Caffeine-Mediated Presynaptic Long-Term Potentiation in Hippocampal CA1 Pyramidal Neurons

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Martín, Eduardo D. and Washington Buño. Caffeine-mediated presynaptic long-term potentiation in hippocampal CA1 pyramidal neurons. J Neurophysiol 89: 3029–3038, 2003; 10.1152/jn.00601.2002. We report a new form of long-term potentiation (LTP) in Schaffer collateral (SC)-CA1 pyramidal neuron synapses that originates presynaptically and does not require N-methyl-D-aspartate (NMDA) receptor activation nor increases in postsynaptic-free Ca2+. Using rat hippocampal slices, application of a brief “pulse” of caffeine in the bath evoked a nondecremental LTP (CAF LTP) of SC excitatory postsynaptic currents. An increased probability of transmitter release paralleled the CAF LTP, suggesting that it originated presynaptically. The P1 adenosine receptor antagonist 8-cyclopentyltheophylline and the P2 purinoceptor antagonists suramin and piridoxal-5’-phosphate-azophenyl 2’,4’-disulphonate blocked the CAF LTP. Inhibition of Ca2+ release from caffeine/ryanodine stores by bath-applied ryanodine inhibited the CAF LTP, but ryanodine in the pipette solution was ineffective, suggesting a presynaptic effect of ryanodine. Previous induction of the “classical” LTP did not prevent the CAF LTP, suggesting that the LTP and the CAF LTP have different underlying cellular mechanisms. The CAF LTP is insensitive to the block of NMDA receptors by 2-amino-5-phosphonoptyanoic acid and to Ca2+ chelation with intracellular 1,2-bis (2-aminoophenoxy) ethane-N,N,N’,N’-tetraacetic acid, indicating that neither postsynaptic NMDA receptors nor increases in cytosolic-free Ca2+ participate in the CAF LTP. We conclude that the CAF LTP requires the interaction of caffeine with presynaptic P1, P2 purinoceptors, and ryanodine receptors and is caused by an increased probability of glutamate release at SC terminals.

INTRODUCTION

It is generally agreed that in the hippocampus the induction of long-term potentiation (LTP) at Schaffer collateral (SC)-CA1 pyramidal neuron synapses originates postsynaptically and requires Ca2+ influx through N-methyl-D-aspartate (NMDA) receptor channels, but it is unclear whether the expression of SC LTP is caused by a postsynaptic increase in glutamate receptor sensitivity or a presynaptic rise in glutamate release probability (e.g., Bekkers and Stevens 1990; Bolshakov and Siegelbaum 1994; Collingridge and Bliss 1987; Issac et al. 1998; Kullmann 1994; Kullmann et al. 1996; Malinow and Tsien 1990; Nicoll and Malenka 1999; Poncer and Malinow 2001; Stevens and Wang 1994).

Among the possible presynaptic mechanisms in LTP, extracellular adenosine triphosphate (ATP) has recently received considerable attention because of its suggested participation as an extracellular diffusible messenger (e.g., Fields and Stevens 2000). The regulation of synaptic transmission by ATP is through purinergic P2 receptors, both of the ionotropic P2X and metabotropic P2Y varieties (Fredholm et al. 1994). In the hippocampus, ATP released by SCs (Wieraszko et al. 1989) enhances the population spike (Nishimura et al. 1990) and contributes to the maintenance of LTP (Wieraszko and Ehrlich 1994). These effects of ATP have been suggested to be presynaptic (e.g., Robertson et al. 2001) and are blocked by extracellular ecto-protein kinase inhibitors (Chen et al. 1996).

In addition, the end product of ATP hydrolysis is adenosine that inhibits synaptic transmission through activation of presynaptic P1 adenosine receptors (Burnstock 1990; Daly et al. 1983; Fredholm et al. 1999; Nishimura et al. 1990). Therefore ATP may regulate synaptic transmission both directly and indirectly via activation of P1 and P2 receptors, respectively (e.g., Daly et al. 1983; Greene et al. 1985; Zimmermann 1994) and also by the simultaneous activation of P1 and P2 receptors (O’Kane and Stone 2000).

Our aim was to investigate the mechanisms that contribute to the LTP in CA3–CA1 pyramidal neuron synaptic contacts through the interactions with P1 and P2 receptors. We show that a brief caffeine application evoked a nondecremental LTP of SC excitatory postsynaptic currents (EPSCs; CAF LTP) that originates presynaptically. This CAF LTP does not require activation of postsynaptic NMDA receptors or an increase in postsynaptic cytosolic-free Ca2+ and is prevented by specific blockers of P1, P2, and ryanodine receptors. The results are consistent with the CAF LTP being caused by caffeine-mediated interactions with presynaptic P1, P2 purinoceptors, and ryanodine receptors and originated by an increased probability of glutamate release at SC terminals.

METHODS

Full details of most of the procedures have been described previously (Fernández de Sevilla et al. 2002; Martín et al. 2001); therefore it suffices to briefly delineate them here. Young Wistar rats (13–17 days old) were anesthetized with ether and decapitated immediately after disappearance of the pinch reflex, and the brain was removed and submerged in artificial cerebrospinal fluid (ACSF; see following text) at 4°C and maintained at pH 7.4 by gassing with a 95% O2-5% CO2 mixture. All experiments conformed to International Guidelines on the ethical use of animals, and every effort was made to minimize the suffering and number of animals used.

