptic connection by passing a depolarizing pulse that evoked an action potential in the CA3 cell. Monosynaptic EPSPs were then accepted based on the criteria of brief (typically less than 3 ms) and constant latencies after the peak of the presynaptic action potential (Fig. 1*A*). Bath application of Sp-cAMPS (50 $\mu$M, 15–20 min) facilitated synaptic transmission at CA3–CA1 synapses (Fig. 1*B*). The increase in amplitude of evoked EPSPs reached a plateau within 30 min after treatment with Sp-cAMPS and lasted for at least 2 h (150 ± 3%, $n$ = 5). Paired-pulse facilitation is a widely accepted index of presynaptic function (19); this parameter decreased from 29% under baseline conditions to 10–16% after exposure to Sp-cAMPS (Fig. 2), suggesting that a presynaptic mechanism may contribute to the cAMP-induced LTP.

**The Late Phase of LTP Requires Protein Synthesis.** Previous reports showed that the expression of the late phase of LTP requires protein synthesis (3, 20). We therefore tested whether the LTP induced by Sp-cAMPS in our experimental conditions required protein synthesis. Anisomycin (20 $\mu$M) was included in the perfusion solution for 30 min before Sp-cAMPS was applied and for at least 40 min after Sp-cAMPS had been washed out. We found that LTP induced by Sp-cAMPS could be characterized by two phases, one independent of protein synthesis and one dependent on protein synthesis. In measurements of evoked EPSP amplitude, the early phase (i.e., 40–60 min after Sp-cAMPS treatment) of LTP was unaffected by anisomycin (130 ± 5%, $n$ = 4), whereas the late phase (i.e., 100–120 min after Sp-cAMPS application) of LTP was blocked (107 ± 4%, $n$ = 4) (Fig. 3 *A* and *C*).

**cAMP-Induced LTP Is PKA-Dependent.** To investigate whether both early and late phases of LTP induced by Sp-cAMPS are mediated through PKA, slices were treated with the PKA inhibitor Rp-cAMPS (100 $\mu$M), for at least 30 min before and during application of Sp-cAMPS. In the presence of Rp-cAMPS, both early and late phases of LTP were blocked (114 ± 3%, $n$ = 4; and 111 ± 2%, $n$ = 4, respectively) (Fig. 3 *B* and *C*). These results show that, under our conditions, the induction of cAMP-mediated early and late phases of LTP requires activation of PKA.

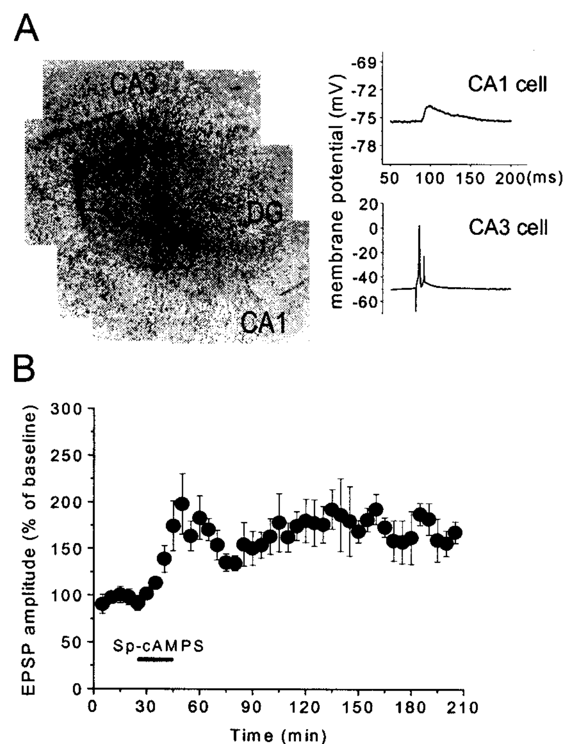


**Fig. 1.** cAMP-induced long-term synaptic potentiation at a monosynaptic CA3–CA1 cell pair in hippocampal slice cultures. (*A*) Paired recordings of presynaptic and postsynaptic cells were obtained from CA3 and CA1 regions, respectively, of a hippocampal slice culture. DG, dentate gyrus. A typical EPSP of a CA1 cell could be evoked by an action potential elicited in a synaptically connected CA3 cell. (*B*) Summary graph illustrating a long-lasting synaptic enhancement of EPSP amplitude (measured as percentage of the baseline) when Sp-cAMPS (50 $\mu$M, 15 min) was applied to the bath. Each point is the average of five experiments. Error bars indicate ± SEM.

