

# Inositol 1,4,5-Trisphosphate (IP<sub>3</sub>)-Mediated Ca<sup>2+</sup> Release Evoked by Metabotropic Agonists and Backpropagating Action Potentials in Hippocampal CA1 Pyramidal Neurons

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We examined the properties of [Ca<sup>2+</sup>]<sub>i</sub> changes that were evoked by backpropagating action potentials in pyramidal neurons in hippocampal slices from the rat. In the presence of the metabotropic glutamate receptor (mGluR) agonists *t*-ACPD, DHPG, or CHPG, spikes caused Ca<sup>2+</sup> waves that initiated in the proximal apical dendrites and spread over this region and in the soma. Consistent with previously described synaptic responses (Nakamura et al., 1999a), pharmacological experiments established that the waves were attributable to Ca<sup>2+</sup> release from internal stores mediated by the synergistic effect of receptor-mobilized inositol 1,4,5-trisphosphate (IP<sub>3</sub>) and spike-evoked Ca<sup>2+</sup>. The amplitude of the changes reached several micromoles per liter when detected with the low-affinity indicators fura-6F, fura-2-FF, or fura-2. Repetitive brief spike trains at 30–60 sec intervals generated increases of constant amplitude. However, trains at intervals of 10–20 sec evoked smaller increases, suggesting that the stores take 20–30 sec to refill. Release evoked by mGluR

agonists was blocked by MCPG, AIDA, 4-CPG, MPEP, and LY367385, a profile consistent with the primacy of group I receptors. At threshold agonist concentrations the release was evoked only in the dendrites; threshold antagonist concentrations were effective only in the soma. Carbachol and 5-HT evoked release with the same spatial distribution as *t*-ACPD, suggesting that the distribution of neurotransmitter receptors was not responsible for the restricted range of regenerative release. Intracellular BAPTA and EGTA were approximately equally effective in blocking release. Extracellular Cd<sup>2+</sup> blocked release, but no single selective Ca<sup>2+</sup> channel blocker prevented release. These results suggest that IP<sub>3</sub> receptors are not associated closely with specific Ca<sup>2+</sup> channels and are not close to each other.

**Key words:** pyramidal neuron; dendrite; IP<sub>3</sub> receptor; metabotropic receptor; BAPTA; EGTA; carbachol; bis-fura-2; fura-2; endoplasmic reticulum

Synaptically activated action potentials backpropagate over the dendrites of hippocampal pyramidal neurons (Turner et al., 1991; Jaffe et al., 1992; Spruston et al., 1995). There is evidence that the action potentials and the [Ca<sup>2+</sup>]<sub>i</sub> increases that are associated with them may be important in the induction of long-term potentiation (LTP) and long-term depression (LTD; Magee and Johnston, 1997; Markram et al., 1997).

When the spikes are activated intrasomatically or antidromically, the associated [Ca<sup>2+</sup>]<sub>i</sub> increase is primarily attributable to Ca<sup>2+</sup> entry through voltage-sensitive Ca<sup>2+</sup> channels. The magnitude and spatial distribution of the [Ca<sup>2+</sup>]<sub>i</sub> increase depends on the distribution of different Ca<sup>2+</sup> channels in the dendrites (Magee and Johnston, 1995) and the amplitude and propagation pattern of the backpropagating spikes (Callaway and Ross, 1995; Spruston et al., 1995). Synaptic input, targeted to the dendrites, can enhance (Magee and Johnston, 1997) or inhibit (Buzsáki et al., 1996; Tsubokawa and Ross, 1996) spike backpropagation by shifting the membrane potential or causing a conductance shunt in the dendrites. Some modulators like carbachol (CCh, a muscarinic receptor agonist; Tsubokawa and Ross, 1997), isoproterenol (a β-adrenergic agonist; Hoffman and Johnston, 1999), and serotonin (5-HT; Ross and Sandler, 1998) affect spike propagation by modulating dendritic conductances. Serotonin (Sandler and Ross, 1999) and possibly other agonists can affect spike-associated [Ca<sup>2+</sup>]<sub>i</sub> increases by modulating directly the Ca<sup>2+</sup> channels that are opened by the action potentials.

