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Calcium-Induced Calcium Release Contributes to Action Potential-Evoked Calcium Transients in Hippocampal CA1 Pyramidal Neurons

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Calcium-induced calcium release (CICR) is a mechanism by which local elevations of intracellular calcium (Ca^{2+}) are amplified by Ca^{2+} release from ryanodine-sensitive Ca^{2+} stores. CICR is known to be coupled to Ca^{2+} entry in skeletal muscle, cardiac muscle, and peripheral neurons, but no evidence suggests that such coupling occurs in central neurons during the firing of action potentials. Using fast Ca^{2+} imaging in CA1 neurons from hippocampal slices, we found evidence for CICR during action potential-evoked Ca^{2+} transients. A low concentration of caffeine enhanced Ca^{2+} transient amplitude, whereas a higher concentration reduced it. Simultaneous Ca^{2+} imaging and whole-cell recordings showed that membrane potential, action potential amplitude, and waveform were unchanged during caffeine application. The enhancement of Ca^{2+} transients by caffeine was not affected by the L-type channel blocker nifedipine, the phosphodiesterase inhibitor IBMX, the adenylyl cyclase activator forskolin, or the PKA antagonist H-89. However, thapsigargin or ryanodine, which both empty intracellular Ca^{2+} stores, occluded this effect. In addition, thapsigargin, ryanodine, and cyclopiazonic acid reduced action potential-evoked Ca^{2+} transients in the absence of caffeine. These results suggest that Ca^{2+} release from ryanodine-sensitive stores contributes to Ca^{2+} signals triggered by action potentials in CA1 neurons.

Key words: hippocampus; slices; fura-2; patch clamp; caffeine; thapsigargin

Calcium ion (Ca^{2+}) is an important second messenger that participates in the triggering and regulation of many neuronal processes, including neurotransmitter release (Mulkey and Zucker, 1991; Borst and Sakmann, 1996), synaptic plasticity (Bear and Malenka, 1994; Malenka, 1994), and transcription control (Hardingham et al., 1997). In central neurons a fast change in $[Ca^{2+}]_i$ (free intracellular Ca^{2+}) can be triggered in the soma and dendrites by sodium action potentials (Jaffe et al., 1992; Spruston et al., 1995). Although it generally is agreed that action potentials cause $[Ca^{2+}]_i$ elevations by opening voltage-gated Ca^{2+} channels (Christie et al., 1995), it remains unclear whether such influx is the only source of Ca^{2+}.

Ca^{2+}-induced Ca^{2+} release (CICR), a process of Ca^{2+} mobilization involving ryanodine receptor (RyR) channels, has been described as a major contributor of action potential-evoked Ca^{2+} signals in muscles (Nabauer et al., 1989; Cannell et al., 1995; Lopez-Lopez et al., 1995) and in peripheral sensory neurons (Usachev and Thayer, 1997). Depolarization-induced Ca^{2+} influx also has been suggested to cause CICR in cerebellar Purkinje cells (Llano et al., 1994), but no clear demonstration of CICR during action potentials has been documented in central neurons.

Several requirements for CICR occurring in neurons can be predicted. Theoretical calculations estimate that high concentrations of Ca^{2+} can be reached only at distances of tens of nanometers from the mouth of a Ca^{2+} channel within microseconds (Chow et al., 1994; Cannell and Soeller, 1997; Klingauf and Neher, 1997; Soeller and Cannell, 1997). Therefore, a close proximity of RyR channels to voltage-gated Ca^{2+} channels is probably important and required for their opening. In addition to RyRs, endoplasmic reticulum (ER) Ca^{2+}-ATPases (SERCA), coexpressed with RyRs, are also necessary for CICR.

These structural requirements for CICR have been documented in CA1 hippocampal pyramidal neurons. These cells have the highest levels of expression of the brain-type RyR3 (Furuichi et al., 1994), which is expressed in the soma, dendrites, and axon. Similarly, the highest expression levels of the SERCA-2, found in the brain, cardiac, and slow-twitch muscle, occur in the hippocampus as well as in the cerebellum, cortex, and thalamus (Miller et al., 1991). In addition, it has been shown that RyRs in central neurons are located mostly in close vicinity to the plasmalemma (for review, see Berridge, 1998). Moreover, they are colocalized together with the SERCA in the smooth ER (Sah et al., 1993; Sah and Dulhunty, 1994). Equally important is that the ER of CA1 pyramidal neurons is filled with Ca^{2+} at rest (Garaschuk et al., 1997).

Here, we tested whether Ca^{2+} influx evoked by either a single or a few action potentials triggers CICR and whether this could influence significantly the overall magnitude of action potential-induced Ca^{2+} signals. Using fast optical imaging (Lasser-Ross et al., 1991) in fura-2 AM-loaded hippocampal slices of the rat (Grynkiewicz et al., 1985; Garaschuk et al., 1997) and whole-cell patch-clamp recordings, we provide evidence in favor of this hypothesis.

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MATERIALS AND METHODS

Slice preparation. Transverse hippocampal slices (300 μm thick) were prepared from 10- to 17-d-old Sprague Dawley rats as previously described (Tsubokawa and Ross, 1997), except that cutting was performed between 0 and 1°C. The cutting solution was composed of (in mM): 120 choline-Cl, 3 KCl, 8 $MgCl_2$, 1.25 NaH_2PO_4, 26 $NaHCO_3$, and 10 glucose, pH 7.4, when bubbled with 95% O_2/5% CO_2, 300–315 mOsm/kg. After cutting, the slices were warmed to 30–32°C for 30 min and then maintained at room temperature in normal saline composed of (in mM): 124 NaCl, 2.5 KCl, 2 $CaCl_2$, 2 $MgCl_2$, 1.25 NaH_2PO_4, 26 $NaHCO_3$, and 10 glucose, pH 7.4, when bubbled with 95% O_2/5% CO_2.

Fura-2 AM loading procedure. CA1 pyramidal neurons were loaded with acetoxymethyl ester of fura-2 (fura-2 AM, Molecular Probes, Eugene, OR) similar to the procedure described in Garaschuk et al. (1997). Briefly, hippocampal slices were incubated for 13–15 min in a plastic tube containing 1 ml of fura-2 AM (15 μM) filled with 95% O_2/5% CO_2, at 35–36°C. Stock solutions of fura-2 AM (3.3 mM) were prepared in dimethyl sulfoxide. After loading, the slices were transferred to the recording chamber where they were washed for at least 30 min. The loading of neurons was restricted to the cytoplasm because the fluorescence measured at 600 nm disappeared within 2–3 min on the application of 30 μg/ml saponin (Golovina and Blaustein, 1997).

Recording of Ca^{2+} transients. $[Ca^{2+}]_i$ measurements were made on pyramidal neurons from the CA1 region loaded either with fura-2 AM or bis-fura-2 through a patch pipette. High-speed digital fluorescence image sequences (25–30 msec frame intervals) were recorded with a cooled CCD camera (Lasser-Ross et al., 1991) on an upright Olympus BX50WI microscope equipped with a 40× water immersion objective, numerical aperture 0.8. Fura-2 fluorescence (F) was measured by using an excitation of 382 ± 6 nm and an emission >455 nm. Changes in $[Ca^{2+}]_i$ are presented as the spatial averages of $\Delta F/F$ (in %) over cell bodies or dendrites, in which F is the fluorescence intensity at resting $[Ca^{2+}]_i$ and ΔF is the time-dependent change in fluorescence corrected for bleaching. Maximal $\Delta F/F$ pseudocolor images were computed when antidromic action potentials were evoked. Regions of high $\Delta F/F$ matched the position of loaded neurons. Boxes of 5 × 5 pixels were chosen for the calculation of $\Delta F/F$. The center of each box was located on the basis of the $\Delta F/F$ pseudocolor images. The positions of maximal $\Delta F/F$ regions were controlled throughout the experiments. To determine which box size was optimal to measure signals from single cells, we calculated $\Delta F/F$ values in the box containing the cell and in all of the surrounding boxes. $\Delta F/F$ values in the 5 × 5 pixels box containing the cell were 3.17 ± 0.5 times larger than in surrounding 5 × 5 pixels boxes (n = 4 cells). This value was 2.6 and 2.5 for box sizes of 3 × 3 and 7 × 7, respectively. All recordings were performed at 30°C in the presence of APV (50–100 μM) and CNQX (5–20 μM) to prevent the activation of excitatory postsynaptic potentials. Data are given as mean ± SEM throughout.