Transverse slices (350 μm) of the dorsal hippocampus were cut...
with a Vibratome (Pelco 1000, St. Louis, MO) and incubated in gassed ACSF (>1 hr at 20–22°C). The ACSF contained (in mM) 124 NaCl, 2.69 KCl, 1.25 KH2PO4, 2 MgSO4, 26 NaHCO3, 2 CaCl2, and 10 glucose. Bicuculline or picrotoxin (50 μM) was added to block GABA_A-mediated inhibitory synaptic transmission. Ryanodine (20 μM; from a stock solution in DMSO at 0.001%) and d-2-amino-5-phosphonovaleric acid (AP5; 50 μM) were added to the ACSF when necessary.

Slices were transferred to an immersion recording chamber placed in an inverted microscope stage (WPI, Sarasota, FL) and superfused (2.5 ml/min) with gassed ACSF at room temperature (20–22°C). Bipolar nichrome wire (80 μm diam) electrodes were placed in the stratum radiatum near the border of CA1 pyramidal layer to stimulate SCs. Stimuli were single or paired pulses (50- to 100-ms delay) delivered at 0.3 s−1 via a stimulus/isolation unit (Cibertec, Madrid, Spain). Patch electrodes were fabricated from borosilicate glass capillaries (1B150F-4, WPI, Saratoga, FL) with a Brown-Flamming puller (Model P-80, Sutter Instruments, CA). Electrodes had resistance of 4–8 MΩ when filled with the internal solution that contained (in mM) 97.5 K-glucuronate, 32.5 KCl 5 EGTA, 10 HEPES, 1 MgCl2, and 4 ATP (added immediately before recordings). In some experiments, K-glucuronate was equimolarly substituted with CsCl in the pipette solution, and no differences were found in the responses evoked by stimulation of SCs. Occasionally, the Ca2⁺ chelator 1,2-bis(2-aminophenoxy)ethane-N,N',N',N''-tetraacetic acid (BAPTA) was added to the internal pipette solution at a concentration of 200 μM. All pipette solutions were adjusted to pH 7.2–7.3 with KOH or COOH and had osmolarities between 280 and 290 mOsm/L.

Patch pipettes were positioned with a mechanical micromanipulator under visual control on the CA1 pyramidal layer. Whole cell recordings, either in the single-electrode current-clamp or voltage-clamp modes, were obtained with a PC-ONE amplifier (Dagan Corporation, MN). Pyramidal neurons were identified by the characteristic responses evoked by transmembrane current pulses under current clamp. Fast and slow capacitances were neutralized, and series resistance was compensated (~80%). Recordings were rejected when the series resistance (12.6 ± 4 MΩ; n = 101) changed >20% during the experiment. The membrane potential (V_m) was held at −70 mV in voltage-clamp experiments except when indicated otherwise. Data were filtered at 3 kHz and transferred to the hard disk of a Pentium based computer using a DigiData 1200 interface and the pCLAMP 6.0 software (Axon Instruments, Foster City, CA).

Caffeine (anhydrous, stock solution in distilled water) was added directly to the chamber with an automatic calibrated microsyringe through a pipette (tip diameter: 400 μm) positioned with a mechanical micromanipulator close to the recording electrode tip. A single volume of 100 μl of the caffeine solution (10 mM) was delivered (total delivery time was in <1 s) 10–20 min after intracellular access was obtained. We estimated that the washout of caffeine from the recording chamber was in <10 min by measuring the spread and clearance, respectively, of similar volumes of concentrated solutions of methyl blue. The washout of caffeine from the bath was also evaluated during continuous superfusion with adenosine (50 μM, n = 4) by the decay to previous values (in <11.0 ± 0.5 min) of the EPSC amplitude increase caused by the antagonistic effect of the caffeine “pulse” on the presynaptic inhibition evoked by adenosine. Glutamate (dissolved in distilled water at 0.7 M; pH adjusted to 8.2 with NaOH) was iontophoretically applied through a micropipette (10–50 MΩ), placed with a micromanipulator close to the recording electrode under visual guidance (<100 μm). A voltage pulse delivered by a stimulator (Cibertec, Madrid, Spain) to the voltage command input of a micro-electrode voltage-clamp amplifier (Biologic, Echirelles, France) provided the iontophoretic current. Drugs were purchased from Sigma except ryanodine, which was from Calbiochem (La Jolla, CA), suramin (stock solution in H2O), 8-cyclopentylephylamine (CPT; stock solution in DMSO at 0.001%), L-glutamic acid, and AP5, which were from Tocris Cookson (Bristol, UK).

The pre- or postsynaptic origin of the observed regulation of EPSC amplitudes by caffeine was tested by both estimating changes in the paired-pulse facilitation (PPF), that are considered of presynaptic origin (e.g., Clark et al. 1994; Creager et al. 1980) and the modifications in the variance of EPSC amplitudes that parallel the EPSC amplitude changes. We first calculated the noise-free coefficient of variation (νEPSC) of the synaptic responses under potentiated and control conditions using the formalism νEPSC = √(δEPSC2−δbaseline2)/m, where δEPSC and δbaseline are the variance of the peak EPSC and baseline, respectively, and m is the mean EPSC amplitude. Assuming that a binomial process governs release probability (see following text), the variance of the EPSC should vary less than the square of the mean (e.g., Kullmann 1994). Therefore if the mean EPSC amplitude increases as a consequence of the number of quanta released, the νEPSC should decrease. We also constructed plots comparing variation in the normalized m (termed M), to the change in response variance of the EPSC amplitude measured during the potentiation and normalized to the respective control values (i.e., 1/CV2) in each cell (Bekkers and Stevens 1990; Malinow and Tsien 1990). In these plots, that show the relationship between the 1/CV2 and M values should follow the diagonal and the 1/CV2 values remain under 1.0 if the excitatory effect has a presynaptic origin. This method requires a binomial EPSC amplitude distribution, a condition that must be met for the synaptic variance to reflect the probability of transmitter release (i.e., the quantal variance). We could not directly test whether our data fits a binomial distribution, but synaptic fluctuations were always evident, and we assumed that synaptic release followed a binomial distribution.