**Effect of Sp-cAMPS on Spontaneous EPSPs.** In addition to potentiation of evoked EPSPs, we also observed increased spontaneous activities in CA1 cells after application of Sp-cAMPS. The average increases in amplitude of spontaneous EPSPs (sEPSPs) during the early and late phases of LTP were 194 ± 43% ($n$ =

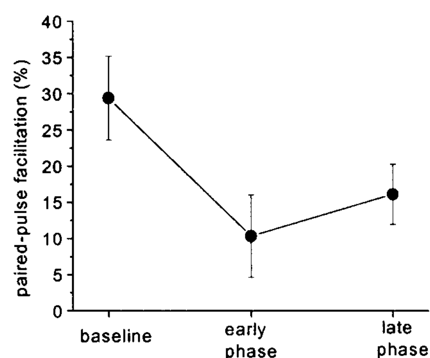


**Fig. 2.** cAMP-induced LTP is associated with a reduction in paired-pulse facilitation during both early (40–60 min after Sp-cAMPS) and late phases (100–120 min after Sp-cAMPS). Each point is the average of three experiments. Error bars indicate ± SEM.

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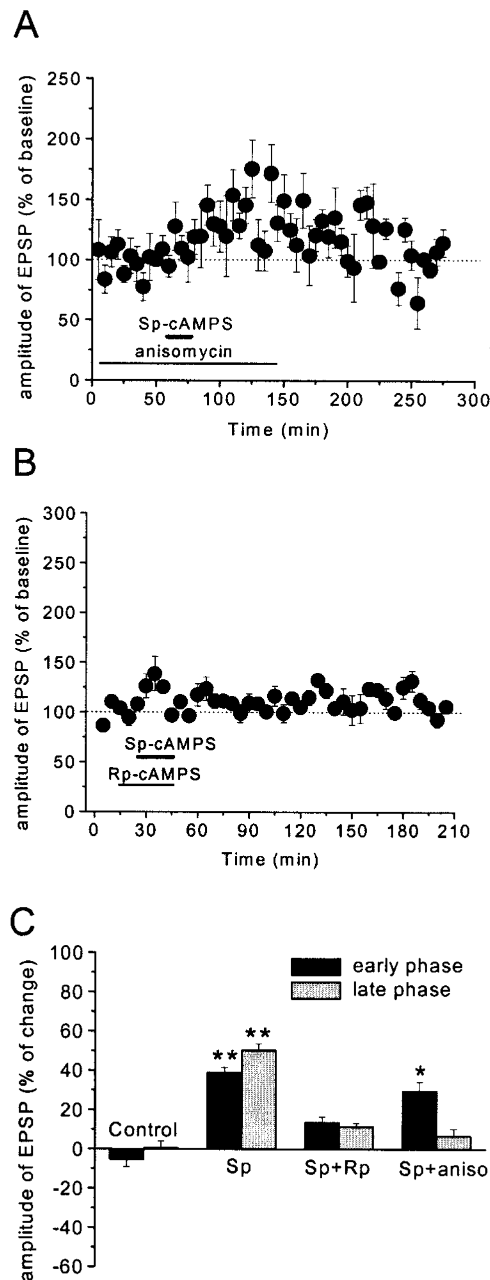


**Fig. 3.** Induction of cAMP-mediated LTP requires PKA and the late phase of LTP depends on protein synthesis. (*A*) Application of anisomycin (20 $\mu$M) blocked the late phase of Sp-cAMPS-induced LTP without affecting the early phase. Data are the average of four experiments. (*B*) Sp-cAMPS (50 $\mu$M) could not induce synaptic enhancement in slices treated with Rp-cAMPS (100 $\mu$M). (*C*) Summary of the effect of Sp-cAMPS (Sp) on synaptic transmission in slices treated with Rp-cAMPS (Rp) or anisomycin (aniso), compared with the control (pseudo perfusion). Values are percent changes in amplitude of EPSP measured at the early phase (40–60 min after Sp) and the late phase (100–120 min after Sp). *, $P < 0.05$; **, $P < 0.01$, compared with the respective control. (*A*–*C*) Error bars indicate ± SEM.