Background fluorescence and background signals in fura-2 AM-loaded slices. Background fluorescence was sampled in regions devoid of loaded cells in the stratum radiatum. Autofluorescence of the tissue, recorded in slices not loaded with fura-2 AM, accounted for 60.7% of the background fluorescence measured in slices loaded with fura-2 AM. The other part of background fluorescence was attributable to residual fura-2 AM that could not be washed from the slice and from stained cellular elements that could not be resolved visually. Background fluorescence was typically 40–60% of the fluorescence in loaded neurons (average 58.5 ± 2.6%; n = 7 slices). Background fluorescence in the fura-2 AM-loaded slices was compared with the background fluorescence in the whole-cell experiments when a neuron was loaded with bis-fura-2 (100–200 μM). In the latter case the background fluorescence accounted for only 10.8 ± 3.4%. In these experiments the background fluorescence originated predominantly from the autofluorescence of the tissue, because at ~380 nm excitation the fluorescence of the residual (spilled) dye in the presence of 2 mM $[Ca^{2+}]_o$ may be considered negligible (Grynkiewicz et al., 1985). Because the purpose of the experiments was to compare the $[Ca^{2+}]_i$ changes in response to the application of different pharmacological agents rather than to calculate absolute calcium concentrations, no correction was made for background fluorescence. Fluorescence changes therefore are underestimated. When antidromic action potentials were evoked, $\Delta F/F$ signals were measured both in loaded cells and in surrounding regions in which background fluorescence was detected. These background signals contributed to 18.5 ± 2.8% (n = 5 slices) of signals in CA1 neurons. They probably originated from loaded fibers or fine dendrites. Background signals recorded far from the loaded cells were insensitive to treatments with caffeine, ryanodine, thapsigargin, or cyclopiazonic acid (CPA).

Stability of fluorescence and $\Delta F/F$ signals in fura-2 AM-loaded neurons. Fluorescence values (F), with no stimulation, were recorded throughout the experiments. Assuming that the concentration of the indicator remained constant during the experiments, F was taken as a measure of the resting $[Ca^{2+}]_i$. We noticed a small decrease in F with time occurring over all areas of slices, probably because of bleaching. However, because this decrease in F was linear, changes of resting $[Ca^{2+}]_i$ could be detected as abrupt changes in F during drug applications. In our experiments only 20 μM CPA affected resting $[Ca^{2+}]_i$ in some cells. Moreover, bleaching did not affect measurements of $\Delta F/F$ values significantly. $\Delta F/F$, recorded every 5 min in loaded cells stimulated antidromically, were stable over 1 hr. A control histogram was built for the ratios between the amplitudes of two control Ca^{2+} signals evoked by five action potentials at a 5 min interval. The histogram was fit with a gaussian (see Fig. 3*E*). The average ratio between amplitudes of two control Ca^{2+} signals was 99.9 ± 1.0% (n = 66 cells). For each slice two to four controls of $\Delta F/F$ were recorded at the beginning of each experiment.

Electrical stimulations. Ca^{2+} transients were evoked by using a 1 MΩ monopolar tungsten electrode, which was placed on the alveus for triggering antidromic action potentials. Stimulation pulses 100–500 μA, 200 μsec, 1–10 at 20 Hz, were delivered from an isolated stimulator (World Precision Instruments, Sarasota, FL). Action potentials recorded in some experiments with whole-cell patch clamp and associated Ca^{2+} transients were all-or-none and showed a marked threshold below 500 μA. Stimulus intensity was set to obtain reproducible responses from 1–10 neurons in the field of view.

Electrical recordings. Combined measurements of membrane potential and $[Ca^{2+}]_i$ were performed on individual neurons. Whole-cell tight seals were made onto cell bodies using video-enhanced DIC optics (Stuart and Sakmann, 1994). Bis-fura-2 was allowed to diffuse into cells for at least 15 min before Ca^{2+} recording. Patch pipettes were pulled from 1.5 mm outer diameter thick-walled glass tubing (number 1511-M, Friderick and Dimmock, Millville, NJ). Intracellular solution contained (in mM): 130 K-gluconate, 10 Na-gluconate, 4 NaCl, 2 Mg-ATP, 0.3 Na-GTP, and 10 HEPES, 0.06–0.2 bis-fura-2, pH-adjusted to 7.2 with KOH; osmotic pressure was 300 mOsm/kg. Open resistance of the pipettes was 5–7 MΩ in normal saline. After breaking into the cell, the holding current was always <50 pA and usually zero. No correction was made for the junction potential between the bath and the pipette.

Chemicals and drugs. Fura-2 AM and bis-fura-2 were obtained from Molecular Probes. Thapsigargin and CPA were purchased from Calbiochem (La Jolla, CA). CNQX and APV were from Research Biochemicals (Natick, MA). Other chemicals were obtained from Sigma (St. Louis, MO).

Perfusion system. All drugs were bath-applied through the perfusion system of the recording chamber. Solutions were exchanged at a rate of 1 ml/min, using a peristaltic pump (Rainin Instruments, Woburn, MA). The recording chamber had a volume of 2.4 ml. When a drug entered the chamber, its concentration rose approximately linearly by 10% of its maximal concentration every 9 sec, as assayed by measuring the fluorescence of a 0.4 μM fluorescein solution. Therefore, a solution exchange of 98% could be achieved within 1.5 min. This allowed a slow rise in drug concentration, which was particularly important for drugs applied to empty internal Ca^{2+} stores without inducing resting $[Ca^{2+}]_i$ changes. A complete wash of fluorescein fluorescence required 10–15 min.

RESULTS

Changes in $[Ca^{2+}]_i$ (intracellular Ca^{2+} concentration) were recorded in CA1 pyramidal neurons from hippocampal slices after antidromic stimulations in the alveus. When the stimulus intensity was increased, Ca^{2+} transients occurred in an all-or-none manner in individual somata (Figs. 1, 2) and were blocked entirely by 1 μM TTX (Fig. 2*C*). With a single stimulus (1 pulse, 100–500 μA, 200 μsec) the occurrence of Ca^{2+} signals was correlated with the generation of action potentials in neurons recorded with whole-cell patch clamp (see Fig. 4*A*). This indicates that Ca^{2+} transients were triggered by antidromically evoked action potentials. Ca^{2+} transient amplitudes (% $\Delta F/F$), recorded in cells loaded with fura-2 AM, were, on average, 1.85 ± 0.14% for a single action potential, with decay time constants of 273.2 ± 32.1 msec

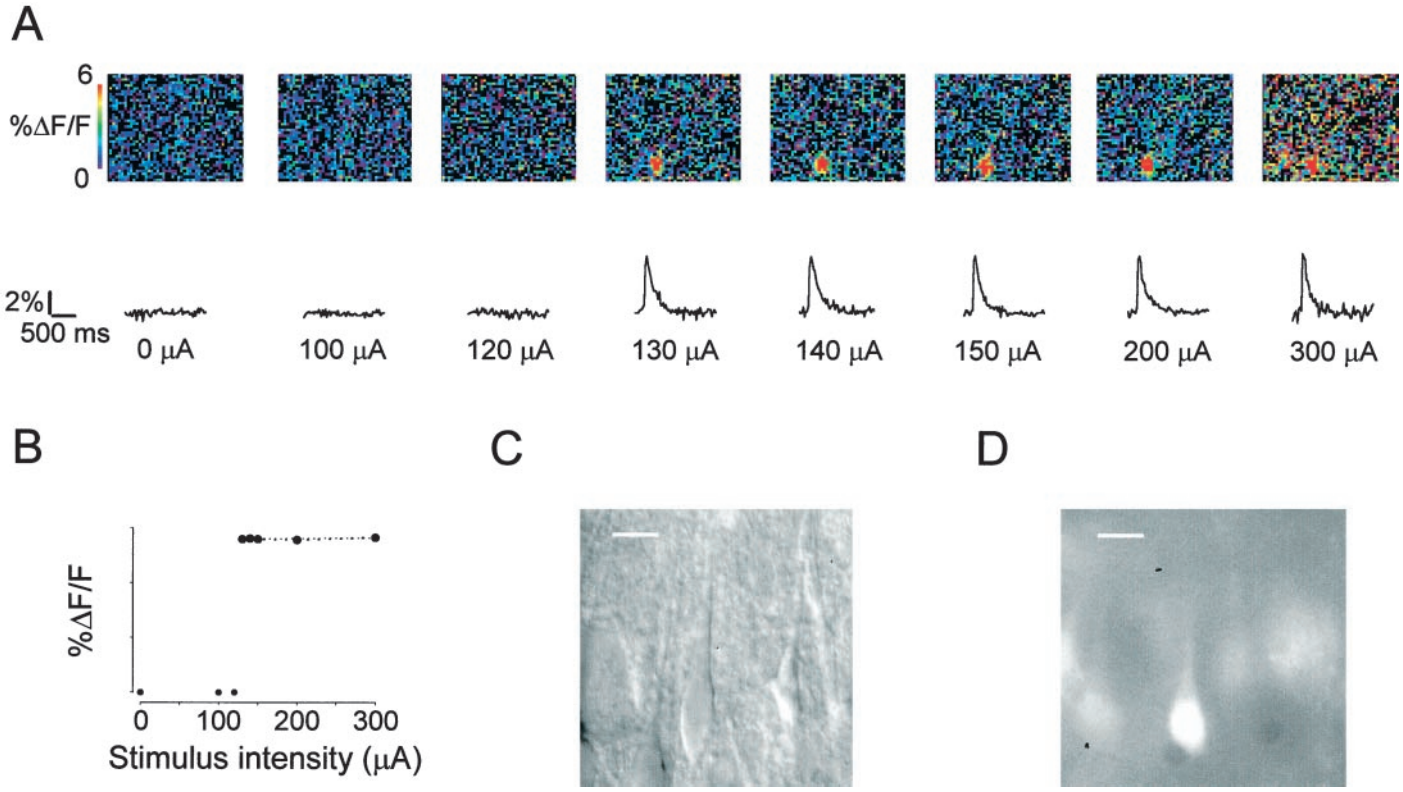

Figure 1. Recording of all-or-none Ca^{2+} transients in a CA1 neuron triggered by a single antidromic stimulation. *A*, Changes of $[Ca^{2+}]_i$ during a single stimulation of increasing intensity in the alveus. *Top panels*, $\Delta F/F$ pseudocolor images during the stimulation show a clear area of high $\Delta F/F$ corresponding to a single CA1 neuron. *Bottom traces* correspond to spatial averages of $\Delta F/F$ over a 5 × 5 pixels area positioned over the stimulated neuron. *B*, Plot of maximal spatial averages of $\Delta F/F$ against the stimulus intensity. *C*, Bright-field image of the stimulated neuron. *D*, Fluorescence image of the same neuron recorded at 380 nm. Scale bars: *C*, *D*, 20 μm.

