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 P2 adenosine receptor antagonist 8-cyclopentyltheophylline and the P1 purino-receptor antagonists suramin and piridoxal-5′-phosphonopentanoic acid and to Ca2+ influx through 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 purino-receptors, 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; Ponce 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 purino-receptors, 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 pincher 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 h 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 GABAₐ-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 (50–100 ms delay) delivered at 0.3 s⁻¹ via a stimulator/isolation unit (Cibertec, Madrid, Spain). Patch electrodes were fabricated from borosilicate glass capillaries (1B150F-4, WPI, Saratosa, 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-glutamate, 32.5 KCl 5 EGTA, 10 HEPES, 1 MgCl₂, and 4 ATP (added immediately before recordings). In some experiments, K-glutamate 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 final concentration of 40 mM. In other experiments, ryanodine (stock solution in ethanol at 0.01%) was included in the internal pipette solution at a final concentration of 200 μM. All pipette solutions were adjusted to pH 7.2–7.3 with KOH or CsOH 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 methylene 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 (<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, Echirroles, 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-cyclopentylephedrine (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 (σEPSC) of the synaptic responses under potentiated and control conditions using the formalism σEPSC ≡ √σEPSC² − σnoise²/µs, where σEPSC and σnoise are the variance of the peak EPSC and baseline, respectively, and µs 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 σEPSC 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 CAF 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 CAF 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. 1A1). A single dose of 10 mM caffeine (100 μl) was then applied close to the recording...
pipette (see METHODS). After a short delay (<5 min) after the "pulse" of caffeine a marked potentiation of the peak EPSC amplitude was evoked that reached a maximum value in ~15 min (a 229 ± 20% increase above control values; n = 7) and remained stable during the rest of the experiment (192 ± 26%; 70 min; n = 7; Fig. 1, A and B). Therefore this CAF-LTP was not reverted by a prolonged washout in control ACSF.

A marked persistent and significant reduction of the PPF index (~0.25 ± 0.06; P = 0.04; same cells) paralleled the CAF-LTP (Fig. 1C). We also constructed plots of the 1/CV² ratio as a function M (see METHODS) 10, 40, and 70 min after caffeine application. The 1/CV² ratios revealed that values grouped following the diagonal and were consistently <1.0. The mean 1/CV² ratios were 0.33 ± 0.07, 0.52 ± 0.2, and 0.51 ± 0.09 measured 10, 40, and 70 min after applying caffeine, respectively (Fig. 1D, same cells). Control recordings in the absence 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 suggest 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 Mg²⁺, whereas at +60 mV, there should also be a slower NMDA_{EPSC} caused by the relief of the Mg²⁺ 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 values 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}. 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

**FIG. 1.** Nondecremental caffeine potentiation; i.e., the caffeine-mediated long-term potentiation (CAF-LTP). A: representative traces of averaged excitatory postsynaptic currents (EPSCs; 10 successive responses, as in all other cases) evoked by paired-pulse (100-ms delay) Schaffer collateral (SC) stimulation, before (1), 15 min after (2), and 70 min after (3) application of a 100 μM of 10 mM caffeine "pulse." B: summary data (n = 7; •) showing the time course of caffeine effects (top, as in other figures) on normalized (to control values) mean EPSC amplitudes. Note the nondrecremental potentiation (CAF-LTP). C: summary data of the persistent reduction of the paired-pulse facilitation (PPF) index. D: plot of normalized 1/CV² (CV = coefficient of variation of peak EPSC amplitudes; n = 7) vs. the normalized M (mean EPSC peak amplitudes; see METHODS) 10, 40, and 70 min after caffeine. E: in the absence of caffeine there were no significant changes in the average peak EPSC amplitude (n = 7). Holding potential was ~70 mV and vertical bars show SE (as in all other cases).
resumed after a 60-min waiting period (Fig. 3, A) when the CAF LTP was growing to reach its maximum conditions and from 10 min after the caffeine challenge (Fig. 3, B). Stimulation was resumed after a control recording of SC EPSCs, stimulation was stopped and the caffeine pulse (100 μM) was delivered. 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 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 Vm by injecting hyperpolarizing current pulses. The Vm depolarized 2.5 ± 0.3 mV and the Rm 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

