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Forskolin-induced LTP in the CA1 hippocampal region is NMDA receptor-dependent

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Abstract

Chemically induced long-term potentiation (cLTP) could potentially work by directly stimulating the biochemical machinery that underlies synaptic plasticity, bypassing the need for synaptic activation. Previous reports suggested that agents that raise cAMP concentration might have this capability. We examined the cLTP induced in acute slices by application of Sp-cAMPS or a combination of the adenylyl cyclase activator, Forskolin, and the phosphodiesterase inhibitor, Rolipram. Under our conditions, cLTP was induced, but only if inhibition was reduced. We found that this form of cLTP was blocked by an NMDAR antagonist and required the low-frequency test stimulation typically used to monitor the strength of synapses. Interestingly, similar LTP could be induced by lowering the Mg^{2+} concentration of the ACSF during Forskolin/Rolipram or Sp-cAMPS application or even by just lowering Mg^{2+} concentration alone. This LTP was also NMDAR-dependent and required only a few (around five) low frequency stimuli for its induction. The finding that even low frequency synaptic stimulation was sufficient for LTP induction indicates that a highly sensitized plasticity state was generated. The fact that some stimulation was required means that potentiation is probably restricted to the stimulated axons, limiting the usefulness of this form of cLTP. However, when similar experiments were conducted using slice cultures, potentiation occurred without test stimuli, probably because the CA3-CA1 connections are extensive and because presynaptic spontaneous activity is sufficient to fulfill the activity requirement. As in acute slices, the potentiation was blocked by an NMDAR antagonist. Our general conclusion is that the induction of LTP caused by elevating cAMP requires presynaptic activity and NMDA channel opening. The method of inducing cLTP in slice cultures will be useful when it is desirable to produce NMDAR-dependent LTP in a large fraction of synapses.

Key words: cAMP, culture, slice, long-term potentiation, NMDA

Introduction.

The induction of LTP is normally performed by intensively stimulating a group of axons that converge on a target neuron. The resulting postsynaptic depolarization and Ca^{2+} entry then sets in motion biochemical changes that lead to enhanced transmission. There has been considerable interest in inducing LTP by chemical rather than electrical means for several reasons. First, if direct chemical activation of specific biochemical processes induces potentiation, this provides important evidence relevant to the involvement of these processes in LTP. Second, for biochemical and morphological studies of LTP it is desirable to maximize the number of synapses that undergo the plasticity changes. This cannot be achieved by standard induction methods in which LTP is induced by electrical stimulation of a small fraction of the synaptic inputs to pyramidal cell, as is typically the case. The stimulated synapses are randomly distributed along the dendritic tree and cannot be easily identified. Induction of LTP by application of chemicals that directly trigger biochemical LTP mechanisms holds the promise of affecting a large fraction of the synapses onto a large fraction of cells in the preparation.

Many methods for directly inducing LTP have been explored. Direct elevation of postsynaptic Ca^{2+} in an single CA1 pyramidal cell can lead to synaptic potentiation without the need of presynaptic activity, indicating that Ca^{2+} elevation is effectively coupled to downstream synaptic strengthening processes (Neveu and Zucker 1996). For many purposes, however, it would be preferable to induce LTP by bath application methods because this would affect all cells in the slice. Several such methods have been studied (Breakwell et al. 1996; Collins et al. 1995; Del Olmo et al. 2000; Fujii et al. 2002; Huber et al. 1995; Kang and Schuman 2000; Levine et al. 1998; Musleh et al. 1997; Yamazaki et al. 2003), but many require low-frequency electrical presynaptic stimulation during drug application or spontaneous presynaptic CA3 cell firing (Breakwell et al. 1996; Collins et al. 1995; Huber et al. 1995; Musleh et al. 1997), a requirement that limits LTP to only the activated synapses. The requirement for presynaptic cell firing is problematic in acute slices because most CA3 neuronal projections to CA1 cells have been cut off from CA1 during slice preparation (Li et al. 1994). Other protocols produced

only rather small LTP because a substantial part of the increase in response is due to an increase in axon excitability (Chinestra et al. 1994; Del Olmo et al. 2000). Several protocols have been reported to induce cLTP without any need for presynaptic activity. For example BDNF application results in LTP in slices with CA1 and CA3 cell bodies detached (Kang and Schuman 1996). This induction method depends on intracellular Ca^{2+} release and protein synthesis (Kang and Schuman 2000, 1995). However, this method has been difficult to reproduce (Figurov et al. 1996), perhaps because of problems with delivery of BDNF into slices (Kang and Schuman 1996). A recent, single report indicates that cLTP can be induced by a cocktail of ATP, tACPD and low concentrations of NMDA (Fujii et al. 2002).