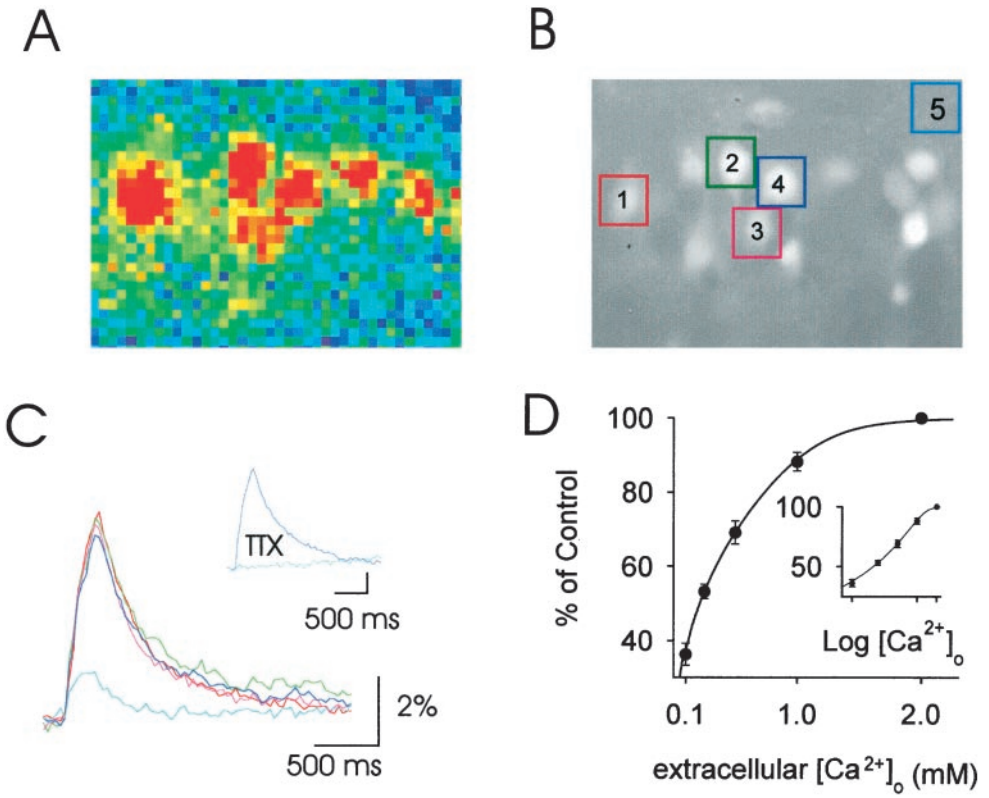

Figure 2. Properties of Ca^{2+} transients recorded simultaneously in several pyramidal neurons from the CA1 layer of an hippocampal slice. *A*, $\Delta F/F$ pseudocolor images during five antidromic stimulating pulses. Note that at least six neurons are stimulated. *B*, Corresponding fluorescence image (380 nm) showing that high $\Delta F/F$ regions in *A* correspond to the fura-2-loaded neurons in the CA1 pyramidal cell layer. The alveus is *upward* in the micrograph. *C*, Ca^{2+} transients corresponding to the neurons shown in *B*. The *light blue trace*, sampled from region *5*, was taken to illustrate a background signal and showed no visible loaded neuron. *Inset*, Example of a Ca^{2+} transient abolished with 1 μM TTX. *D*, Dependence of Ca^{2+} transients evoked by five action potentials on external $[Ca^{2+}]_o$ (n = 5–20 cells for each concentration). *Inset*, Log plot of the same data.

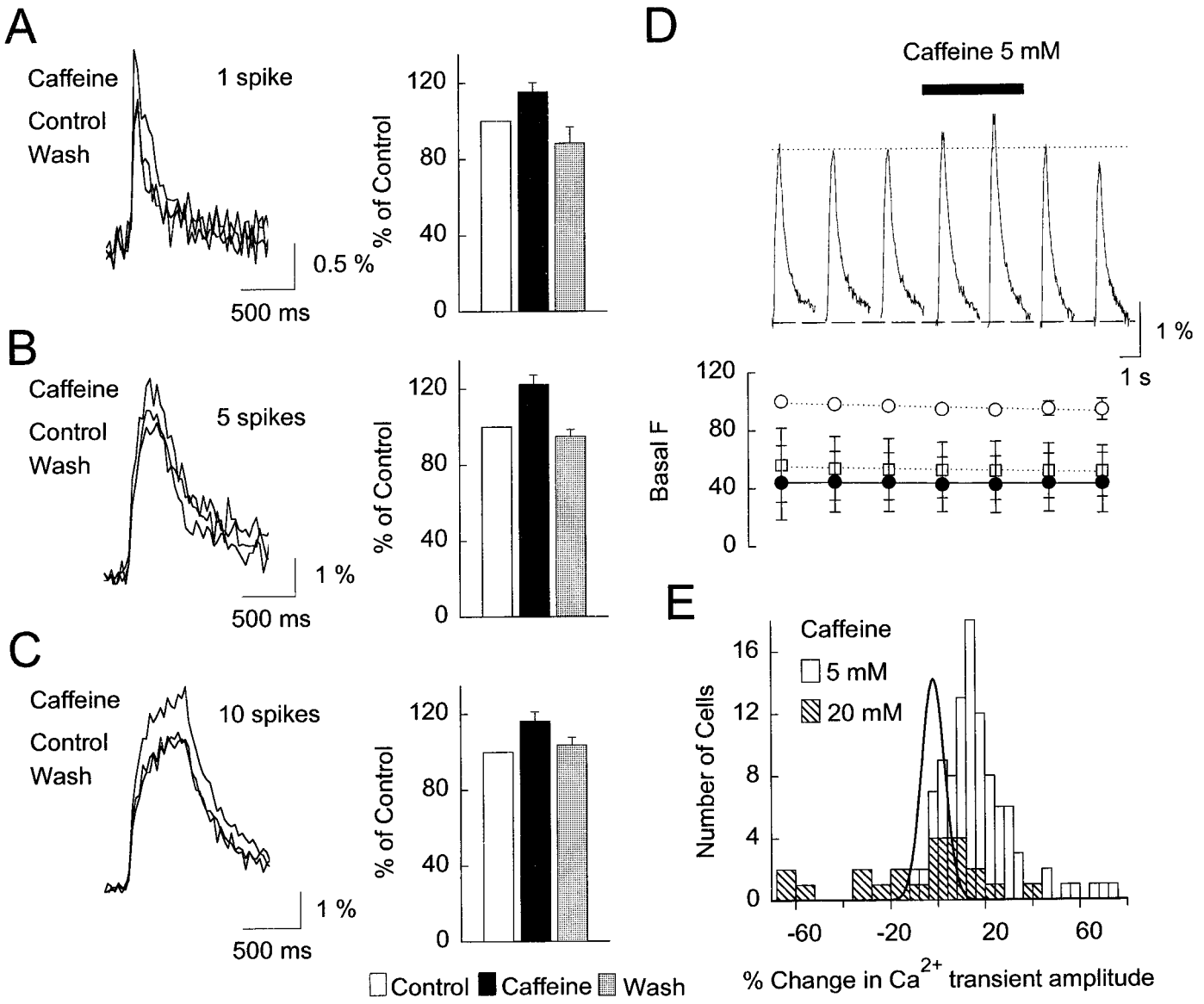

Figure 3. Effect of caffeine on Ca^{2+} transients evoked by 1–10 antidromic stimulations. *A*, Ca^{2+} transients evoked by a single action potential were potentiated by the application of caffeine (5 min, 5 mM). Average data are illustrated on the *right*. *B*, Same as in *A*, except that five action potentials were evoked. *C*, Same as in *A*, except that 10 action potentials were evoked. Average data are from 10–16 cells. *D*, Time course of the action of caffeine (5 mM). Traces were recorded at 5 min intervals, except for the *fourth trace*, which was recorded after 1 min of caffeine application. Basal *F* values, with no stimulation, are plotted correspondingly to each trace. (○), Basal *F* at the location of the cell; □, background *F* (see Materials and Methods); (●), difference between the two fluorescence values. *E*, Histograms of the changes of Ca^{2+} transient amplitudes by 5 and 20 mM caffeine. The *line* in the histogram of caffeine action represents a fit of the control histogram (see Materials and Methods).