Analysis of the spontaneous EPSC activity was performed with the ACSPLOUF software (obtained from Dr. Pierre Vincent, University of California, San Diego, CA). We estimated the cumulative probabilities of the amplitude and frequency of the spontaneous EPSCs recorded during 10 min in control conditions and 10 min after the caffeine challenge during the caffeine LTP. Statistically significant differences were established at P < 0.05, using the Kolmogorov-Smirnov test. Data were compared using the Student’s t-test and values are given as the mean ± SE.

**RESULTS**

Results are based on 101 neurons that exhibited a stable V_m between −59 and −65 mV throughout an experiment (>70 min). The V_m was monitored, and the input resistance (R_p) was tested at specified times through experiments by injecting hyperpolarizing current pulses under current clamp. Cells that fired unclamped action currents in response to synaptic stimulation at any time during the experiment were rejected to eliminate the possible induction of activity-dependent plastic phenomena that may interfere with the analysis.

Brief caffeine “pulse” evokes the Caffeine LTP

Control recordings of SC EPSCs evoked by paired-pulse stimulation (100-ms interval) were obtained after checking their stability during 10–20 min (Fig. 1A). A single dose of 10 mM caffeine (100 μl) was then applied close to the recording
of caffeine were stable during >80 min without EPSC potentiation or rundown (Fig. 1E, n = 7).

The decreased PPF that paralleled the CAF LTP is consistent with an increased probability of transmitter release and thus suggests that a presynaptic mechanism is involved. Although the variance analysis suggests a presynaptic origin for the CAF LTP, other interpretations are also possible. The changes in the variance of EPSC amplitudes reduce the possibility of a postsynaptic participation but do not rule out the possible insertion of functional AMPA receptors at silent synapses that only express NMDA receptors. The freshly inserted AMPA receptors would signal an increased number of quanta by allowing the freshly incorporated receptors to sample release at terminals that were not tested before the potentiation (Kullmann 1994; Montgomery et al. 2001; Nicoll and Malenka 1999; Poncer and Malinow 2001).

Both AMPA and NMDA EPSC components are potentiated during the CAF LTP

A potentiation of the AMPA EPSC (AMPA_{EPSC}) component, without changes in the NMDA EPSC (NMDA_{EPSC}) component, characterizes the classical LTP of SC EPSCs in CA1 pyramidal neurons (e.g., Kullmann 1994; Nicoll and Malenka 1999; Poncer and Malinow 2001), which is thought to be triggered by the insertion of AMPA receptors at silent synapses (Isaac et al. 1995, 1998; Malenka and Nicoll 1999; Nicoll and Malenka 1999). However, if the CAF LTP has a presynaptic origin, the magnitude of the potentiation of both AMPA_{EPSC} and NMDA_{EPSC} should be similar. The AMPA_{EPSC} and NMDA_{EPSC} can be isolated because at ~60 mV, only the fast AMPA_{EPSC} is recorded due to the voltage-dependent block of the NMDA channel by extracellular Mg^{2+} whereas at +60 mV, there should also be a slower NMDA_{EPSC} caused by the relief of the Mg^{2+} block of NMDA channels (e.g., Collingridge et al. 1983; Hestrin et al. 1990). At +60 mV and at delays >50 ms, the AMPA_{EPSC} has completely vanished, whereas the slower NMDA_{EPSC} is peaking and its amplitude can be estimated in isolation (e.g., Hestrin et al. 1990).

We found that the potentiation evoked by the caffeine pulse was similar at ~60 and +60 mV (Fig. 2, A and B). The mean increases of peak AMPA_{EPSC} from controls were 255 ± 33, 217 ± 33, and 202 ± 21% 10, 40, and 70 min, respectively, after applying caffeine and the NMDA_{EPSC} (measured at delays of 50 ms) increased 289 ± 47, 231 ± 25, and 207 ± 39% 10, 40, and 70 min (n = 4) after caffeine, respectively (Fig. 2, A and B).

The “classical” SC LTP is paralleled by a reduction of the coefficient of variation (CV) of the AMPA_{EPSC} without changes in the CV of the NMDA_{EPSC}, a result that is also interpreted to indicate a postsynaptic LTP expression caused by the insertion of AMPA receptors at silent synapses (e.g., Kullmann 1994). However, the NF CV of AMPA_{EPSC} and NMDA_{EPSC} changed in similar proportions during the CAF LTP. The NF CV-AMPA decreased 0.23 ± 0.01, 0.19 ± 0.06, and 0.27 ± 0.05, 10, 40, and 70 min, respectively, after caffeine and NF CV-NMDA (measured at delays of 50 ms) decreased 0.10 ± 0.02, 0.11 ± 0.02, and 0.23 ± 0.06, 10, 40, and 70 min (same cells) after caffeine, respectively (Fig. 2C). These results are consistent with the CAF LTP being caused by a presynaptic
increase in the number of quanta released (see Methods and Discussion).