4) and 373 ± 47% ($n = 4$), respectively (Fig. 4*A*). The frequency of sEPSPs increased significantly from 1.2 ± 0.2 Hz (the baseline, $n = 4$) to 3.3 ± 0.3 Hz (the early phase, $n = 4$, $P < 0.05$) and 8.1 ± 3.2 Hz (the late phase, $n = 4$, $P < 0.05$) (Fig. 4*B*). The increases in amplitude of sEPSPs associated with Sp-cAMPS-induced LTP were inhibited by Rp-cAMPS (Fig. 4*C*) and by anisomycin (Fig. 4*D*).

**Sp-cAMPS Induces LTD in the Presence of Picrotoxin.** In one experiment, we fortuitously impaled a presynaptic interneuron in the CA3 area. The induced action potential evoked an inhibitory postsynaptic potential in a CA1 cell. We then observed that Sp-cAMPS decreased the amplitude of the evoked inhibitory postsynaptic potential. This depression recovered within 1 h after Sp-cAMPS was washed out with normal ACSF (data not shown). This result drew our attention to involvement of inhibitory synaptic transmission in Sp-cAMPS-induced LTP and to the modulation of $GABA_A$ receptors by phosphorylation (21, 22). We therefore examined the effect of Sp-cAMPS on synaptic transmission in the presence of picrotoxin, a $GABA_A$ receptor antagonist.

Slices were pretreated with 20–50 $\mu$M picrotoxin before application of Sp-cAMPS. Under these conditions, Sp-cAMPS no longer induced LTP; instead, a persistent LTD gradually developed and reached the maximum depression about 60 min after the treatment (to 57 ± 2% of baseline EPSP, $n = 5$; Fig. 5 *A* and *D*). In contrast, application of picrotoxin alone caused little or no depression of synaptic transmission (87 ± 4%, $n = 4$; Fig. 5*D*). During this form of LTD, single presynaptic action potentials in CA3 cells led to 35% of failed EPSPs in CA1 cells, compared with 4% during baseline recordings (Fig. 5*B*). This result suggests that an increased failure rate may partially account for LTD.

**LTD Requires Activation of Protein Phosphatases and Protein Synthesis.** Several investigators have proposed that hippocampal LTD involves activation of protein phosphatases (23, 24). We therefore tested whether the LTD induced by concurrent application of Sp-cAMPS and picrotoxin was mediated through protein phosphatases. Slices were pretreated with 1 $\mu$M calyculin A, an inhibitor of protein phosphatases 1 and 2A, for 1 h before application of Sp-cAMPS and picrotoxin. The results showed modest facilitation in slices pretreated with calyculin A (119 ± 2%, $n = 3$), compared with the robust LTD in slices exposed to Sp-cAMPS and picrotoxin but not exposed to calyculin A (57 ± 2%, $n = 5$, $P < 0.01$) (Fig. 5 *A* and *D*).

We also examined whether LTD induced by concurrent application of Sp-cAMPS and picrotoxin required protein synthesis. In these experiments, anisomycin was included in the ACSF for 10 min before application of picrotoxin, during perfusion of picrotoxin and Sp-cAMPS, and for 40 min after picrotoxin and Sp-cAMPS had been washed out. In slices pretreated with anisomycin, combined application of Sp-cAMPS and picrotoxin did not induce LTD, either at the early stage (i.e., 40–60 min after Sp-cAMPS treatment, 110 ± 3%, $n = 5$) or at the late stage (i.e., 100–120 min after Sp-cAMPS treatment, 116 ± 1%, $n = 5$) (Fig. 5 *C* and *D*).

## Discussion

There are two main findings in this study. First, two distinct phases of cAMP-mediated LTP can be characterized at monosynaptic CA3–CA1 synapses by the requirement for protein synthesis: a protein-synthesis-independent early phase of LTP and a protein-synthesis-dependent late phase of LTP. Both phases require activation of PKA. Second, a form of LTD can be induced by cAMP in slices pretreated with a $GABA_A$ receptor antagonist. This form of LTD requires protein synthesis and activation of protein phosphatases. These results demonstrate an important role of $GABA_A$ receptors in modulating and determining the direction of cAMP-induced synaptic plasticity.