($n = 17$ cells). These fast kinetics suggest that the fura-2 concentration inside neurons was below 50 μM (Helmchen et al., 1996). Thus, dye buffering was probably low in our Ca^{2+} recordings.

Action potential-evoked Ca^{2+} signals were dependent on $[Ca^{2+}]_o$ (external Ca^{2+}). When $[Ca^{2+}]_o$ was reduced from 2 to 1 mM or 100 μM, amplitudes of Ca^{2+} transients were 88.2 ± 2.5% ($n = 9$ cells) and 36.3 ± 3.0% ($n = 14$ cells) of control signals, respectively (Fig. 2*D*).

Caffeine induces a transient increase in action potential-evoked Ca^{2+} signals

To examine whether CICR contributes to Ca^{2+} signals evoked by action potentials, we used the xanthine derivative caffeine. Caffeine readily crosses plasma membranes, where it binds intracellularly to RyRs. If CICR were triggered during action potentials, caffeine either would increase Ca^{2+} signals by sensitizing RyR channels (Sitsapesan and Williams, 1990) or would decrease them by a partial depletion of internal Ca^{2+} stores (Usachev et al., 1993; Shmigol et al., 1996).

In a first series of experiments a low concentration of caffeine (5 mM) was found to induce a small and reversible potentiation of Ca^{2+} signals evoked by action potentials (Fig. 3). When caffeine was bath-applied for 5 min, Ca^{2+} signal amplitudes were increased by 15.4 ± 4.9, 22.5 ± 4.8, and 16.2 ± 4.9% within 1–3 min for 1, 5, and 10 action potentials, respectively (Fig. 3*A–C*). Neurons that did not show the potentiation with caffeine were included in the statistics. The potentiation reached up to 78% and was observed in >89.3% of cells (see histogram, Fig. 3*E*) (ANOVA, $p < 0.001$; $n = 103$ cells). Ca^{2+} transients returned to

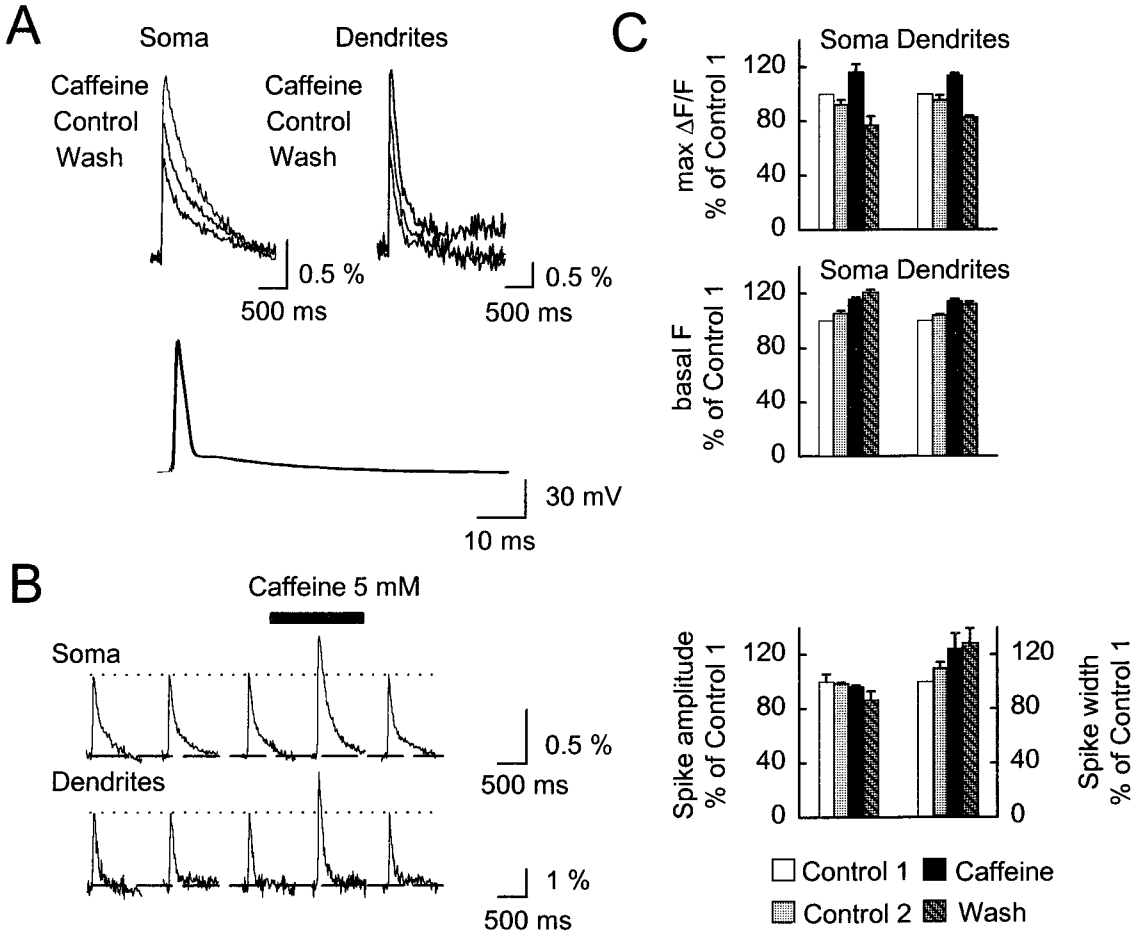

Figure 4. The caffeine-induced increase in Ca^{2+} transients does not depend on a change in action potentials, which are recorded with whole-cell patch clamp. *A*, Caffeine (5 mM) potentiates Ca^{2+} transients both in the soma and in the proximal dendrites of a CA1 neuron recorded in current-clamp mode (*top panels*). Action potentials before, during, and after caffeine application are superimposed in the *bottom panel*. No significant change in action potential waveform is observed during the caffeine application. *B*, Time course of caffeine effect in a neuron recorded in whole-cell configuration. Three controls are shown separated by 5 min intervals. The *fourth traces* were recorded after 1 min of caffeine application. *C*, Average data are from five neurons. Maximal $\Delta F/F$ values were recorded 10 and 5 min before caffeine application (*Control 1* and *Control 2*, respectively), during the caffeine application, and after wash of caffeine. Maximal $\Delta F/F$ values were increased in the presence of caffeine (*top plot*). Basal fluorescence (F) recorded at 380 nm slightly and linearly increased in the soma but did not change when caffeine was applied or removed. Spike amplitude and spike width were unaffected by the application of caffeine (*bottom plot*).

control values after 10 min of wash. The effect of caffeine was associated with no change in either basal F recorded in somata or a change in background fluorescence (Fig. 3*D*).

In contrast, a higher concentration of caffeine (20 mM) caused a reduction of Ca^{2+} signals of 8.9 ± 6.1% (n = 21 cells) (Fig. 3*E*), which partially recovered. Control Ca^{2+} signals were recorded in a saline in which sucrose (20 mM) was used instead of caffeine to mimic the change in osmolarity. This result is consistent with an expected reduction of Ca^{2+} signals by caffeine depleting the ryanodine-sensitive internal Ca^{2+} stores. It suggests a contribution of CICR to action potential-evoked Ca^{2+} transients in CA1 neurons.

Caffeine-induced potentiation of Ca^{2+} transients is not attributable to changes in action potentials properties

Simultaneous whole-cell recordings and Ca^{2+} imaging were performed to determine whether caffeine changed the action potential properties in pyramidal neurons. CA1 pyramidal neurons were filled with bis-fura-2 (200 μM) through a patch pipette. Single antidromic stimulations (200 μsec, <500 μA) evoked action potentials and associated Ca^{2+} transients of 2.0 ± 0.3% in somata and 3.5 ± 0.7% in the proximal apical dendrites (n = 5 cells) (Fig. 4*A*,*B*). Caffeine application led to a potentiation of these Ca^{2+} signals both in somata and in the dendrites (Fig. 4*A*,*B*). Small changes in action potential amplitude and width did occur, but they were not significant (t test, p = 0.79 and p = 0.39, respectively; n = 5 cells) (Fig. 4*C*) and were not dependent on the application of caffeine. These changes did not affect Ca^{2+} signals significantly. Although the effect of caffeine was 16.4 ± 5.7% in somata and 13.8 ± 1.6% in the dendrites, action potential amplitudes were 101.5 ± 6.4 mV and 99.1 ± 6.3 mV before and during caffeine application, respectively (n = 5 cells) (Fig. 4*C*). Furthermore, the effect of caffeine was not associated with a change in basal F in these experiments (data not shown). We conclude that caffeine potentiates Ca^{2+} transients without modification of the amplitude or shape of the action potentials.