A frequently used method for inducing cLTP utilizes bath application of agents that raise cAMP levels (Bolshakov et al. 1997; Frey et al. 1993; Huang and Kandel 1995; Nguyen et al. 1994; Patterson et al. 2001; Yu et al. 2001). It is thought that this procedure directly activates transcription/translation processes resulting in development of protein synthesis-dependent late phases of LTP. Some studies suggest that this form of cLTP can be induced without electrical stimulation of presynaptic fibers (Bolshakov et al. 1997; Frey et al. 1993) or NMDAR activation (Lu and Gean 1999). In contrast, other reports indicate that synaptic NMDAR activation might be needed. For instance, one study showed that the LTP induced by Sp-cAMPS could be blocked by a combination of AMPA and NMDA receptor antagonists (Bozdagi et al. 2000). Another study showed that the increase in synapse number produced by Sp-cAMPS was NMDA receptor-dependent (Ma et al. 1999). Finally, a recent paper on mutant mice showed that a mutation activating the cAMP regulated transcription factor, CREB, is not sufficient to induce late LTP by itself and requires an NMDA receptor-dependent process (“synaptic tagging”) to occur (Barco et al. 2002). These considerations suggest that stimulation of cAMP dependent transcription/translation processes might not be sufficient to induce cLTP and that synaptic activity might be required. Here we have tested whether this is the case. An additional goal of our work was to find a method for reliably inducing cLTP in a large fraction of synapses.

Experimental Procedures

Electrophysiology

Transverse hippocampal slices (350- to 400-µm-thick) were prepared from 17- to 25-d-old Long-Evans rats using a Vibratome-1000 (Technical Products International Inc, St. Lous, MO). After preparation, slices were preincubated for at least 2 hrs in an interface chamber before an experiment. Field potential recordings were performed on slices placed on nylon net and superfused on both sides with ACSF at the flow rate 2-2.3 ml/min in a chamber of 0.5 ml volume. The ACSF contained (in mM): NaCl 120, $NaHCO_4$ 26, NaH_2PO_4 1, KCl 2.5, $MgSO_4$ 1.3, $CaCl_2$ 2.5, and D-glucose 10, saturated with 95%O_2/5%CO_2 and preheated to 29-30^oC. Glass pipettes (resistance, 0.2-0.3 MΩ when filled with ACSF) were used as stimulating and recording electrodes and were placed in the stratum radiatum of the CA1 region (two monopolar stimulating electrodes were positioned on both sides of the recording electrode ~200-250 µm apart from each other). Synaptic field potentials (fEPSP) were amplified 1,000-10,000 fold, filtered (1 Hz- 5 kHz), digitized (10 kHz), and stored on a PC using a program written in AXOBASIC (Axon Instruments, Foster City, CA). Stimuli (100 µs) were delivered using current isolation units (Axon Instruments, Foster City, CA) once every 6 s in most experiments. In experiments summarized in Fig. 1 C and 2 A, B the test stimuli were given once every 30 s. Stimulus strength was adjusted to produce a fEPSP amplitude ~50% of that which reached threshold for a population spike. In experiments reported in Fig. 1 C and 2, the CA3 region of the slice was not removed and the Mg^{2+} concentration in ACSF was 1 mM. This was done in an attempt to reproduce conditions of published experiments in which the cLTP produced by elevating cAMP was studied: specifically,

the CA3 region of the slice was present and the Mg^{2+} concentration slightly lowered to 1 mM (Bolshakov et al. 1997). Whole-cell recording was performed using an Axopatch-1D amplifier with low-pass filter set at 1 kHz. The pipette solution contained (in mM): Cs-methanesulfonate, 120; CsCl, 20; HEPES, 10; MgATP, 4; Na_3GTP, 0.3; EGTA, 0.2; phosphocreatine, 10; pH, 7.3; osmolarity, 300 mOsm. Whole-cell currents were measured in voltage-clamp mode at a holding potential of -65 mV. Series and input resistances were monitored after each stimulus by measuring the peak and steady-state currents in response to 2 - 4 mV, 40 ms hyperpolarizing steps.

Slice culture

Hippocampal slice cultures were prepared and maintained similarly to the protocol described in (Hayashi et al. 2000). Briefly, 200 μm slices were prepared from P6-8 Sprague – Dawley rats using a Leica vibratome. Sterile, cold (4^oC) modified ACSF saturated with $95\%O_2 + 5\%CO_2$ was used during the preparation. Modified ACSF contained (in mM): sucrose, 248; KCl, 4; $CaCl_2$, 1; $MgCl_2$, 5; $NaHCO_3$, 26; pH 7.3, osmolarity, 320 mOsm. Slices were plated on the membrane of 6 well plate inserts (Falcon, 0.4 μm pore size) and incubated in a CO_2 incubator at 36^o C in culture medium containing MEM (Cellgro #50-019-PB) supplemented with: horse serum (Sigma), 20%; insulin, 1μg/ml; glutamine, 1mM; HEPES, 30 mM; $CaCl_2$, 1mM; $MgSO_4$, 2mM; $NaHCO_3$, 5 mM; D-Glucose, 16.5 mM; ascorbate, 0.5 mM. One half of the medium volume was changed 2-3 times per week. During experiments slice cultures were incubated in ACSF containing in (mM): NaCl, 124; KCl, 2.5; $CaCl_2$, 4; $MgCl_2$, 4; NaH_2PO_4, 1.25; $NaHCO_3$, 26; 2-Cl- adenosine, 0.006; saturated with $95\%O_2 + 5\%CO_2$,

pH 7.4. Cultures were placed directly on the glass bottom of the recording chamber (0.5 ml) and superfused at the flow rate 2-2.3 ml/min. 2-Cl- adenosine, an A1 adenosine receptor agonist, was used to decrease polysynaptic responses and spontaneous cell bursting (Hayashi et al. 2000). For LTP induction Forskolin and Rolipram were prepared using this modified ACSF, but without $MgSO_4$ and 2-Cl-adenosine (0 Mg^{2+} ACSF). The test electrical stimulation of stratum radiatum fibers was delivered once every 5 min. In the preliminary experiments we found that if test stimulation was faster (1/10 s, 1/30s or 1/60 s) or 2Cl-adenosine was included in ACSF during the induction, the magnitude and duration of LTP decreased.