![FIG. 2. Both AMPAEPSCs and N-methyl-D-aspartate (NMDA)EPSCs are similarly potentiated during the CAF LTP. A: representative EPSCs averages recorded at -60 and +60 mV (i.e., AMPAEPSCs and NMDAEPSCs, respectively) in control and 10 and 70 min following the caffeine pulse. B: summary data showing mean AMPAEPSCs peak amplitudes (-60 mV; ○) and NMDAEPSCs amplitude (+60 mV; ●) at 50-ms delay (n = 4). C: summary data showing mean sCV values (see METHODS) of AMPAEPSCs, (-60 mV; ○) and NMDAEPSCs (+60 mV; ●) same cells). Note similar CAF-LTP and sCV reductions of both AMPAEPSCs and NMDAEPSCs.](image-url)

![FIG. 3. 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 peak EPSC amplitudes, the caffeine application (↑), 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.](image-url)
iontophoretic 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; Poncer 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.

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 micro-

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 (↑↑; 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).

**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 ± 23% 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 postsynaptic K+ conductances can account for the observed effects of caffeine.

**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 micro-

FIG. 5. The CAF-LTP is insensitive to intracellular 1,2-bis (2-aminophenoxy) ethane-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).

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Block of adenosine P1 and purinergic P2 receptors inhibits the \( \text{CAF LTP} \)

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 \( \text{CAF LTP} \), by increasing \( \text{Ca}^{2+} \) influx during the action potential (see Discussion). Therefore we blocked adenosine P1 receptors with CPT (10 \( \mu \text{M} \); \( n = 5 \)), which is an A1 type adenosine receptor antagonist, and applied the caffeine pulse 20 min latter. The CPT treatment inhibited the \( \text{CAF LTP} \) (Fig. 6A), suggesting that inhibition of adenosine P1 receptors by caffeine plays a major role in the induction of the \( \text{CAF LTP} \). An increase of the EPSC peak amplitude was evoked by superfusion of 10 \( \mu \text{M} \) CPT (141 \pm 16%; \( <40 \text{ min} \); \( n = 4 \)) 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 piridoxal-5'-phosphate-azophenyl \( 2',4' \)-disulphonate (PPADS) and of suramin, which are P2 purinergic receptor antagonists. Superfusion with PPADS (20 \( \mu \text{M} \)) starting 10 min before the caffeine challenge abolished the late sustained phase of the \( \text{CAF LTP} \) without changing the amplitude of initial phase (Fig. 6B). The magnitude of the initial caffeine potentiation was 223 \pm 22\% (5 min; \( n = 5 \)), and EPSCs amplitudes decreased to control values in \( <40 \text{ min} \). (Fig. 6B). Suramine, at a concentration of 10 \( \mu \text{M} \) 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 \pm 29\%; \( \approx 5 \text{ min} \); \( n = 5 \)) and also abolished the late phase of the \( \text{CAF LTP} \) (Fig. 6C).

To test if block of P2 purinoreceptors inhibited the late phase of the \( \text{CAF LTP} \) by interaction with processes occurring during the initial phase or conversely if the block occurred during the late phase of the \( \text{CAF LTP} \), we first evoked the \( \text{CAF LTP} \) (248 \pm 33\%; \( \approx 10 \text{ min} \)) and 10 min after superfused suramin (10 \( \mu \text{M} \); \( n = 4 \)). The potentiated EPSCs gradually decreased to reach control values in \( <30 \text{ min} \) (Fig. 6D). Therefore these results suggest that activation of P2 purinoreceptors is essential for the genesis of the late phase of the \( \text{CAF LTP} \). Suramine did not modify the EPSC from control values (96.9 \pm 5\%; 40 min; \( n = 4 \)) in the absence of caffeine.

The preceding results taken together suggest that the \( \text{CAF LTP} \) requires the inhibition of P1 and the subsequent activation of P2 purinoreceptors (see Discussion).

Ryanojine superfusion blocks the \( \text{CAF LTP} \)

The preceding results suggest that caffeine acts presynaptically probably by increasing the release of \( \text{Ca}^{2+} \) from caffeine/ryanodine-sensitive \( \text{Ca}^{2+} \) stores. We tested the effects of blocking the release of \( \text{Ca}^{2+} \) 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 \( \text{Ca}^{2+} \) influx through voltage-gated channels because it has been proposed that a rise in the intracellular \( \text{Ca}^{2+} \) concentration is required for ryanodine to interfere with release from intracellular \( \text{Ca}^{2+} \) 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 \( \mu \text{M} \) ryanodine, a concentration that applied extracellular irreversibly blocks the ryanodine-sensitive \( \text{Ca}^{2+} \) 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).