Therefore the preceding results taken together are consistent with a presynaptic origin of the CAF LTP caused by an increased release probability. In addition, the results reduce the possible contribution of the insertion of AMPA receptors at silent synapses during the CAF LTP that characterizes the "classical" SC LTP.

A contribution of activity-dependent synaptic modifications to the CAF LTP was minimized because the selected cells did not fire action potentials during the experiments. However, evoked transmitter release and presynaptic action potentials were always present during stimulation and could be involved in the genesis of the CAF LTP. To verify or disprove the contribution of presynaptic activity evoked by stimulation, we tested the effects of a pulse of caffeine in the absence of SC stimulation. After a control recording of SC EPSCs, stimulation was stopped and caffeine was applied. The CAF LTP was present (213 ± 28%, n = 3) when synaptic stimulation was resumed after a 60-min waiting period (Fig. 3, A and B). Therefore neither synaptic activity evoked by SC stimulation or postsynaptic action potential activity are necessary for the induction of the CAF LTP.

We also checked if caffeine was modifying the spontaneous synaptic activity by monitoring in voltage-clamp conditions the frequency and amplitude of spontaneous EPSCs in control conditions and from 10 min after the caffeine challenge (Fig. 3C) when the CAF LTP was growing to reach its maximum amplitude (see Fig. 1A). Pyramidal neurons in the CA1 region are known to display few spontaneous EPSCs in control conditions (e.g., Martín et al. 2001), and there were no significant differences from control values in the frequency and amplitude of spontaneous EPSCs and 10 min after caffeine application as indicated by comparing the corresponding cumulative probability plots of spontaneous EPSCs (Fig. 3D, n = 5). Therefore these results suggest that an increased "spontaneous" glutamate release is not contributing to the CAF LTP.

Caffeine may also modify the membrane conductance of the postsynaptic neuron and hence change the voltage control at the dendritic sites where EPSCs are generated, thus introducing possible artifacts in the recordings of EPSCs. However, there were no differences in the results obtained when K⁺ conductances were blocked with Cs⁺ in the pipette solution (n = 3). In addition, we found no significant changes in the rise and decay time constants (τ) of EPSCs (fits to single exponential functions) that may reflect membrane conductance modifications at, or near, the sites of EPSC generation (e.g., Mainen et al. 1996). Rise and decay τ were 5.6 ± 0.6 and 17 ± 1 ms in control and 6.6 ± 1.2 and 13 ± 2 ms 80 min after caffeine application (n = 7), and these values were not statistically significant. We also checked the effects of caffeine on the V_m and R_m by injecting hyperpolarizing current pulses. The V_m depolarized 2.5 ± 0.3 mV and the R_m changed from 124 ± 14 to 139 ± 15 MΩ in control and caffeine conditions, respectively (n = 15). These changes were not statistically significant. Therefore neither changes in voltage control nor in the

![FIGURE 2](http://jn.physiology.org/)

The CAF LTP is evoked in the absence of stimulus-evoked synaptic activity. A: representative traces showing averaged EPSCs evoked by SC stimulation, immediately before application of caffeine (1), then SC stimulation was stopped and the caffeine pulse (100 μM of 10 mM) was delivered. Stimulation was resumed after a 60-min waiting period (2). B: summary data showing the control and caffeine application (τ), and the CAF LTP when synaptic stimulation was resumed. C: representative records showing the spontaneous EPSC activity in control conditions and 10 min after caffeine. D: cumulative probability plots of the spontaneous EPSCs interevent interval and amplitude, recorded during 1–3 min in control (●), and 10 min after caffeine (○). Each value in the plots represents the mean of ≥5 cells.

![FIGURE 3](http://jn.physiology.org/)

Both AMPA_{EPSC} and N-methyl-D-aspartate (NMDA)_{EPSC} are similarly potentiated during the CAF LTP. A: representative EPSCs averages recorded at −60 and +60 mV (i.e., AMPA_{EPSC} and NMDA_{EPSC}, respectively) in control and 10 and 70 min following the caffeine pulse. B: summary data showing mean AMPA_{EPSC} peak amplitudes (−60 mV; ○) and NMDA_{EPSC} amplitude (+60 mV; ●) at 50-ms delay (n = 4). C: summary data showing mean NF CV values (see Methods) of AMPA_{EPSC}, −60 mV; ○) and NMDA_{EPSC}, +60 mV; ●) same cells. Note similar CAF LTP and NF CV reductions of both AMPA_{EPSC} and NMDA_{EPSC}.

![FIGURE 3](http://jn.physiology.org/)

The CAF LTP is evoked in the absence of stimulus-evoked synaptic activity. A: representative traces showing averaged EPSCs evoked by SC stimulation, immediately before application of caffeine (1), then SC stimulation was stopped and the caffeine pulse (100 μM of 10 mM) was delivered. Stimulation was resumed after a 60-min waiting period (2). B: summary data showing the control and caffeine application (τ), and the CAF LTP when synaptic stimulation was resumed. C: representative records showing the spontaneous EPSC activity in control conditions and 10 min after caffeine. D: cumulative probability plots of the spontaneous EPSCs interevent interval and amplitude, recorded during 1–3 min in control (●), and 10 min after caffeine (○). Each value in the plots represents the mean of ≥5 cells.
post synaptic K⁺ conductances can account for the observed effects of caffeine.