Solutions

"0 Mg^{2+}" ACSF was identical to regular ACSF, except $MgSO_4$ was omitted. Forskolin (Alomone Labs, Jerusalem, Israel) was prepared as a stock solution (50 mM, in DMSO) and stored at –80°C until used at final concentration of 50 µM in the bath ACSF. Rolipram (Sigma) stock solution (0.1 mM in DMSO) was stored at –80°C until used at final concentration of 0.1 µM. This concentration was chosen because it was optimal in facilitating LTP induction (Barad et al. 1998). D, L-2-Amino-5-phosphonovaleric acid (APV) (Sigma) was stored at 4°C as a 20 mM stock solution and used at final concentration of 100µM. Sp-cAMPS (Alexis Corporation, San Diego, CA) stock solution (50mM, in distilled water) was stored at –80°C until used at final concentration of 50 µM. Picrotoxin (PTX) (Sigma) was stored as a stock solution (5 mM, in distilled water) at 4°C and used at 50 µM concentration. In experiments with APV on acutely prepared

slices, APV was present in ACSF from 15 min before and until 15 min after drug application.

Statistics

Initial data analysis was performed using a custom-made program written in AXOBASIC, followed by analysis using Microsoft “Excel” and Microcal “Origin”. Plots were made in “Origin”. Every data point represents an average of all collected data points in a 2 min interval (unless noted otherwise). Statistical differences of values after drug application (Student’s t-test) were calculated in comparison to values in the baseline in the same experimental series unless stated otherwise. Data are presented as mean ± standard error.

Results

Suppressed inhibition facilitates induction of cAMP-dependent cLTP.

There have been many reports that brief application of agents that raise cellular cAMP in acute hippocampal slices can produce LTP, implying that the biochemical mechanisms of plasticity could be directly activated (Bolshakov et al. 1997; Frey et al. 1993; Huang and Kandel 1995; Nguyen et al. 1994; Patterson et al. 2001; Yu et al. 2001). We first tried to reproduce these results using standard ACSF with the CA3 region of slices removed. Surprisingly, bath application of Sp-cAMPS (100 μM) did not produce long -lasting potentiation (n = 3, Fig. 1 A); the only effect was a depression of transmission during drug application, an effect previously reported by others (Bolshakov et al. 1997; Frey et al. 1993). We next tried to elevate endogenous cAMP by stimulating its production with the adenylate cyclase activator, Forskolin (50 μM), and preventing its degradation by the phosphodiesterase inhibitor, Rolipram (0.1 μM). Rolipram was chosen instead of the IBMX often used in previous studies (Chavez-Noriega and Stevens 1994; Duffy et al. 2001; Duffy and Nguyen 2003; Huang and Kandel 1994; Woo et al. 2002), because IBMX also strongly inhibits adenosine receptors (Chavez-Noriega and Stevens 1992). This induction procedure produced potentiation, but it was only transient (n = 5, Fig. 1B). This transient potentiation lasted on averaged only 22 ± 2.2 min after drug washout and was in part accounted for by an increase in presynaptic excitability, as indicated by the increase in fiber volley; the fEPSP increased 154 ± 8 % of the baseline and the fiber volley increased 111 ± 2.8 % at 14 min of drug application. One difference from previous

work was that in our experiments the CA3 region was removed. It has been reported that for some methods of cLTP induction, intact connections between CA3 and CA1 neurons are required (Breakwell et al. 1996; Collins et al. 1995; Makhinson et al. 1999). When we conducted experiments with the CA3 region present, potentiation was longer in duration but still decayed in less than an hour (58 ± 14.8 min, $n = 5$, $p < 0.01$, Fig.1C).

The reason for the inability of these procedures to induce stable LTP was unclear. It is known that the state of inhibition can vary dramatically, depending on the condition of slices and that strong inhibition can prevent LTP induction. We therefore tested whether the combination of Forskolin/Rolipram could induce LTP when applied in the presence of PTX, a GABA-A inhibitor (50 μM). Fig. 2A shows that under these conditions a stable LTP was produced that lasted for at least 120 min after drug washout (158 ± 11.6 %, $n = 6$, $p < 0.01$). In these experiments Forskolin/Rolipram also produced a transient increase in the fiber volley magnitude (Fig. 2 B), indicating that increased axon excitability contributes to the early phase of the observed fEPSP potentiation, but not to the later phase. PTX itself produced no long-lasting effect: after a small initial increase (120 ± 13.5%) the response returned to the baseline by 25 min after drug washout (110 ± 11.3%, $n = 5$, $p < 0.05$, Fig. 2A). We conclude that suppression of inhibition greatly facilitates induction of stable cAMP-dependent cLTP.

cLTP produced by cAMP elevation is NMDAR-dependent.

We next tested whether this form of cLTP depends on NMDA channel activation. Fig. 2A shows that in the presence of an NMDAR antagonist, APV (100 μM) the peak of the initial potentiation significantly decreased and by 60 min the response returned to the baseline level (107 ± 5.31, n = 4, $p > 0.05$). Therefore, stable LTP was entirely blocked by NMDA receptor antagonist ($p < 0.01$).