The effect of caffeine is not mediated by protein phosphorylation

Caffeine is a known antagonist of phosphodiesterases (PDEs) (Butcher and Sutherland, 1962) and can trigger protein phosphor-

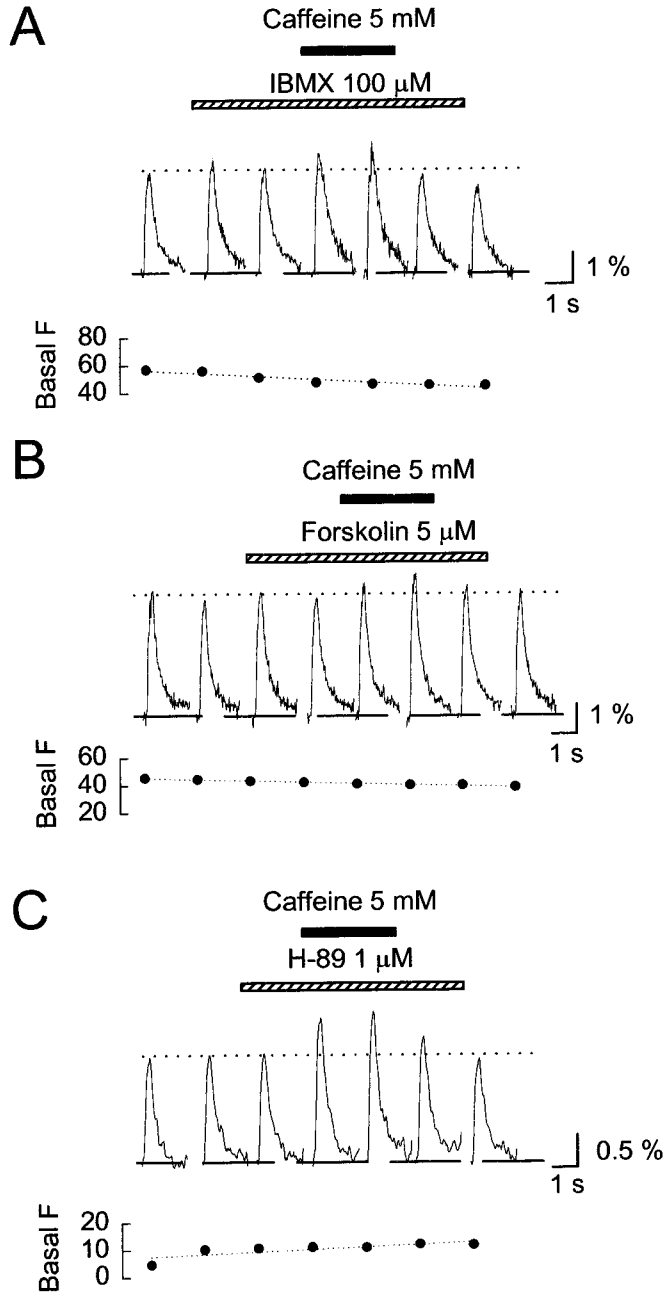

Figure 5. The caffeine-induced increase in Ca^{2+} transients is not mediated by a rise in cyclic nucleotides. *A*, IBMX (100 μM) has little effect on Ca^{2+} transients (*second* and *third traces*) and does not occlude caffeine action (*fourth* and *fifth traces*). *B*, Same as in *A* except that forskolin (5 μM) was applied. *C*, Same as in *A* except that H-89 was used. *Dashed lines* indicate the time of application of IBMX, forskolin, or H-89. *Solid lines* indicate the 5 min caffeine application. Ca^{2+} transients in the presence of caffeine were recorded after 1 and 3 min of caffeine application. Except during the caffeine application, the traces are separated by 5 min intervals. Background *F* is plotted correspondingly to each Ca^{2+} transient in the *bottom panels*. The traces for *A–C* were recorded from three representative cells. See Table 1 for average results.

ylation by increasing cAMP and/or cGMP levels (Beavo and Reifsnyder, 1990). Thus, a caffeine-induced potentiation of Ca^{2+} signals could be attributable to an increased Ca^{2+} influx mediated by a phosphorylation of voltage-dependent Ca^{2+} channels, as recently reported in CA1 neurons (Kavalali et al., 1997).

Table 1. Pharmacology of the effect of caffeine on action potential-evoked Ca^{2+} transients

Drugs	Change from control (%)	Effect of caffeine (%)	Number of cells
IBMX (100 μM)	12.2 ± 5.6*	10.5 ± 2.5	19
Forskolin (5 μM)	0.7 ± 3.1	21.8 ± 7.1*	16
H-89 (1 μM)	−1.1 ± 1.8	19.5 ± 2.4*	14
Nifedipine (20 μM)	−10.8 ± 2.5	14.0 ± 2.3*	16
Ryanodine (20 μM)	−46.0 ± 1.5	−5.4 ± 3.3	30
Thapsigargin (3 μM)	−20.2 ± 3.0	−13.4 ± 3.0	32

Effects of drugs (second column) are given as percentage changes of control Ca^{2+} transient amplitudes. Ca^{2+} transients in these experiments were all triggered by five action potentials. The effect of caffeine was calculated as the percentage change compared with the treated Ca^{2+} transients. *ANOVA, $p < 0.001$.

To exclude this possibility, we used 1-methyl-3-isobutylxanthine (IBMX), a nonspecific PDE inhibitor related to caffeine, but two orders of magnitude more potent (Wells et al., 1975). When IBMX (100 μM) was bath-applied for 5 min, a transient potentiation of Ca^{2+} signals was observed (12.2 ± 5.6%; $n = 19$ cells; ANOVA, $p = 0.01$) (Fig. 5*A*, Table 1). In the presence of IBMX, Ca^{2+} signals returned to control values within 15 min (86.7 ± 4.6%; $n = 19$ cells) (Fig. 5*A*, Table 1). Caffeine was bath-applied consecutively in the presence of IBMX. This protocol did not prevent the potentiation of Ca^{2+} signals by caffeine, which was 10.5 ± 2.5% for five action potentials (ANOVA, $p < 0.001$; $n = 19$ cells) (Fig. 5*A*, Table 1).

Using forskolin, an activator of adenylyl cyclase (Seamon et al., 1985), we examined the contribution of cAMP to the potentiation of Ca^{2+} signals. When forskolin (5 μM) was bath-applied for 10 min, no increase in Ca^{2+} signals was detected after 5–10 min of incubation (0.7 ± 3.1%; $n = 16$ cells) (Fig. 5*B*, Table 1). This finding strongly supports that an increase in cAMP cannot account for a potentiation of action potential-evoked Ca^{2+} signals recorded in our conditions. Furthermore, after a 10 min preincubation with forskolin (5 μM), caffeine applied in the presence of forskolin caused a potentiation to 21.8 ± 7.1% (ANOVA, $p < 0.001$; $n = 16$ cells) (Fig. 5*B*, Table 1). This result demonstrates that caffeine can potentiate Ca^{2+} signals independently of a cAMP production.

Finally, the possible involvement of the cAMP–PKA and cGMP–PKG pathways was assessed further with H-89, an antagonist of PKA and PKG at 1 μM (Chijiwa et al., 1990). H-89 alone had no effect on Ca^{2+} signals during a 10 min incubation (1.1 ± 1.8%; $n = 14$ cells) (Fig. 5*C*, Table 1). However, caffeine applied in the presence of H-89 caused a potentiation of Ca^{2+} signals of 19.5 ± 2.4% (ANOVA, $p < 0.001$; $n = 14$ cells) (Fig. 5*C*, Table 1). We conclude that caffeine potentiates action potential-evoked Ca^{2+} signals independently of protein phosphorylation mediated by either cAMP or cGMP.

L-type channels are not needed for caffeine-induced potentiation of Ca^{2+} signals

The previous experiments suggest that the effect of caffeine cannot be accounted for by a modulation of voltage-gated Ca^{2+} channels, because the most likely upregulation of Ca^{2+} influx by caffeine would involve PKA. Furthermore, caffeine acting on adenosine receptors would rather inhibit Ca^{2+} influx (Zhu and Ikeda, 1993). However, another pathway independent of kinases and involving L-type channels could be implicated. It recently has been proposed that caffeine modifies a direct interaction between

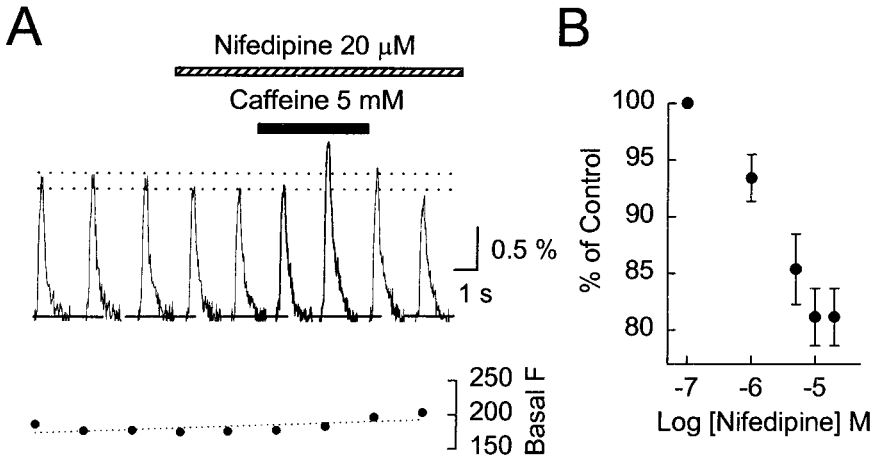

Figure 6. L-type Ca^{2+} channels are not required for the effect of caffeine on Ca^{2+} transients. *A*, Nifedipine (20 μM) reduces Ca^{2+} transient amplitude (*fourth* and *fifth traces*) but does not occlude caffeine action (*sixth* and *seventh traces*). Three controls are shown separated by 5 min intervals. The *sixth* and *seventh traces* were recorded after 1 and 3 min of caffeine application, respectively. No change in basal fluorescence (*F*) was observed when the drugs were applied. *B*, Dose–response curve of nifedipine on the reduction of Ca^{2+} transient amplitude. Each *point* is from five to six cells.