Several reports have shown that cAMP can selectively induce a late phase of LTP in CA1 of acute hippocampal slices (3, 4, 20). The measurements made at monosynaptic CA3–CA1 synapses in the present study reveal a protein-synthesis-independent early phase (40–60 min) of cAMP-induced LTP. There is a general agreement that a late phase of LTP (100–120 min) requires the

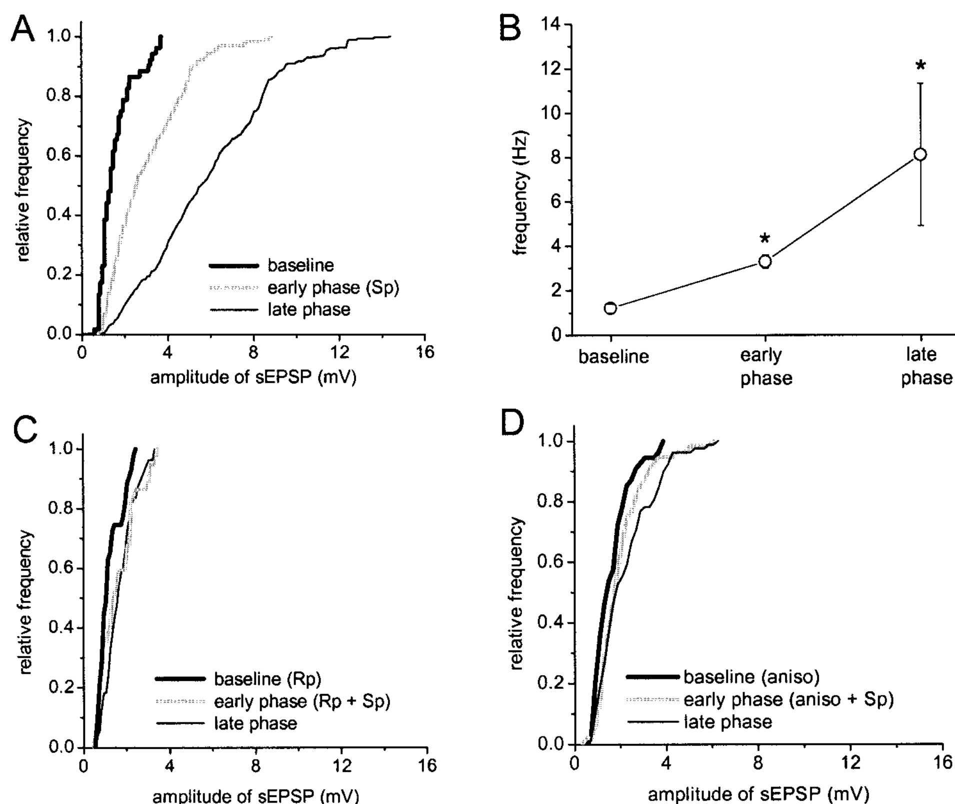


**Fig. 4.** Effect of Sp-cAMPS on sEPSP. (*A*) Cumulative sEPSP amplitude distributions show that cAMP-mediated LTP is associated with significant increases in amplitudes of sEPSPs recorded both during the early phase (40–60 min after Sp, shaded line) and during the late phase (100–120 min after Sp, thin line), compared with the baseline (thick line). (*B*) Frequency of sEPSPs was increased during both early and late phases of LTP. *, $P < 0.05$, compared with the baseline. (*C*) No significant increase in the amplitude of sEPSPs was observed when Rp-cAMPS (Rp, 100 μM) was coapplied with Sp-cAMPS (50 μM). (*D*) Anisomycin (aniso, 20 μM) prevented the Sp-cAMPS-associated increase in the amplitude of sEPSPs. Error bars indicate ± SEM.

activation of adenylyl cyclase and cAMP-dependent PKA. However, a role of PKA in an early phase of LTP has been confirmed only recently (15). By using a broad range of inhibitors, combinations of inhibitors, and direct methods for introducing inhibitors into the postsynaptic cell, Otmakhova *et al.* (15) reported that inhibition of the cAMP pathway indeed decreased an early phase of LTP. The early phase of cAMP-induced LTP observed in the present study can be blocked by a PKA inhibitor, and this result adds support to the notion that the PKA signaling pathway participates in induction of the early phase of LTP.