The requirement for NMDA receptor activation suggested an explanation of our results (Fig. 2) showing the requirement for PTX: inhibition might prevent the depolarization required to open NMDA channels. If this is the case, removing Mg^{2+} should allow Ca^{2+} influx through these channels without strong depolarization and thus PTX should not be required. Fig. 3A shows that this was the case for both the LTP induced by Sp-cAMPS or by Forskolin/Rolipram. The level of potentiation at 60 min after drug washout was 142 ± 3.68 %, n = 3 and 156 ± 6.5 % respectively (n = 6, $p < 0.05$ for each). This LTP could not be attributed to changes in presynaptic excitability because this was only transient: at 40 min after drug washout the fiber volley amplitude was not different from the baseline in experiments with Forskolin/Rolipram (96 ± 3.1 %) or slightly below (92 ± 10.1 %) the baseline in experiments with Sp-cAMPS (Fig. 3 B). Again, this form of potentiation was completely blocked by APV: at 60 min after washout of Forskolin/Rolipram/0Mg^{2+} stable LTP was completely blocked (108 ± 6.49 %, n = 6, $p > 0.05$, Fig. 3 C).

In acute slices low-frequency stimulation of presynaptic fibers is required for induction of cAMP-dependent cLTP.

If NMDA channel activation is required for induction of cLTP, what causes the release of glutamate that activates these channels? We checked whether the low-frequency test stimuli used to monitor synaptic strength might be responsible. Such stimuli normally have no effect on potentiation. However, there have been reports that application of some neuromodulators can bring cells into such a sensitized state that even low-frequency test stimuli become sufficient for the induction of LTP (Huerta and Lisman 1993). Fig. 3D indicates that this was also the case under our experimental conditions. If test stimuli were turned off during application of Forkolin/Rolipram/0Mg^{2+}, and kept off for 30 min after drug washout (or at least for 5 min of the washout, data are not shown), no LTP was induced (111 ± 7.2 % at 60 min of washout, $n = 4$, $p > 0.05$). However, if we turned synaptic stimulation back at the onset of drug washout, the LTP was similar to that in experiments with continuous stimulation (160 ± 13.2 % at 60 min of washout, $n = 8$, $p > 0.05$). These results suggest that the interaction of even a few stimuli with the residual effect of drugs was sufficient for LTP induction. Indeed, a plot of responses to individual test stimuli demonstrates that potentiation developed very quickly during the first 5 stimuli after the start of drug washout. (Fig.3 E). Remarkably, after these five stimuli (delivered within 30 s) the potentiation reached the same level as that with continuous stimulation during the entire period of drug application (375 ± 27.5 and 399 ± 37.2 respectively, $p < 0.01$, Fig 3 D, E). Therefore, only five low-frequency stimuli given immediately after drug application are sufficient for cLTP induction. Notably, the first of

these responses was already potentiated (239 ± 10.4 %). This potentiation was only marginally different from the level of potentiation in experiments with NMDAR blocked by APV (204 ± 11 %, Fig. 3 C, $p = 0.044$) suggesting that NMDAR-independent transient potentiation does not require evoked synaptic activation. The potentiation induced by this protocol was similar in magnitude and was still stimulation-dependent in slices with CA3 region present (data not shown). In control experiments, in which drugs were not applied, but test stimulation was stopped for 15 min, responses were slightly elevated right after the break in stimulation and then slowly recovered to near the baseline level (81 ± 5.6 %, $n = 9$, Fig. 3 D). We also tested whether the LTP induced by application of Forskolin/Rolipram/PTX in normal ACSF required electrical presynaptic stimulation and found that it did: when electrical stimulation was stopped during and for 30 min after the drug application LTP was not induced (107 ± 8 %, at 80 min of washout $n = 4$, $p > 0.05$). We conclude that the potentiation induced by either application of Forskolin/Rolipram/PTX or Forskolin/Rolipram/0Mg^{2+} is activity-dependent. Notably, the frequency of electrical synaptic activation used in the first of these protocols was very low (1/30s) and the number of stimuli required for LTP induction using the sond protocol was also remarkably small (around 5). Apparently the chemical induction procedure produces a highly sensitized state in which LTP induction is greatly facilitated.

Lowering Mg^{2+} concentration is sufficient to produce sensitized plasticity state.

To better understand the potentiation produced by Forskolin/Rolipram/0Mg^{2+} solution, we tested whether merely lowering Mg^{2+} was sufficient to induce the sensitized state. In

the experiments shown in Fig. 4, we removed Mg^{2+} for 15 min but did not apply the cAMP elevating drugs. Stimulation was stopped at the beginning of 0 Mg^{2+} application and was resumed at 1 min before 0 Mg^{2+} solution was replaced by normal ACSF. Surprisingly, the LTP was large (151 ± 2.5 %, n = 4; 60 min) and not different from that induced by application of cAMP elevating drugs in 0 Mg^{2+} ACSF ($p > 0.05$). Furthermore, LTP developed with a similarly rapid time course: only 5 test stimuli potentiated responses to a saturated level (Fig. 4 C, D). To further test if these 5 stimuli were sufficient to induce stable LTP, we stopped stimulation in one pathway for 30 min after delivering only these five stimuli (Fig. 4 A, B). At 60 min after removal of 0 Mg^{2+} solution the LTP in this pathway was indistinguishable (153 ± 7.0 %, $p > 0.05$) from that in the first pathway in which stimulation was continuous from the time it was resumed at the end of the 0 Mg^{2+} treatment. We conclude that transient removal of Mg^{2+} from ACSF alone is sufficient to produces the sensitized state.