Classical LTP at SC-CA1 pyramidal neuron synapses does not occlude the CAF LTP

To further investigate the cellular mechanisms of the CAF LTP, we analyzed the effects of the caffeine pulse after evoking a classical LTP. After a brief control stabilization period, we induced the LTP either by tetanizing SCs (sequential delivery of 4 100-Hz 1-s-duration barrages repeated every 20 s; n = 2) or by simultaneously stimulating SCs at 2 Hz and depolarizing the recorded pyramidal cell to 0 mV during 60 s (n = 4). The LTP resulted in a 205 ± 17% increase of peak EPSC amplitude from controls 15 min after induction. Sixty minutes later we tested the effects of caffeine and a CAF LTP was evoked that resulted in a 228 ± 23% increase of peak EPSC amplitude (same cells) superimposed on the classical LTP (Fig. 4A). Therefore the preceding results suggest that different cellular mechanisms trigger both nondecremental po-
tations.

Caffeine does not potentiate postsynaptic glutamate currents

To further test the presynaptic nature of the CAF LTP, we examined the effects of caffeine on currents evoked by microio-
tophoretic glutamate applications. Control currents evoked by microiontophoresis of glutamate and control SC EPSCs were recorded in the same neurons (n = 6). Application of caffeine, evoked the CAF LTP of SC EPSCs (216 ± 21%) but did not significantly modify the glutamate-induced current (110 ± 13%; Fig. 4B). Therefore these results suggest that a rise in postsynaptic glutamate receptor sensitivity does not contribute to the CAF LTP.

Inhibition of NMDA receptors with AP5 does not prevent the CAF LTP

It is generally agreed that induction of the classical LTP at SC-CA1 pyramidal neuron synapses requires the activation of postsynaptic NMDA receptors (e.g., Collingridge and Bliss 1987; Kullmann et al. 1996; Nicoll and Malenka 1999; Ponder and Malinow 2001; Siegelbaum and Kandel 1991). We therefore investigated the effects of blocking NMDA receptors with AP5 (50 μM) that prevented the classical LTP but did not modify CAF LTP in any of the cells tested (n = 3; Fig. 4C). Therefore the results exclude a contribution of postsynaptic NMDA receptors in the genesis of the CAF LTP.

CAF LTP is insensitive to intracellular BAPTA

The classical SC LTP requires an increase in the intracellular postsynaptic Ca²⁺ concentration (e.g., Malenka 1991; Nishiyama et al. 2000). To establish if changes in the cytosolic-free Ca²⁺ concentration due to Ca²⁺ inflow were involved in the genesis of the CAF LTP, we added the fast Ca²⁺ chelator BAPTA to the internal pipette solution (e.g., Williams and Johnston 1989; Yeckel et al. 1999). Intracellular BAPTA (40 mM), did not block the CAF LTP (269 ± 24%; n = 8) but prevented the classical SC LTP evoked by tetanization of SCs in the same neurons (Fig. 5). Therefore postsynaptic increases in the intracellular-free Ca²⁺ concentration due to Ca²⁺ inflow do not contribute to the CAF LTP.

The preceding results are consistent with caffeine mediating the CAF LTP acting through presynaptic mechanisms, not depending on evoked synaptic activity, on postsynaptic action potentials, or on increases in the postsynaptic concentration of free Ca²⁺ and therefore supported by entirely different cellular mechanisms than the classical LTP at SC-CA1 pyramidal neuron synapses.

FIG. 4. The CAF LTP may be superimposed on a previously evoked classical LTP. A: representative EPSCs averages and summary data showing LTP induced by SC tetanization (↑↑) followed by caffeine application (↑↑; n = 7). B: averaged EPSCs (left) and averaged currents (right) evoked by glutamate microiontophoresis (horizontal bar) before (1) and 70 min after (2) caffeine in the same cell. C: averaged EPSCs and summary data showing CAF LTP in the presence of bath applied 2-amino-5-phosphonopentanoic acid (50 μM; n = 3).

FIG. 5. The CAF LTP is insensitive to intracellular 1,2-bis (2-aminophenoxy) ethane-N,N,N',N'-tetraacetic acid (BAPTA). The classical LTP evoked by SC tetanization (↑↑) was blocked with 40 mM BAPTA in the pipette solution but the CAF LTP was evoked (↑↑) in the same cells (n = 8).
Block of adenosine P₁ and purinergic P₂ receptors inhibits the CAF LTP

Adenosine is the end product of ATP hydrolysis and blocks synaptic responses by interacting with specific presynaptic P₁ receptors (e.g., Burnstock 1990; Miras-Portugal et al. 2000). Moreover, caffeine blocks P₁ receptors (Daly et al. 1983; Fredholm et al. 1999; see following text), suggesting that interactions between caffeine and P₁ receptors may contribute to the CAF LTP, by increasing Ca²⁺ influx during the action potential (see DISCUSSION). Therefore we blocked adenosine P₁ receptors with CPT (10 µM; n = 5), which is an A₁ type adenosine receptor antagonist, and applied the caffeine pulse 20 min latter. The CPT treatment inhibited the CAF LTP (Fig. 6A), suggesting that inhibition of adenosine P₁ receptors by caffeine plays a major role in the induction of the CAF LTP. An increase of the EPSC peak amplitude was evoked by superfusion of 10 µM CPT (141 ± 16%; <40 min; n = 4) in the absence of the caffeine challenge, suggesting that in control conditions extracellular adenosine was inhibiting glutamate release (not shown).