LTD may have distinguishable phases that are analogous to LTP. The form of LTD induced by our protocol, namely, concurrent application of Sp-cAMPS and picrotoxin, develops gradually and reaches the maximum depression 60 min after the treatment. Although we did not directly address whether multiple phases of LTD could be induced by our protocol, the time course of induction and the complete blockade of LTD by the protein synthesis inhibitor anisomycin appear to suggest a late phase of LTD induced selectively in our experimental condition. In fact, a late phase protein-synthesis-dependent LTD in hippocampal slice cultures has been reported (25). Nevertheless, we cannot rule out the possibility that LTD induced by concurrent application of Sp-cAMPS and picrotoxin may include an early phase of LTD that requires protein synthesis, presumably locally and within 30 min after the drug treatment (26–28).

Activation of the cAMP cascade enhances evoked and spontaneous release of neurotransmitter in Schaffer/commissural terminals in CA1 of acute hippocampal slices (29), as indicated by a significant increase in frequency of miniature excitatory postsynaptic currents. Quantal analysis of unitary synaptic transmission between CA3–CA1 synapses also suggests that the cAMP-induced late phase of LTP involve an increase in the number of quanta released in response to a single presynaptic action potential (20). Such an increase may be a consequence of new release sites resulting from membrane insertion into a preexisting presynaptic terminal of new active zones that can synchronously release transmitters (20). This hypothesis has been further supported by the finding of Sp-cAMPS-induced increases in the number of functional presynaptic terminals at dissociated CA3–CA1 neuronal cultures (30). In the present study, at monosynaptic CA3–CA1 synapses studied by intracellular recordings from single CA3 and CA1 cells, the cAMP-induced LTP was associated with a reduction of paired-pulse facilitation, which also supports the notion that Sp-cAMPS induces LTP by increasing neurotransmitter release from presynaptic terminals.

Although we interpret our results in terms of activation of an intracellular PKA pathway by Sp-cAMPS, it remains possible that extracellularly applied Sp-cAMPS or a metabolite may affect synaptic transmission by acting extracellularly to activate or inhibit adenosine receptors (31–36). This hypothesis is unlikely in view of our observations that the PKA inhibitor Rp-cAMPS completely blocked the induction of LTP by Sp-cAMPS, indicating that Sp-cAMPS is acting intracellularly on the PKA pathway.

Our results also suggest that Sp-cAMPS may act on inhibitory synaptic transmission in addition to excitatory synaptic transmission. If Sp-cAMPS indeed inhibits $GABA_A$ receptors and thus subsequently enhances synaptic transmission, one expects no further facilitation of synaptic transmission induced by Sp-cAMPS when the action of $GABA_A$ receptors is removed by application of picrotoxin. As expected, in the presence of