In slice culture, cLTP is NMDA receptor-dependent but does not require electrical stimulation.

The dependence of cLTP on electrical synaptic stimulation means that potentiation will only be induced in the small fraction of stimulated synapses. As mentioned previously, it would be desirable for many purposes to induce LTP in a large fraction of synapses. It has been found that in some protocols for cLTP induction there is a requirement for bursting of presynaptic CA3 cells (Makhinson et al. 1999). In acute slices, this requirement cannot be met in a large fraction of synapses because the majority of CA3 –

CA1 connections are severed during slice preparation. In cultured slices, however, cut axons are replaced by collaterals from CA3 cells, suggesting that most synapses could be affected by CA3 activity. We therefore tested whether the Forskolin/Rolipram/0 Mg^{2+} solution could induce LTP without electrical stimulation in cultured slices. Raising cAMP induced strong synchronized bursting, as indicated by large amplitude field potentials (data not shown), consistent with previous reports of such bursting (Makhinson et al. 1999). Under these conditions, strong LTP was induced even when test stimuli were stopped during and for 30 min after drug application (at 180 min of drug washout LTP was 161 ± 12.5 %, n = 14, p >0.01, Fig. 5 A). This potentiation was entirely dependent on the NMDAR-channel activation: 180 min after drug application in the presence of APV the fEPSP slope was 89 ± 7.9 % (n = 10), a value not significantly different from the response in control experiments, in which no drugs were applied (94 ± 9.8 %, n = 9). Similarly, the same induction procedure produced stable cLTP under conditions of whole-cell voltage-clamp (Fig. 5 C, D). At 100 min after induction LTP magnitude was 157 ± 7.7 % (n = 3, p < 0.01). Therefore, in slice cultures application of Forskolin/Rolipram/0 Mg^{2+} induces LTP, which does not required electrical synaptic stimulation but is still NMDAR-dependent.

Discussion

Our principle finding is that the induction of cLTP by agents that elevate cAMP cannot be attributed fully to a *direct* activation of biochemical plasticity processes, but requires presynaptic activity and opening of NMDA channels. In acute slices, NMDAR activation during induction requires evoked electrical stimulation, but the frequency of presynaptic stimuli needed for induction is very low (1/30 s, Fig. 2) indicating that a sensitized state of plasticity has been generated. Because of this heightened sensitivity, LTP can be triggered inadvertently by low frequency test stimuli. In the cultured slice preparation the required synaptic activation appears to arise from spontaneous activity in presynaptic neurons. Such spontaneous activity can be used to one's advantage in slice culture to produce LTP in a large fraction of synapses.

Previous work has suggested that cAMP elevating agents induced LTP due to direct activation of transcription/translation processes without a need for presynaptic activation (Bolshakov et al. 1997; Frey et al. 1993). This suggestion was based on the fact that normal LTP-inducing stimuli did not have to be applied. However, no explicit tests were done to check whether lower levels of activity might be triggering the plasticity. The experiments of Frey et al. (Frey et al. 1993) were performed on slice cultures where there are extensive connections between CA3 and CA1 cells. Thus, spontaneous firing of presynaptic CA3 neurons could have provided the needed synaptic stimulation. The results of Bolshakov et al. (Bolshakov et al. 1997) are more difficult for us to explain because they were done on acute slices in which most inputs to CA1 from CA3 are

severed by the slicing procedure and could thus not provide spontaneous input. Furthermore, no electrical stimulation was given during the induction period. However, this study (Bolshakov et al. 1997) specifically examined CA1 cells with preserved connections from CA3, and it is therefore possible that spontaneous presynaptic firing could have contributed to potentiation at this subset of connections. Since the induction of cLTP can depend on subtle changes in the condition of the experiment, we cannot exclude the possibility that induction would be activity-independent under other conditions. In any case, our results emphasize the importance of directly testing the activity-dependence of cLTP induction under the particular conditions used.

The idea that raising cAMP might *not* be sufficient to trigger strengthening processes directly is consistent with several other studies. For instance, it was shown that combined inhibition of both AMPA and NMDA channels blocks induction of LTP by Sp-cAMPS (Bozdagi et al. 2000). It was also reported that NMDA channel inhibition decreases the magnitude of the LTP induced by activation of dopamine receptors, which are coupled to cAMP production (Huang and Kandel 1995). In another study, the long-lasting increase in the number of active presynaptic terminals induced by Sp-cAMPS was shown to be NMDA dependent (Ma et al. 1999). Finally, a constitutively active CREB mutation does not by itself produce potentiation, but requires synaptic NMDAR activity to generate a "tag" (Barco et al. 2002). All these results suggest that direct activation of transcription/translation by cAMP is not sufficient to induce potentiation and that activation of NMDA channels is also required.

Generally the induction of LTP requires repetitive, high frequency stimulation involving hundreds of stimuli. However, during cholinergic modulation there is a sensitized state in which LTP can be induced by single bursts involving only 5 stimuli given at high frequency (Huerta and Lisman 1995). Our results indicate that a sensitized state is also induced by either Forskolon/Rolipram/0 Mg^{2+}, or just in 0 Mg^{2+}. However, in this case, the 5 stimuli need not be given at high frequency: one every 6 s is sufficient. Moreover, when inhibition is lowered, Forskolin/Rolipram produces LTP even at a stimulation rate of 1/30 s.