Caffeine may evoke ATP release from neurons and glia (see DISCUSSION), therefore we tested the effects of pirdoxal-5'-phosphate-azophenyl 2',4'-disulphonate (PPADS) and of suramin, which are P₂ purinergic receptor antagonists. Superfusion with PPADS (20 µM) starting 10 min before the caffeine challenge abolished the late sustained phase of the CAF LTP without changing the amplitude of initial phase (Fig. 6B). The magnitude of the initial caffeine potentiation was 223 ± 22% (5 min; n = 5), and EPSCs amplitudes decreased to control values in <40 min. (Fig. 6B). Suramine, at a concentration of 10 µM that does not inhibit glutamate receptors (e.g., Nakatsuka and Gu 2001), reduced the magnitude and duration of the initial potentiation induced by caffeine (148 ± 29%; ∼5 min; n = 5) and also abolished the late phase of the CAF LTP (Fig. 6C).

To test if block of P₂ purinoreceptors inhibited the late phase of the CAF LTP by interaction with processes occurring during the initial phase or conversely if the block occurred during the late phase of the CAF LTP, we first evoked the CAF LTP (248 ± 33%; ∼10 min) and 10 min after superfused suramin (10 µM; n = 4). The potentiated EPSCs gradually decreased to reach control values in <50 min (Fig. 6D). Therefore these results suggest that activation of P₂ purinoreceptors is essential for the genesis of the late phase of the CAF LTP. Suramine did not modify the EPSC from control values (96.9 ± 5%; 40 min; n = 4) in the absence of caffeine.

The preceding results taken together suggest that the CAF LTP requires the inhibition of P₁ and the subsequent activation of P₂ purinoreceptors (see DISCUSSION).

Ryanojine superfusion blocks the CAF LTP

The preceding results suggest that caffeine acts presynaptically probably by increasing the release of Ca²⁺ from caffeine/ryanodine-sensitive Ca²⁺ stores. We tested the effects of blocking the release of Ca²⁺ from stores with extra- and intracellular-applied ryanodine. We reasoned that bath-applied ryanodine would block both pre- and postsynaptic stores, whereas ryanodine in the pipette solution would only block postsynaptic receptors. When ryanodine was superfused or added to the patch pipette cells were repeatedly depolarized (5 200-ms duration pulses to 0 mV) to induce Ca²⁺ influx through voltage-gated channels because it has been proposed that a rise in the intracellular Ca²⁺ concentration is required for ryanodine to interfere with release from intracellular Ca²⁺ stores (e.g., Caillard et al. 2000). To avoid possible activity-dependent poteniations, presynaptic stimulation was absent during these depolarizations, which were applied 5–10 min before the caffeine challenge.

We superfused with ACSF containing 20 µM ryanodine, a concentration that applied extracellular irreversibly blocks the ryanodine-sensitive Ca²⁺ stores (McPherson et al. 1991), and >40 min later we tested the effects of the caffeine challenge.
Caffeine induced a transient increase (136 ± 52%; ≈10 min.; n = 5) of the EPSC amplitude followed by a sustained decrease below control values (68 ± 18%) during the rest of the experiment, indicating that extracellular ryanodine blocked the CAF-LTP (Fig. 7A).

To test if the block of the late phase of the CAF-LTP by extracellular ryanodine was due to interaction with presynaptic Ca²⁺ release from stores occurring during the early or the late phase of the CAF-LTP, we superfused with ryanodine (20 µM) 10 min after inducing the CAF-LTP (Fig. 7B). The potentiation reached values of 260 ± 24% immediately before the ryanodine challenge (n = 4) and then gradually decreased without reaching control values (209 ± 21, 130 ± 16, and 131 ± 25%, 30, 50, and 70 min, respectively, after caffeine; Fig. 7B). Therefore ryanodine totally inhibited the CAF-LTP when superfused before but not if applied after the caffeine challenge. Ryanodine did not modify the EPSC from control values (102 ± 6%; 40 min; n = 4) in the absence of caffeine.

The preceding results suggest that ryanodine blocks the CAF-LTP by interacting with the release of Ca²⁺ from presynaptic stores during the early phase of the CAF-LTP. However, the reduction of the CAF-LTP by delayed application of ryanodine (Fig. 7B), may also suggest an important contribution of Ca²⁺ release intracellular presynaptic stores to the late phase. However, intracellular ryanodine (200 µM in the pipette solution; see METHODS) applied >20 min after attaining access to the intracellular compartment, had no effect on the CAF-LTP (240 ± 20%; n = 5; Fig. 7C).

Therefore this analysis suggests that presynaptic Ca²⁺ stores contribute to the CAF-LTP, whereas postsynaptic Ca²⁺ stores are not involved.

**Discussion**

We describe a new form of LTP of synaptic transmission at SC-CA1 pyramidal neuron synapses that is evoked by a brief pulse of caffeine and requires interactions with presynaptic P₁ adenosine, P₂ purino, and ryanodine receptors. This CAF-LTP does not entail the activation of NMDA receptors nor rises in the postsynaptic cytosolic concentration of free Ca²⁺. In addition, this potentiation does not require postsynaptic depolarization, is activity-independent, and does not follow the Hebbian rule because neither evoked pre- nor postsynaptic action potential activity is required to generate the CAF-LTP.