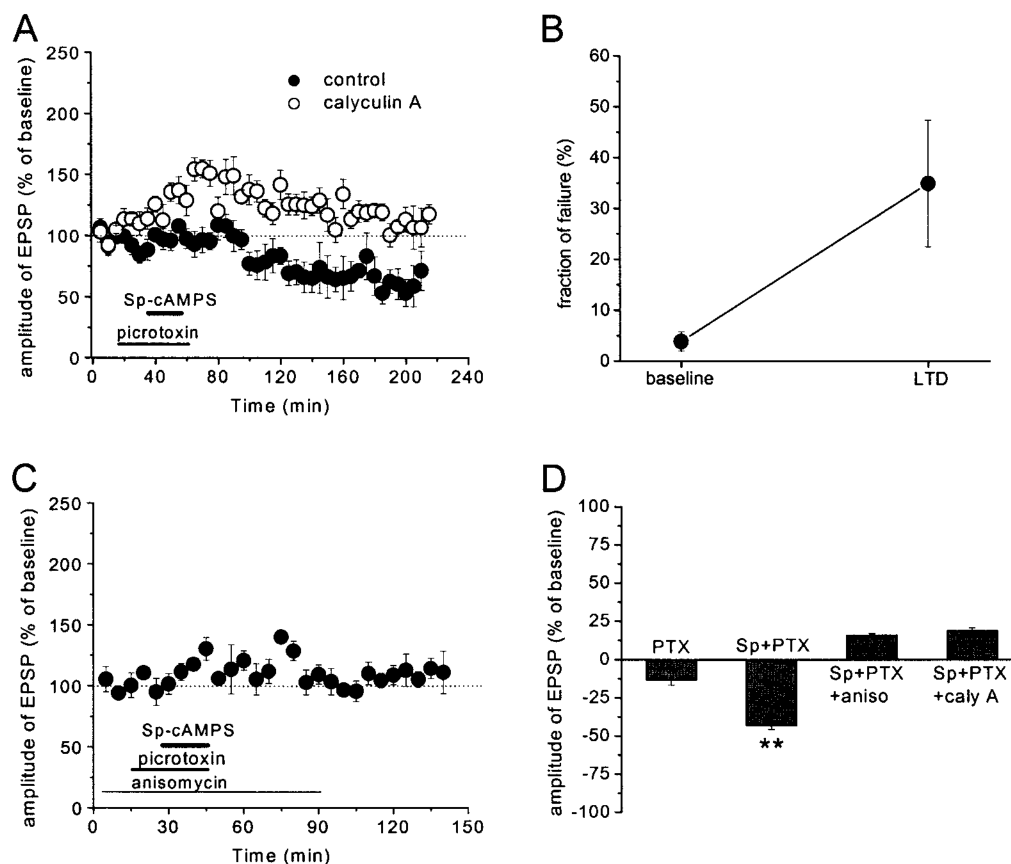


**Fig. 5.** Sp-cAMPS induces phosphatase-dependent LTD in the presence of picrotoxin. (*A*) When slices were perfused with normal ACSF (control, ●, $n = 5$), LTD was induced after concurrent application of Sp-cAMPS (Sp) and picrotoxin (PTX, 20–50 $\mu$M). After slices were pretreated with 1 $\mu$M calyculin A (caly A) for 1 h, coapplication of Sp-cAMPS and picrotoxin no longer induced LTD (○, $n = 3$). (*B*) This form of LTD is accompanied by an increase in failure rate. (*C*) In the presence of anisomycin (aniso, 20 $\mu$M), Sp-cAMPS and picrotoxin did not produce LTD ($n = 4$). (*D*) Summary of data shown in *A* and *C*. Picrotoxin (PTX) applied alone did not cause significant depression ($n = 4$). *, $P < 0.01$, compared with the PTX group. Error bars indicate ± SEM.

picrotoxin, Sp-cAMPS no longer induced an early phase of LTP. To our surprise, LTD was induced in this condition. Although several reports have shown that blockade of $GABA_A$ receptor-mediated transmission can attenuate neuronal firing and tetanus-induced LTP in hippocampus (37, 38), the induction of LTD has not been reported in the presence of a $GABA_A$ receptor antagonist. Our result shows that the early phase of cAMP-mediated LTP requires the function of $GABA_A$ receptors. Moreover, when the early phase of LTP was blocked, the late phase of LTP was also prevented. This suggests that the early phase of LTP may act to induce the late phase of LTP.

Recently, it was suggested that a reversible change in phosphorylation of the $\alpha$-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor subunit GluR1 could contribute to bidirectional synaptic plasticity at CA3–CA1 synapses (39). Other evidence also suggests that activation of protein phosphatases favors the induction of LTD, whereas activation of protein kinases such as PKA, CaMK (calcium calmodulin kinase)-II, or CaMKIV promotes LTP (6, 40). Furthermore, modulations of $GABA_A$ receptors by PKA and protein phosphatases can eliminate and enhance the inhibitory function of $GABA_A$ receptors, respectively (21, 22). It is therefore possible that PKA-activated phosphorylation of both $GABA_A$ and AMPA receptors can subsequently inhibit activation of protein phosphatases, ensuring that the direction of synaptic plasticity is primarily toward potentiation. Likewise, dephosphorylation of $GABA_A$ receptors by protein phosphatases in the presence of a $GABA_A$ receptor antagonist may directly or indirectly facilitate dephosphorylation of PKA sites on GluR1, leading to LTD. Our results apparently support the involvement of protein phosphatases in LTD induced by concurrent application of Sp-cAMPS and picrotoxin. Further studies will be needed to explore how modulations of $GABA_A$ receptors by PKA and protein phosphatases in the presence and absence of a $GABA_A$ receptor antagonist determine the direction of cAMP-induced synaptic plasticity.

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