A variety of factors could contribute to a sensitized state. There are several reports that cAMP-dependent processes may directly facilitate NMDA channel function (Cerne et al. 1993; Huang and Gean 1995; Raman et al. 1996; Westphal et al. 1999), increase spontaneous transmitter release (Chavez-Noriega and Stevens 1994), and increase membrane excitability (Pockett et al. 1993). However, it appears critical that conditions that reduce the Mg^{2+} block of NMDA channels must also be present: either Mg^{2+} must be lowered or inhibition reduced. Because we find that the sensitized state can be produced by lowering Mg^{2+} in ACSF for 15 min without addition of cAMP raising drugs, it could be argued that cAMP elevation is not required for induction of the sensitized state. However, such a conclusion would be premature since lowering Mg^{2+}can lead to Ca^{2+} entry through the NMDA channel which, in turn, activates adenylyl cyclase (Chetkovich and Sweatt 1993). Furthermore, the fact that LTP can be induced by low-frequency stimulation in the presence of cAMP elevating drugs even without removing Mg^{2+} (Fig. 2) indicates that raising cAMP is a critical factor for induction of the sensitized state.

One important reason for studying cLTP is to find a method for potentiating a large fraction of synapses in the slice. This requirement is important in experiment that seeks to identify the biochemical and morphological correlates of potentiation. If this is the goal, any form of stimulation requirement is undesirable because only the small, stimulated fraction of synapses will be potentiated. There have been reports that in some methods of cLTP induction, drug-induced firing of presynaptic CA3 cells can substitute electrical stimulation of presynaptic axons (Makhinson et al. 1999). This may lead to potentiation in the fraction of CA1 synapses that remain connected to CA3 neurons, but in the acute slice preparation the majority of CA3-CA1 connections will inevitably be severed (Li et al. 1994). A way around this problem is the use of slice culture, because cut axons regrow, making it possible to potentially activate all CA3-CA1 synapses. Our results indicate that the potentiation induced by Forskolin/Rolipram 0 Mg^{2+} produces NMDA channel activation without the need for electrical stimulation and is therefore a useful method for determining the morphological and biochemical changes during NMDAR-dependent LTP.

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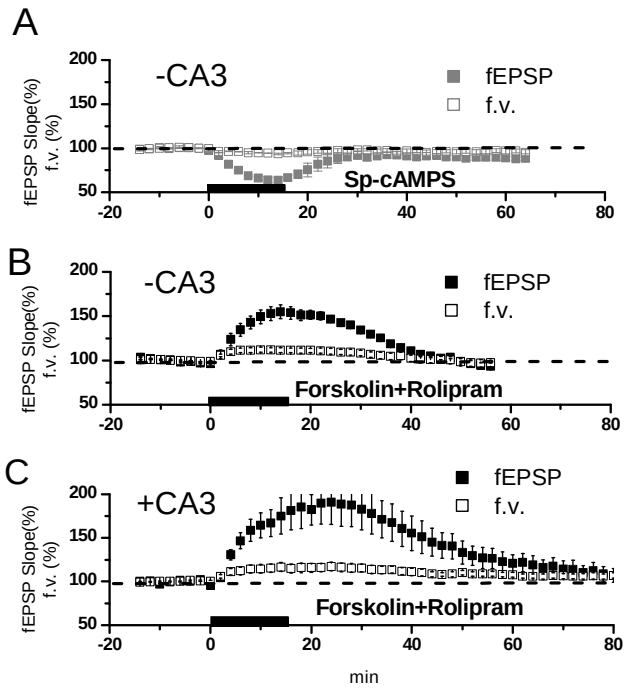

Fig. 1. Raising cAMP levels in acute slices bathed in standard ACSF is not sufficient to produce LTP. Summary graphs of average fEPSP slope and fiber volley amplitude over the time. Drug application is marked by thick horizontal bar. CA3 region was removed in A and B but not in C.

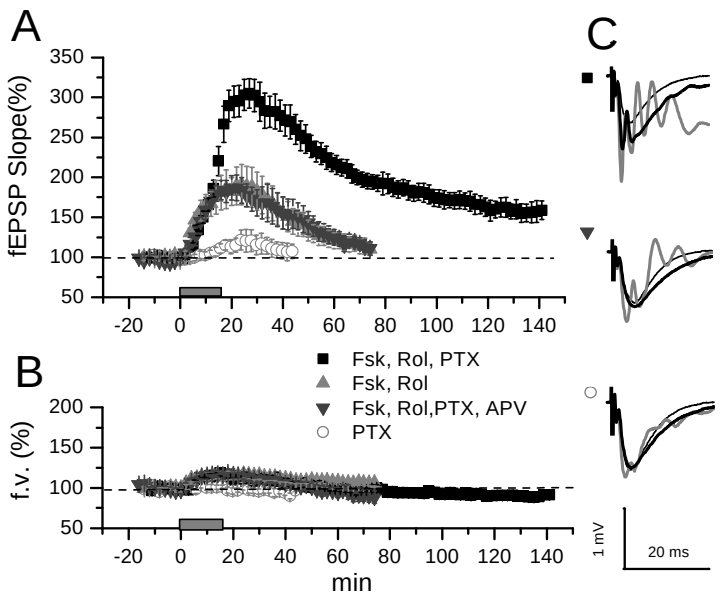

Fig. 2. When inhibition is decreased by pictrotoxin (PTX), raising cAMP produces NMDAR-dependent LTP. A: Addition of PTX during Forskolin/ Rolipram application resulted in stable LTP, which was converted to decrementing potentiation when drugs were applied in the presence of APV. APV was applied from15 min before until 15 min after drug application. PTX alone did not produce significant potentiation. B: Summary graphs of fiber volley amplitudes for the experiments in A. Horizontal bar marks the period of drug application. C: A representative fEPSPs (average of 10) before (thin black), during (grey) and 60 min after drug application (thick black) for the experiments shown in A, B. CA3 was not removed.