**Presynaptic origin of the CAF-LTP**

We show that the CAF-LTP is associated with a nondecremental reduction of the PPF, a similar and persistent potentiation of both AMPAEPSC and NMDAEPSC, and comparable and sustained modifications in the variance of the amplitudes of both AMPAEPSC and NMDAEPSC. All the preceding effects have been linked with an increased probability of transmitter release (Atwood and Karunanithi 2002; Bekkers and Stevens 1990; Clark et al. 1994; Creager et al. 1980; Isaac et al. 1998; Kullmann 1994; Malinow and Tsien 1990; Poncer and Malinow 2001; Schulz et al. 1994; Stevens and Wang 1994). Therefore the increased probability of release during the CAF-LTP reinforces the reliability of transmission between individual SC terminals and CA1 pyramidal neurons.

The PPF (1st point in the preceding paragraph) is a form of short-term presynaptic plasticity characterized by an increased peak amplitude of the second EPSC when it is elicited shortly after (<100 ms) a preceding EPSC (Clark et al. 1994; Creager et al. 1980; Fernández de Sevilla et al. 2002; Poncer and Malinow 2001). The increased second EPSC is thought to be caused by the raise in release probability that results from Ca²⁺ remaining in the terminal after the first action potential (Kamiya and Zucker 1994). Moreover, changes in the PPF are considered of presynaptic origin (e.g., Creager et al. 1980; Clark et al. 1994).

Solid support in favor of a presynaptic origin of the CAF-LTP was provided by the similar potentiation of both AMPAEPSC and NMDAEPSC (2nd point in the preceding paragraph) and by the similar reductions of the CV of both AMPAEPSC and NMDAEPSC (3rd point in the preceding paragraph) during the CAF-LTP.

The parallel changes of both AMPAEPSC and NMDAEPSC during the CAF-LTP contrast with the classical SC LTP of CA1 neurons.
pyramidal neurons, where a potentiation of the AMPA EPSC and a reduction of the CV of the AMPA EPSC amplitude take place without changes in the NMDA EPSC. The isolated changes in the AMPA EPSC that parallel the classical SC LTP are interpreted to result from the insertion of functional AMPA receptors at silent synapses (e.g., Issac et al. 1995, 1998; Kullmann 1994; Malenka and Nicoll 1999; Poncer and Malinow 2001).

The parallel potentiation and CV reductions of both AMPA EPSC and NMDA EPSC during the CAF LTP (present results) are consistent with a presynaptic increase in the number of quanta released because the variance of the signal must increase less than the square of the mean peak EPSC amplitude if the increment has a presynaptic origin (Kullmann 1994) (see METHODS). Therefore taken together the preceding results can be explained by a presynaptic origin of the CAF LTP mediated by an increased release probability (e.g., Atwood and Karunanithi 2002). In addition, the findings reduce the possibility of a postsynaptic participation triggered by the insertion of functional AMPA receptors at silent synapses.

A CAF LTP can be superimposed on a previously evoked classical LTP (present experiments), suggesting different cellular mechanisms and sites of origin for both types of sustained potentions. Activation of postsynaptic NMDA receptors is required to induce the classical SC LTP in CA1 pyramidal cells (e.g., Collingridge and Bliss 1987; Nicoll and Malenka 1999), an effect that we can exclude in our experiments because blocking NMDA receptors with AP5 did not modify the CAF LTP. In addition, the response evoked by glutamate iontophoresis is not potentiated by caffeine (present results), an effect that contrasts with the finding that responses evoked by exogenous AMPA are potentiated during the LTP in CA3 pyramidal neurons (e.g., Montgomery et al. 2001). Therefore an increased postsynaptic AMPA receptor sensitivity is not involved in the CAF LTP.

The classical LTP requires an increase in the postsynaptic intracellular concentration of free Ca$^{2+}$ and is blocked by intracellular BAPTA that chelates Ca$^{2+}$ and prevents the rise in cytosolic Ca$^{2+}$ (Clark et al. 1994; Williams and Johnston 1989; Yeckel et al. 1999). However, BAPTA in the patch pipette did not block the CAF LTP but prevented the classical LTP (present results), ruling out a postsynaptic effect due to an increase in the cytosolic-free Ca$^{2+}$ concentration in the genesis of the CAF LTP.

Purinergic receptors mediate the CAF LTP

Inhibition of A1 type adenosine receptors with CPT prevented the initial potentiation and the subsequent CAF LTP, suggesting that adenosine contributes to the initial induction phase of the CAF LTP. Adenosine inhibits synaptic transmission through the activation of presynaptic P$_1$ adenosine receptors and caffeine acts via an antagonism of adenosine P$_1$ receptors (e.g., Daly et al. 1983; Fredholm et al. 1999). Adenosine is released in the slice preparation and may attain extracellular concentrations that could inhibit glutamate release (Fredholm et al. 1984). Caffeine relieves the presynaptic inhibition by extracellular adenosine (Daly et al. 1983; Fredholm et al. 1999; Greene et al. 1985), thus increasing the inflow of Ca$^{2+}$ and the probability of transmitter release. Consequently, superfusion with CPT would block P$_1$ adenosine receptors at the presynaptic terminals of SCs, thus preventing the increased quantal release that caffeine would otherwise elicit by inhibiting presynaptic P$_1$ adenosine receptors (see following text).