RyRs and L-type channels, leading to an increase of this open probability of RyRs (Chavis et al., 1996). We investigated this possibility in an experiment in which caffeine was applied while L-type channels were blocked by nifedipine (Tombaugh and Somjen, 1997).

Nifedipine (20 μM) maximally and partially inhibited action potential-evoked Ca^{2+} signals (Fig. 6*B*). A 10 min preincubation with nifedipine did not prevent the caffeine-induced potentiation of Ca^{2+} transients (14.0 ± 2.3%; $n = 16$ cells; ANOVA, $p < 0.001$) (Fig. 6*A*, Table 1). This result demonstrates that the caffeine-induced enhancement of action potential-evoked Ca^{2+} signals does not require L-type Ca^{2+} channels. Thus, an interaction between the RyRs and L-type channels is probably not responsible for the effect of caffeine in CA1 pyramidal neurons.

Ryanodine decreases action potential-evoked Ca^{2+} transients and occludes the effect of caffeine

The findings presented thus far suggest that caffeine potentiated action potential-evoked Ca^{2+} transients independently of a modulation of Ca^{2+} influx by PKA or activation of RyR. A possible

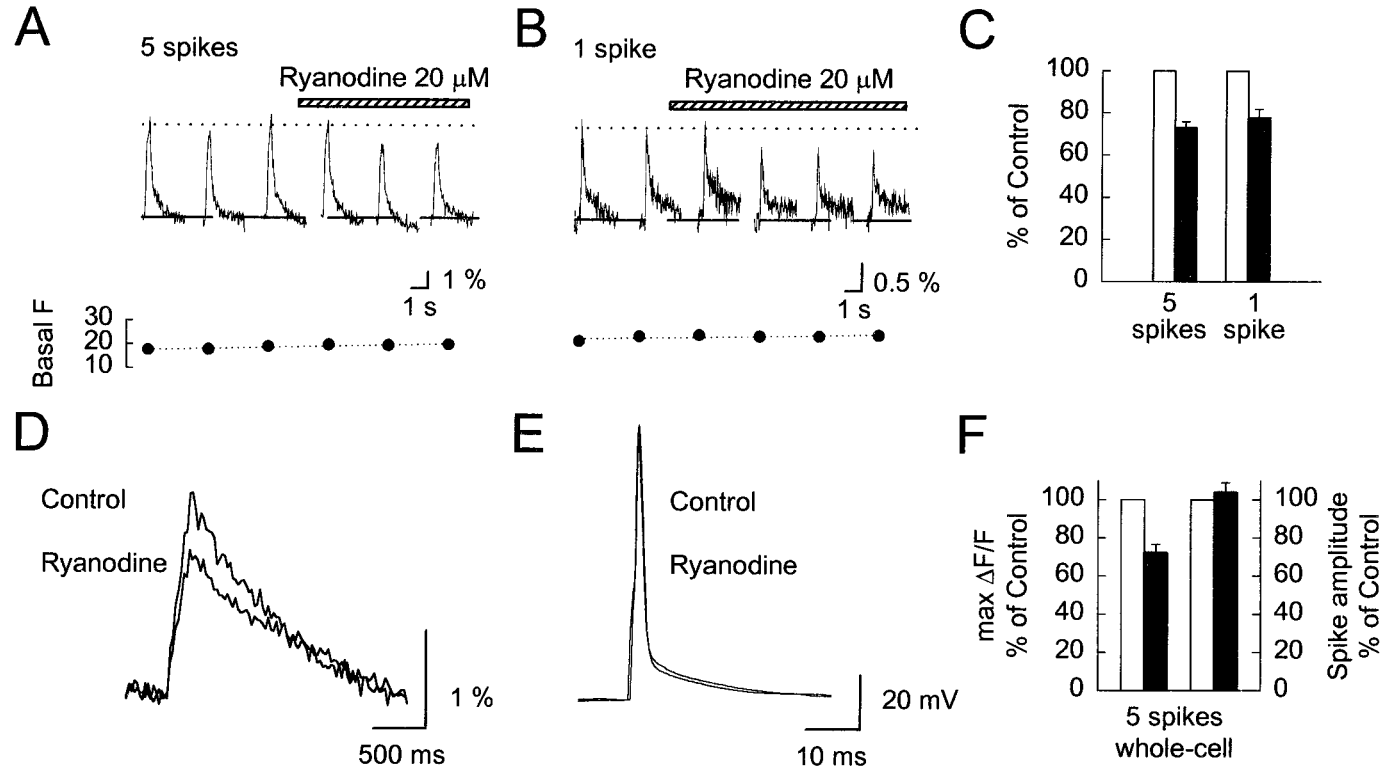

Figure 7. Ryanodine reduces Ca^{2+} transient amplitude. *A*, Ryanodine (20 μM) reduced Ca^{2+} transient amplitude to a stable level. Traces were recorded every 5 min. Ca^{2+} transients were evoked by five action potentials. No change in basal fluorescence (*F*) was associated with the application of ryanodine. *B*, Same as in *A* except that Ca^{2+} transients were evoked by a single action potential. *C*, Average data of the effect of ryanodine on Ca^{2+} transients evoked by five spikes (*left bars*) and one spike (*right bars*). *Open bars*, Controls; *filled bars*, ryanodine (20 μM). Data are from 20–24 cells. *D*, Ryanodine (20 μM) reduced Ca^{2+} transient amplitude in a whole-cell recorded neuron. *E*, No change in action potential occurred when ryanodine was applied. Spikes and Ca^{2+} transients were recorded simultaneously. *F*, Average data of the effect of ryanodine (20 μM) in whole-cell recorded neurons on Ca^{2+} transient amplitude evoked by five spikes (*left bars*) and on the first spike amplitude (*right bars*). *Open bars*, Controls; *filled bars*, ryanodine (20 μM).

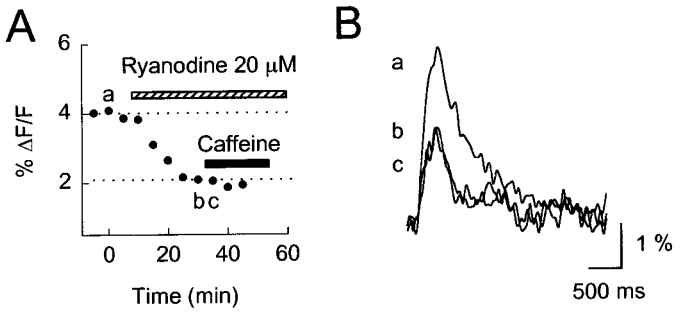

Figure 8. Ryanodine occludes the effect of caffeine. *A*, Ryanodine (20 μM) reduced the amplitude of Ca^{2+} transients evoked with five action potentials and occluded caffeine action on Ca^{2+} transients. *B*, Selected traces (*a–c*) are shown corresponding to the *points* labeled *a–c* in the plot in *A*. The cell was stimulated by five action potentials every 20 sec between measurements of the Ca^{2+} transients. See Table 1 for average results.

explanation of the action of caffeine is that caffeine favors CICR by interacting with the RyR channels, thereby enhancing their open probability (Rousseau and Meissner, 1989; Sitsapesan and Williams, 1990). If this were true, ryanodine, binding to the RyRs and locking them in a low-conductance open state (Coronado et al., 1994), should prevent the action of caffeine (Sitsapesan and Williams, 1990). To test this hypothesis, we used ryanodine, which was shown to have no effect on voltage-gated Ca^{2+} currents and resting potential in CA1 pyramidal neurons (Belousov et al., 1995; Garaschuk et al., 1997).

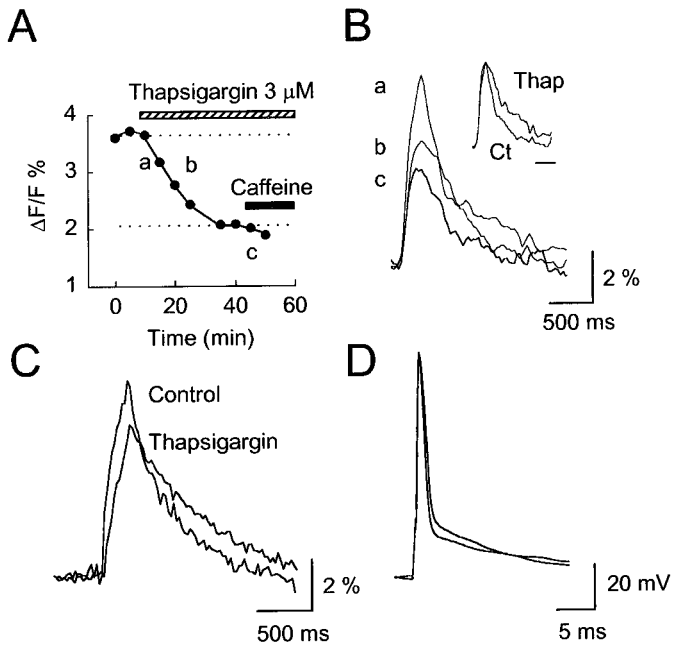

Figure 9. Thapsigargin reduces Ca^{2+} transient amplitude and occludes the effect of caffeine. *A*, Thapsigargin (3 μM) reduced Ca^{2+} transient amplitude and occluded caffeine action on Ca^{2+} transients. *B*, Selected traces (*a–c*) are shown corresponding to the *points* labeled *a–c* in the plot in *A*. *Inset*, Traces *a* and *b* have been scaled to visualize the change in kinetics induced by thapsigargin. Time scale, 500 msec. *C*, Thapsigargin (3 μM) reduced Ca^{2+} transient amplitude in a whole-cell recorded neuron. *D*, No change in action potential occurred when thapsigargin was applied. Ca^{2+} transients were evoked with five action potentials throughout.