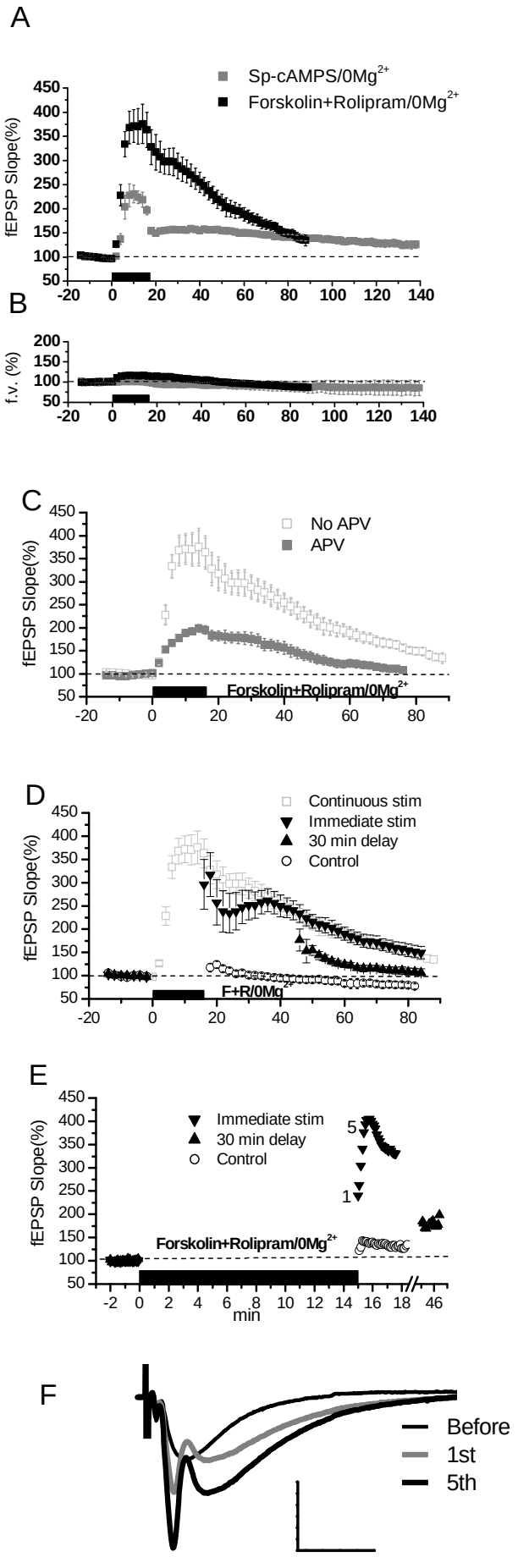
A
Sp-cAMPS/0Mg2+
Forskolin+Rolipram/0Mg2+
fEPSP Slope(%)
B
f.v. (%)
C
No APV
APV
Forskolin+Rolipram/0Mg2+
D
Continuous stim
Immediate stim
30 min delay
Control
F+R/0Mg2+
E
Immediate stim
30 min delay
Control
5
1
Forskolin+Rolipram/0Mg2+
min
F
Before
1st
5th

Fig. 3. When Forskolin/Rolipram or Sp-cAMPS are applied under conditions favorable for the NMDA channels opening (0 mM Mg^{2+}) a sensitized plasticity state is generated so that NMDAR-dependent LTP is induced by only 5 test stimuli, even with inhibition intact. A, B: Summary graphs of fEPSPs and fiber voleys in experiments with application of Sp-cAMPS and Forskolin/Rolipram. C: APV was present during application of Forskolin/Rolipram. D: Test stimulation was stopped at the beginning of drug application and resumed immediately at the drug washout or with 30 min delay. For comparison, data from A during which stimulation was continuous is replotted in C, D. E: Summary graphs showing fast developing potentiation of fEPSP when stimulation was resumed *immediately* after drug application (downward triangles, the same data as in D). When stimulation was resumed with 30 min delay after drug application there were no significant additional increase in fEPSP (upward triangles, the same data as in D). In control experiments response was not significantly changed after stimulation was resumed. Each point represents a slope of response to a single stimulus averaged over several experiments. F: A representitive fEPSPs before drug application and the first and the fifth responses after the interuption as indicated in E. Scale, 0.5 mV, 10 ms. CA3 region was removed in these experiments.