The late persistent expression phase of the CAF LTP was prevented by the block of P$_2$ purinoreceptors with PPADS or suramin, implying that it requires activation of P$_2$ purinergic receptors. However, to our knowledge there is no mention in the literature that caffeine directly interacts with P$_2$ receptors. Therefore caffeine must evoke the release of ATP that activates P$_2$ receptors from neurons and astrocytes. Evidence exists indicating that caffeine induces the release of ATP from neurons (e.g., Queiroz et al. 1999), and it is extremely likely that caffeine also evokes the release of ATP from astrocytes. Indeed, Ca$^{2+}$ signals are evoked by the caffeine challenge in astrocytes and ATP is released and propagates the Ca$^{2+}$ signal to other astrocytes (Cotrina et al. 1998; Golovina et al. 1996; Schipke et al. 2002). Therefore these results suggest that the induction of the CAF LTP requires inhibition of P$_1$ adenosine receptors and the expression of the CAF LTP entails the activation of P$_2$ purinoreceptors by the ATP released from neurons and astrocytes.

Release of Ca$^{2+}$ from presynaptic stores contributes to the CAF LTP

We show that ryanodine in the intracellular pipette solution, at concentrations one order of magnitude higher than those that block Ca$^{2+}$ release in CA3 pyramidal cells (Caillard et al. 2000), had no effect on the CAF LTP, excluding a contribution of the release of Ca$^{2+}$ from postsynaptic caffeine/ryanodine-sensitive stores. However, extracellular ryanodine superfused before the caffeine challenge prevented the CAF LTP, suggesting an action mediated via modifications of the release of Ca$^{2+}$ from presynaptic caffeine/ryanodine-sensitive stores (e.g., Caillard et al. 2000; Empetage et al. 2001; Kriżaj et al. 1999; Liang et al. 2002; McPherson et al. 1991). The presence of a caffeine/ryanodine-sensitive intracellular Ca$^{2+}$ pool has been well established in CA3 pyramidal neurons (e.g., McPherson et al. 1991; Miller et al. 1996), and the release of Ca$^{2+}$ from intracellular stores may regulate glutamate release at the SC terminals in (Caillard et al. 2000; Empetage et al. 2001) mossy fiber terminals in the hippocampus (Liang et al. 2002) and hippocampal neurons in culture (e.g., Križaj et al. 1999) via a Ca$^{2+}$-induced Ca$^{2+}$-release mechanism. Therefore present results are consistent with the view that an increased release of Ca$^{2+}$ from presynaptic stores during the initial phase of the CAF LTP is required for the induction of the CAF LTP. In addition, we show that ryanodine applied after inducing the CAF LTP markedly reduced but did not prevent the CAF LTP, suggesting that intracellular Ca$^{2+}$ release also contributes to the late expression phase of the CAF LTP. The release of Ca$^{2+}$ would result in a higher intracellular Ca$^{2+}$ concentration at presynaptic terminals and an increased probability of release by the arriving action potential. However, contrasting results showing no contribution of calcium-induced calcium release in short term plasticity have also been reported in CA1 pyramidal neurons (Carter et al. 2002), suggesting that different types of presynaptic plasticities may require diverse mechanisms.

Cellular mechanism of the caffeine potentiation

It is generally recognized that the Ca$^{2+}$ concentration at the presynaptic terminal regulates transmitter release (e.g., At-
Caffeine concentrations in the millimolar range release Ca$^{2+}$ from intracellular ryanodine-sensitive stores by increasing the affinity of the ryanodine receptor for cytosolic-free Ca$^{2+}$ (e.g., Fredholm et al. 1999; Hernández-Cruz et al. 1995). Caffeine at millimolar concentrations also inhibits P1 adenosine receptors (Fredholm et al. 1999). At lower micromolar concentrations, caffeine acts exclusively via an antagonism of adenosine P1 receptors (e.g., Daly et al. 1983; Fredholm et al. 1999). Adenosine inhibits synaptic transmission by blocking presynaptic N-type Ca$^{2+}$ channels (e.g., Wu and Saggau 1994). Adenosine is released in the slice preparation and may attain extracellular concentrations that could inhibit glutamate release (Fredholm et al. 1984, 1999). Caffeine by blocking P1 receptors relieves the presynaptic inhibition by extracellular adenosine (Daly et al. 1983; Fredholm et al. 1999; Greene et al. 1985), thus increasing the inflow of Ca$^{2+}$ evoked by the action potential invading the terminal. The increased Ca$^{2+}$ inflow triggers a larger release of Ca$^{2+}$ from the caffeine/ryanodine sensitive stores through the sensitized (by caffeine) Ca$^{2+}$-activated Ca$^{2+}$-release mechanism. These effects of caffeine are initiated without the need of presynaptic action potential activity (present results), but they become manifest as an increased release probability when the action potential invades the terminal (present results).

Although the underlying molecular machinery of the cAFTP remains to be discovered, the cooperative effects of a block of presynaptic adenosine receptors—that would increase Ca$^{2+}$ influx through voltage-gated N channels—and the sustained increase in Ca$^{2+}$ sensitivity of the presynaptic ryanodine-sensitive Ca$^{2+}$ stores could provide the rise in presynaptic Ca$^{2+}$ that evokes the increased transmitter release.

In conclusion, the cAFTP requires the interaction of caffeine with presynaptic P1, P2 purinoreceptors, and ryanodine receptors and is caused by an increased probability of glutamate release at SC terminals. Therefore this cAFTP is mediated by entirely different mechanisms than those that have been proposed to underlie the “classical” LTP.

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Ca$^{2+}$


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