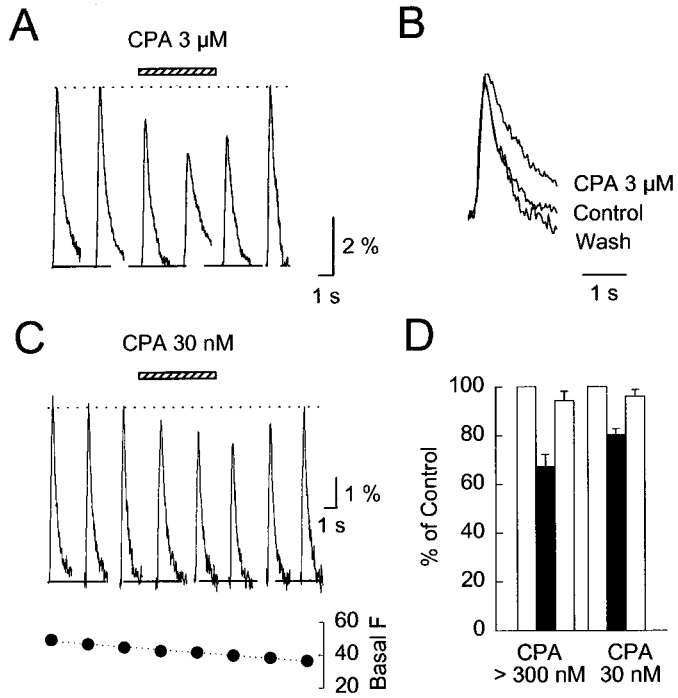

Figure 10. Cyclopiazonic acid (CPA) reversibly reduces Ca^{2+} transient amplitude. *A*, CPA (3 μM) applied for 20 min reduced Ca^{2+} transient amplitudes (*third* and *fourth traces*). A full wash of CPA allows for the complete recovery of Ca^{2+} transient amplitude. The traces were recorded every 10 min. *B*, Effect of CPA (3 μM) on the decay of Ca^{2+} transients. Transients have been scaled for a comparison of their kinetics. *C*, CPA (30 nM) applied for 10 min reduced Ca^{2+} transient amplitudes (*fourth* and *fifth traces*). A full wash of CPA allowed for the complete recovery of Ca^{2+} transient amplitude (*eighth trace*). The traces were recorded every 5 min. No change in basal fluorescence (*F*) was associated with the application of CPA. *D*, Average results for the effect of >300 nM CPA (*left bars*) and 30 nM CPA (*right bars*). For each group of bars the *first bar* is the control, the *second bar* is CPA, and the *third bar* is wash.

Ryanodine (20 μM) irreversibly reduced Ca^{2+} transients evoked by one or five action potentials by 22.2 ± 4.0% (n = 24 cells) and 27.1 ± 2.9% (n = 24 cells), respectively (Fig. 7*A*,*B*). This effect was observed within 10–15 min of ryanodine incubation. Ca^{2+} signal amplitude then reached a steady state (Fig. 7*A*,*B*). A similar effect of ryanodine was observed in neurons loaded with bis-fura-2 (200 μM) by using whole-cell patch clamp (28.0 ± 4.3, n = 5) (Fig. 7*D*), with no change in action potential amplitude (paired t test, p = 0.60; n = 5) (Fig. 7*E*). The percentage of reduction was greater when the cells were stimulated by five action potentials every 20 sec between measurements of Ca^{2+} signals (Fig. 8*A*,*B*). Such stimulation did not interfere with the stability of control Ca^{2+} transients, because their amplitudes were stable for >20 min under these conditions. The application of ryanodine with this protocol caused a reduction of Ca^{2+} transient amplitudes by 46.0 ± 1.5% of control (n = 30 cells) (Fig. 8*A*,*B*, Table 1). This larger reduction of Ca^{2+} transients in stimulated neurons is consistent with the use-dependent block of caffeine-evoked Ca^{2+} transients by ryanodine reported by Garaschuk et al. (1997) and the Ca^{2+} dependence of ryanodine binding to RyRs (Coronado et al., 1994). The reduction of Ca^{2+} transients by ryanodine is unlikely to be attributed to Ca^{2+}-dependent inactivation of Ca^{2+} influx, because no change in basal fluorescence was associated with application of ryanodine (see Fig. 7*A*,*B*). The effect of ryanodine observed here is in

agreement with a contribution of CICR to action potential-evoked Ca^{2+} transients.

Because ryanodine was able to reduce action potential-evoked Ca^{2+} transients, we tested ryanodine on the effect of caffeine. When cells were made to fire five action potentials at 20 Hz every 20 sec in the presence of ryanodine (20 μM) to deplete Ca^{2+} stores, caffeine failed to induce a potentiation of Ca^{2+} signals evoked by five action potentials but rather slightly decreased them ($-5.4 \pm 3.3\%$; $n = 30$ cells) (Fig. 8*A*,*B*, Table 1). These experiments show that ryanodine can occlude the action of caffeine. We therefore conclude that caffeine probably favored CICR by interacting with the RyR channels, whereas ryanodine suppressed CICR induced by action potentials.

Effect of endoplasmic Ca^{2+}-ATPase inhibitors on action potential-evoked Ca^{2+} transients

The involvement of internal Ca^{2+} stores in action potential-evoked Ca^{2+} transients was examined further by blocking endoplasmic Ca^{2+}-ATPases. Thapsigargin, a sesquiterpene lactone, is an effective and irreversible inhibitor of endoplasmic ATPases (Thastrup, 1990; Thastrup et al., 1990) without a known influence on plasma membrane ATPases (Lytton et al., 1991). Thapsigargin (3 μM) was shown to empty efficiently the caffeine-sensitive internal Ca^{2+} stores in CA1 pyramidal neurons (Garaschuk et al., 1997) with no effect on resting potential (Belousov et al., 1995).

Bath application of thapsigargin (500 or 3000 nM) irreversibly reduced Ca^{2+} signals evoked by one or five action potentials by $17.1 \pm 3.4\%$ ($n = 16$ cells) and $20.2 \pm 3.0\%$ ($n = 32$ cells) (Fig. 9*A*,*B*), respectively. The decrease in signal amplitude reached a steady-state level of reduction in 30 min (data not shown). Furthermore, thapsigargin caused a change in Ca^{2+} signal kinetics, with decay phases being slower in the presence of thapsigargin (Fig. 9*B*, *inset*). A similar effect of thapsigargin (3 μM) was observed in neurons loaded with bis-fura-2 (60 μM) by using whole-cell patch clamp (19.2 ± 2.6, $n = 5$) (Fig. 9*C*,*D*). In these experiments the action potential amplitudes were unaffected by the drug (paired *t* test, $p = 0.7$) (see Fig. 8*D*). No change in basal fluorescence was observed during thapsigargin application (data not shown), consistent with the reported lack of effect of thapsigargin on resting $[Ca^{2+}]_i$ in neurons (Shmigol et al., 1995). Taken together, these results suggest that thapsigargin prevents CICR by depleting Ca^{2+} stores.

We tested if thapsigargin could prevent the action of caffeine on action potential-evoked Ca^{2+} signals. Caffeine (5 mM) applied after 30 min of thapsigargin incubation failed to induce a potentiation of Ca^{2+} signals (see Fig. 8*A*, Table 1). On average, caffeine reduced Ca^{2+} signals by $23.5 \pm 5.8\%$ ($n = 5$ cells) and $13.4 \pm 3.0\%$ ($n = 32$ cells) (Fig. 9*A*, Table 1) for one and five action potentials, respectively. This is consistent with the assumption that caffeine further depleted internal Ca^{2+} stores in the presence of thapsigargin. No potentiation by caffeine was ever observed in these experiments. These results further imply that the caffeine action required loaded internal Ca^{2+} stores.

Because thapsigargin is an irreversible antagonist (Thastrup et al., 1990) and because it partially may inhibit Ca^{2+} influx in some cells (Rossier et al., 1993; Nelson et al., 1994; Shmigol et al., 1995), we used CPA, a reversible and more specific blocker of SERCAs (Seidler et al., 1989). CPA (in the micromolar range) has been shown to have no effects on Ca^{2+} channels, resting potential, and action potential amplitude in neurons (Ishii et al., 1992; Nelson et al., 1994). These properties enabled us to study the effect of a reversible Ca^{2+} store depletion on action potential-evoked Ca^{2+} signals.