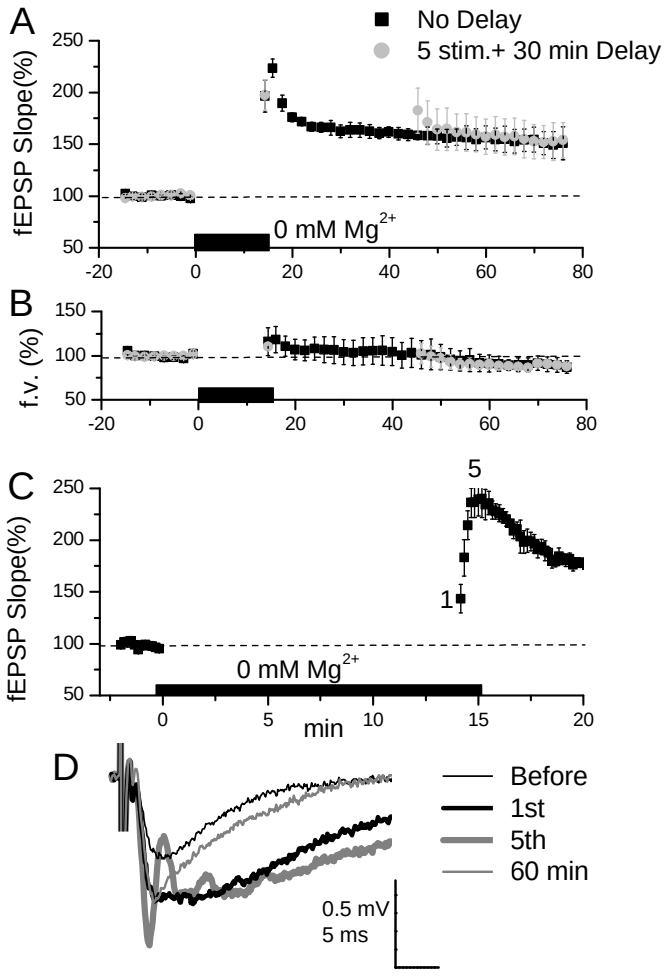

Fig. 4. Application of 0 mM Mg^{2+} ACSF alone sensitizes plasticity so that LTP is rapidly induced by 5 test stimuli. A, B: Summary graphs of fEPSP (A) and fiber volley (B). Stimulation was stopped during application of 0 Mg^{2+} ACSF and resumed 1 min before switching to standard ASCF. After 5 stimuli in one pathway the stimulation was stopped again for the next 30 min. C: Shows one pathway from the experiment in A during the period starting 2 min before and until 5min after 0 mM Mg^{2+} ASCF application. Each point represent the slope of response to a single stimulus averaged over several experiments. D: A representitive fEPSPs before application of 0 mM Mg^{2+} ACSF; the first and fifth responses after the interuption as indicated in C; and a response at 60 min after washout of 0 mM Mg^{2+} ACSF.

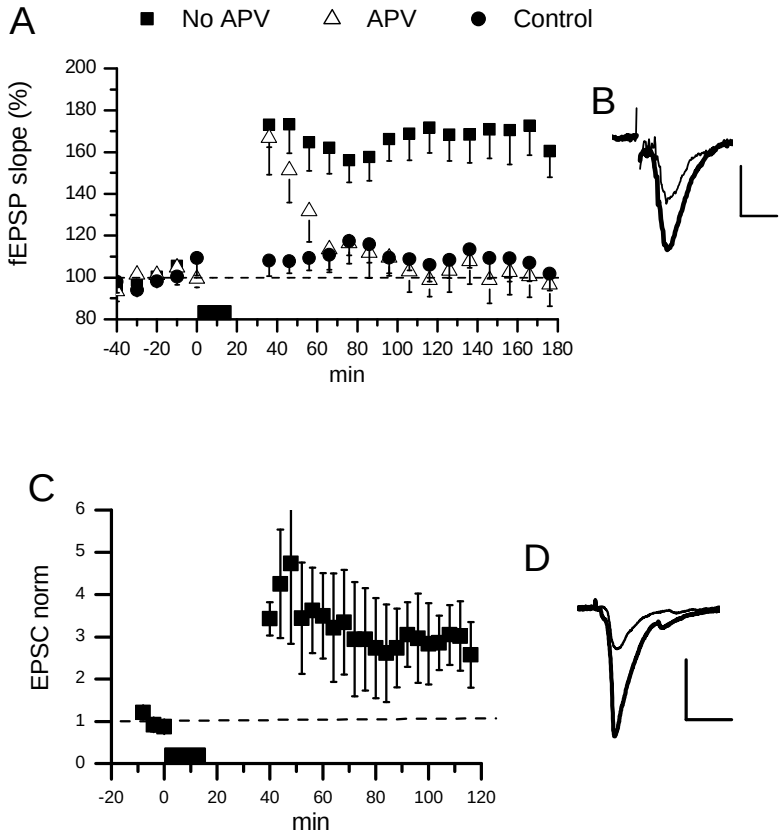

Fig. 5. In slice culure, application of Forskolin/Rolipram/ 0 mM Mg^{2+} ACSF produces NMDAR-dependent, stimulation-independent LTP. A: Field potential recordings; Forskolin/Rolopram/ 0 mM Mg^{2+} produced stable LTP. In the presence of APV, the same induction procedure produced only decrementing potentiation. In control experiments, no drugs were applied; B: Representitive traces of fEPSPs (average of 10) before (thin) and 120 min after (thick) drug application. Scale bars, 50 μV, 20 ms. C: Stable LTP was induced by the same procedure as in **A** but recorded in whole-cell voltage-clamp configuration. D: A representitive traces of EPSCs (average of 10) before (thin) and 60 min after (thick) drug application. Scale bars, 50 pA, 20 ms. A, B: Stimulation was stopped during and for 30 min after drug application.