We show 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 NP-TCV of both AMPAEPSC and NMDAEPSC (3rd point in the preceding paragraph) during the CAF-LTP.

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 NP-TCV 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.
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., Isaac et al. 1995, 1998; Kullmann 1994; Malenka and Nicoll 1999; Ponce 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 potentiations. 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 ionophoresis 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 Ca²⁺ and is blocked by intracellular BAPTA that chelates Ca²⁺ and prevents the rise in cytosolic Ca²⁺ (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 Ca²⁺ 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 P₁ adenosine receptors and caffeine acts via an antagonism of adenosine P₁ 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 Ca²⁺ and the probability of transmitter release. Consequently, superfusion with CPT would block P₁ adenosine receptors at the presynaptic terminals of SCs, thus preventing the increased quantal release that caffeine would otherwise elicit by inhibiting presynaptic P₁ adenosine receptors (see following text).

The late persistent expression phase of the CAF LTP was prevented by the block of P₂ purinoreceptors with PPADS or suramin, implying that it requires activation of P₂ purinergic receptors. However, to our knowledge there is no mention in the literature that caffeine directly interacts with P₂ receptors. Therefore caffeine must evoke the release of ATP that activates P₂ 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, Ca²⁺ signals are evoked by the caffeine challenge in astrocytes and ATP is released and propagates the Ca²⁺ 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 P₁ adenosine receptors and the expression of the CAF LTP entails the activation of P₂ purinoreceptors by the ATP released from neurons and astrocytes.

**Release of Ca²⁺ 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 Ca²⁺ release in CA3 pyramidal cells (Caillard et al. 2000), had no effect on the CAF LTP, excluding a contribution of the release of Ca²⁺ 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 Ca²⁺ from presynaptic caffeine/ryanodine-sensitive stores (e.g., Caillard et al. 2000; Empetage et al. 2001; Krizaj et al. 1999; Liang et al. 2002; McPherson et al. 1991). The presence of a caffeine/ryanodine-sensitive intracellular Ca²⁺ pool has been well established in CA3 pyramidal neurons (e.g., McPherson et al. 1991; Miller et al. 1996), and the release of Ca²⁺ 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., Krizaj et al. 1999) via a Ca²⁺-induced Ca²⁺-release mechanism. Therefore present results are consistent with the view that an increased release of Ca²⁺ 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 Ca²⁺ release also contributes to the late expression phase of the CAF LTP. The release of Ca²⁺ would result in a higher intracellular Ca²⁺ 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 Ca²⁺ concentration at the presynaptic terminal regulates transmitter release (e.g., At-
wood and Karunanithi 2002). Caffeine concentrations in the millimolar range release Ca\(^{2+}\) from intracellular ryanoide-sensitive stores by increasing the affinity of the ryanoide receptor for cytosolic-free Ca\(^{2+}\) (e.g., Fredholm et al. 1999; Hernández-Cruz et al. 1995). Caffeine at millimolar concentrations also inhibits P\(_1\) adenosine receptors (Fredholm et al. 1999). At lower micromolar concentrations, caffeine acts exclusively via an antagonism of adenosine P\(_1\) 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 P\(_1\) receptors relieves the presynaptic inhibition by extracellular adenosine (Daly et al. 1983; Fredholm et al. 1999; Greene et al. 1985), thus increasing the influx of Ca\(^{2+}\) evoked by the action potential invading the terminal. The increased Ca\(^{2+}\) influx triggers a larger release of Ca\(^{2+}\) from the caffeine/ryanoide 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 CAF LTP 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 ryanoide-sensitive Ca\(^{2+}\) stores could provide the rise in presynaptic Ca\(^{2+}\) that evokes the increased transmitter release.

In conclusion, the CAF LTP requires the interaction of caffeine with presynaptic P\(_1\), P\(_2\) purinoreceptors, and ryanoide receptors and is caused by an increased probability of glutamate release at SC terminals. Therefore this CAF LTP is mediated by entirely different mechanisms than those that have been proposed to underlie the “classical” LTP.

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