A 10 min application of CPA (30 nM) reduced Ca^{2+} transients by $19.6 \pm 2.6\%$ ($n = 20$ cells) (Fig. 10*C*,*D*). Recovery by >96% of control signals occurred in ~75% of cells with the washout of CPA. No change in basal fluorescence was detected when CPA was applied (Fig. 10*C*). These results indicate that Ca^{2+} transient amplitudes can be reduced when internal Ca^{2+} stores are emptied in a reversible manner. However, at such low concentration the effect of CPA was probably partial, because the reduction of Ca^{2+} transient amplitudes and the effect on Ca^{2+} signal kinetics were smaller than those observed with thapsigargin. For this reason CPA was used at higher concentrations between 1 and 20 μM. CPA (>300 nM) had a similar effect on Ca^{2+} signal kinetics with that observed in the presence of thapsigargin. Furthermore, a reversible reduction of Ca^{2+} signals by $33.3 \pm 5.2\%$ was observed in 31.3% of cells ($n = 10$ cells) (Fig. 10). This result shows that the effect of CPA is reversible and dose-dependent. We conclude that the amplitude of action potential-evoked Ca^{2+} transients can be reduced in a reversible manner when internal Ca^{2+} stores are emptied slowly with no change in basal $[Ca^{2+}]_i$. These results further establish the contribution of CICR in setting the amplitude of action potential-evoked Ca^{2+} signals in CA1 pyramidal neurons.

DISCUSSION

Our results provide new evidence for a contribution of internal Ca^{2+} stores in elevations of $[Ca^{2+}]_i$ associated with action potentials in CA1 pyramidal neurons. This conclusion is based on pharmacological manipulations of CICR, which either can increase or decrease action potential-evoked Ca^{2+} transients in these cells. Although this contribution does not seem predominant in magnitude, the contribution of internal Ca^{2+} stores to action potential-evoked Ca^{2+} transients may influence the subcellular patterns of $[Ca^{2+}]_i$ increases profoundly. In addition, CICR may present new targets for neuromodulators controlling the amplitude of Ca^{2+} transients in the soma and dendrites of neurons.

A main argument in favor of a contribution of CICR to action potential-evoked change in $[Ca]_i$ was the caffeine sensitivity of Ca^{2+} transients. They were enhanced by a low concentration (5 mM) and reduced by a higher concentration (20 mM) of caffeine. Caffeine was shown to reduce action potential-evoked Ca^{2+} signals in Purkinje cells, but no enhancement with a low dose of caffeine was reported in this study (Kano, 1995). The sensitivity to caffeine found here has been reported in various neuronal cell types (Friel and Tsien, 1992; Usachev et al., 1993; Verkhratsky and Shmigol, 1996) and usually is attributed to the caffeine sensitivity of isolated RyRs (Sitsapesan and Williams, 1990). Low concentrations of caffeine increase the open probability of the RyR channels only when they are activated by Ca^{2+}, thus favoring CICR once a suprathreshold $[Ca^{2+}]_i$ is reached. Higher caffeine concentrations increase the sensitivity of RyRs to Ca^{2+} so that resting $[Ca^{2+}]_i$ becomes sufficient to cause the depletion of Ca^{2+} stores. Our experiments support this view, because low concentrations of caffeine had no effect on resting $[Ca^{2+}]_i$ but enhanced Ca^{2+} transients evoked by action potentials in a ryanodine-sensitive manner. A role of intact internal Ca^{2+} stores in the action of caffeine was established further because, on average, ryanodine and thapsigargin occluded the action of caffeine. However, a minor action of caffeine was observed in the presence of ryanodine or thapsigargin in 25 and 8% of cells,

respectively. Therefore, it should be pointed out that a part of the action of caffeine might not be directly related to CICR. However, because the main effect of caffeine was not mediated by PKA and blocked by ryanodine and thapsigargin, we believe that caffeine revealed CICR in CA1 cells, as previously reported in other neurons (Kano et al., 1995; Usachev and Thayer, 1997). Also, however, CICR reported in peripheral sensory neurons is a large regenerative process that significantly contributes to Ca^{2+} transients (Usachev and Thayer, 1997), whereas the magnitude of CICR induced by caffeine in CA1 pyramidal cells seems rather modest.

Caffeine has been used in CA1 neurons to show that ryanodine-sensitive Ca^{2+} stores contain releasable Ca^{2+} at rest (Garaschuk et al., 1997). This stored Ca^{2+} is required for the caffeine-induced potentiation of Ca^{2+} transients because it was occluded by the depletion of these Ca^{2+} stores. However, because caffeine was slowly bath-applied in our study, it did not induce a significant rise in resting $[Ca^{2+}]_i$. A possible slow Ca^{2+} release caused by caffeine application probably was counteracted effectively by both extrusion and uptake mechanisms. For caffeine-evoked calcium release to be observed, a fast change in RyRs sensitivity to Ca^{2+} is required. Under these conditions Ca^{2+}-dependent inactivation of the RyRs has no time to occur (Hernández-Cruz et al., 1997). This explains why rapidly puffer-applied caffeine releases large amounts of Ca^{2+} (Garaschuk et al., 1997; Hernández-Cruz et al., 1997), whereas slow caffeine applications are ineffective. Other Ca^{2+}-releasing agents such as ryanodine, thapsigargine, or CPA were slowly bath-applied and did not affect resting $[Ca^{2+}]$ either.

Experiments with caffeine did not provide direct evidence that CICR could be triggered during action potentials. Our data obtained with ryanodine, thapsigargin, and CPA further suggested that internal Ca^{2+} stores participate not only in the clearance of Ca^{2+} from the cytoplasm, as shown elsewhere (Markram et al., 1995; Fierro et al., 1998), but also in setting the amplitude of Ca^{2+} transients. Ryanodine has been shown to reduce action potential-evoked Ca^{2+} transients in several peripheral neurons (Cohen et al., 1997; Usachev and Thayer, 1997; Moore et al., 1998), in agreement with an underlying CICR. Our results show that ryanodine reduces Ca^{2+} transients during a single action potential in CA1 neurons. Furthermore, the use-dependent block of Ca^{2+} signals by ryanodine, observed in the present study, fits well with the reported action of ryanodine on RyRs of other central neurons (Kano et al., 1995; Garaschuk et al., 1997). The block of ER-ATPases by thapsigargin or CPA also reduced Ca^{2+} transients and affected their time course. Although thapsigargin was shown to block Ca^{2+} voltage-dependent channels in some cells (Rossier et al., 1993; Nelson et al., 1994; Shmigol et al., 1995), a low concentration reduced depolarization-induced Ca^{2+} transients in dorsal root ganglion neurons with no effect on Ca^{2+} influx (Shmigol et al., 1995). In agreement, we observed that the effect of thapsigargin on the decay and amplitude of Ca^{2+} transients developed in a parallel manner, suggesting that the reduction in Ca^{2+} transient amplitude was related to the depletion of Ca^{2+} stores. Furthermore, the dose-dependent and reversible inhibition observed with CPA, which was shown to block caffeine-induced release in CA1 neurons (Garaschuk et al., 1997), supports our conclusions that store depletion affects action potential-evoked Ca^{2+} transients. Finally, the effects of ryanodine, thapsigargin, and CPA cannot be explained by a Ca^{2+}-dependent modulation of voltage-dependent Ca^{2+} currents because resting $[Ca^{2+}]_i$ was unchanged in our experimental conditions. Such modulation was reported when resting $[Ca^{2+}]_i$ rose above 100 nM by puffer-applying Ca^{2+}-releasing agents (Kramer et al., 1991). Our measurements of basal $[Ca^{2+}]_i$ are not consistent with such rises. We therefore conclude that a CICR component underlies action potential-evoked Ca^{2+} transients with the involvement of ryanodine-sensitive stores. A participation of other stores such as ryanodine-insensitive and $InsP_3$-sensitive Ca^{2+} stores or a novel type of ryanodine-insensitive Ca^{2+} store (Jacobs and Meyer, 1997) is not excluded, nevertheless.

The small contribution of CICR to action potential-evoked Ca^{2+} transients described here raises the question of its role and relevance. The contribution of CICR to global somatic Ca^{2+} transients may be estimated to be between 10 and 30%, according to experiments with thapsigargin, ryanodine, and CPA. However, if CICR occurs in a localized manner, it could be predominant in some subcellular regions. Both experimental evidence and theoretical derivations suggest that CICR might occur in small Ca^{2+} microdomains (Bezprozvanny et al., 1991; Hernández-Cruz et al., 1997; Berridge, 1998; Neher, 1998). Measurements of Ca^{2+} signals that rise in 1 msec also favor the idea that CICR might be triggered locally (Ross et al., 1998). Localized CICR has been suggested to control neuronal excitability by producing slow afterhyperpolarizations in peripheral and central neurons (for review, see Berridge, 1998), including hippocampal CA1 neurons (Torres et al., 1996). Thus CICR, described in this study, is probably important in regulating the excitability of CA1 neurons. In addition, CICR could occur more widely within the cytoplasm and distal dendrites when internal Ca^{2+} stores are sensitized by cADPRibose or $InsP_3$ in the presence of neurotransmitters. In such a case the role of CICR might be relevant to synaptic plasticity and the coupling of synaptic inputs to gene transcription in the nucleus.

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