In addition to these mechanisms, we recently showed that action potential-associated [Ca<sup>2+</sup>]<sub>i</sub> changes could be enhanced by pairing backpropagating spikes with repetitive synaptic activation (Nakamura et al., 1999a). The enhanced [Ca<sup>2+</sup>]<sub>i</sub> increase results from the synergistic action of Ca<sup>2+</sup> entering through spike-opened Ca<sup>2+</sup> channels and metabotropic glutamate receptor (mGluR)-mobilized inositol 1,4,5-trisphosphate (IP<sub>3</sub>) acting on IP<sub>3</sub> receptors (IP<sub>3</sub>Rs) to release Ca<sup>2+</sup> from the endoplasmic reticulum (ER). The magnitude of this increase is much larger than the increase attributable to spikes alone, even when the amplitude of the spikes is increased by neuromodulators.

During the course of these experiments we found that bath application of the mGluR agonist 1-amino-cyclopentyl-1,3-dicarboxylate (*t*-ACPD) appeared to have the same effect as synaptic activation in generating the synergistic release of Ca<sup>2+</sup> by action potentials in pyramidal neurons (Nakamura et al., 1999a). Because other metabotropic agonists coupled to IP<sub>3</sub> mobilization are likely to have the same effect, it was of interest to discover which agonists or neurotransmitters could couple with action potentials to evoke Ca<sup>2+</sup> release. In addition, analytical experiments with bath-applied agonists allow for more precise targeting of different receptors, eliminate the spatial inhomogeneity of synaptic stimulation, and bypass effects on presynaptic terminals or other neurons in the slice. Several questions were of interest. Which kinds of mGluRs participate in Ca<sup>2+</sup> release? Which Ca<sup>2+</sup> channels opened by action potentials promote release? What kinds of mechanisms are responsible for the preferred release of Ca<sup>2+</sup> in the proximal apical dendrites? How close in space are the Ca<sup>2+</sup> channels to the IP<sub>3</sub>R channels and how close together are different IP<sub>3</sub>Rs to each other? What levels of [Ca<sup>2+</sup>]<sub>i</sub> and IP<sub>3</sub> are required to cause regenerative release? These new experiments explore some of these questions.

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form (Ross and Sandler, 1998; Barbara et al., 1999; Nakamura et al., 1999b, 2000).

## MATERIALS AND METHODS

Transverse hippocampal slices (300  $\mu$ m thick) were prepared from 2- to 3-week-old Sprague Dawley rats as previously described (Tsubokawa and Ross, 1997; Nakamura et al., 1999a). Animals were anesthetized with methoxyflurane before decapitation. Slices were incubated for at least 1 hr in normal artificial CSF (ACSF) composed of (in mM): 124 NaCl, 2.5 KCl, 2 CaCl<sub>2</sub>, 2 MgCl<sub>2</sub>, 1.25 NaH<sub>2</sub>PO<sub>4</sub>, 26 NaHCO<sub>3</sub>, and 10–20 glucose, bubbled with a mixture of 95% O<sub>2</sub>/5% CO<sub>2</sub>, making a final pH of 7.4. During experiments the ACSF was superfused over the slice at a rate of 1 ml/min. Tight seals on CA1 pyramidal cell somata and electrophysiological recordings were made as previously described (Sakmann and Stuart, 1995; Nakamura et al., 1999a). The standard patch pipette solution contained (in mM): 140 K-gluconate, 4 NaCl, 4 Mg-ATP, 0.3 Na-GTP, and 10 HEPES, pH-adjusted to 7.2–7.4 with KOH. For some experiments we added 14 mM Na<sub>2</sub>-phosphocreatine. This solution was supplemented with 0.1–1.0 mM of a selected calcium indicator (fura-2, bis-fura, fura-6F, fura-2-FF, fura-2/AM, all from Molecular Probes, Eugene, OR). Typical osmolality was 300 mOsm. AP-5 [( $\pm$ )-2-amino-5-phosphonopentanoic acid], CNQX (6-cyano-7-nitroquinoxaline-2,3-dione), MCPG [(R,S)- $\alpha$ -methyl-4-carboxyphenylglycine], *t*-ACPD, ryanodine, serotonin,  $\alpha$ -methylserotonin, phenylephrine, dopamine, nimodipine, nifedipine, nitrendipine,  $\omega$ -conotoxin GVIA, BAPTA [1,2-bis(2-aminophenoxy)ethane-*N,N,N',N'*-tetra-acetic acid], EGTA, IP<sub>3</sub> (*D*-myo-inositol 1,4,5-trisphosphate), CPA (cyclopiazonic acid), TTX (tetrodotoxin), ruthenium red, and low-molecular-weight heparin were obtained from Sigma/RBI (St. Louis, MO). DHPG [(S)-3,5-dihydroxyphenylglycine], CHPG (2-chloro-5-hydroxyphenylglycine), AIDA (1-aminoindan-1,5-dicarboxylic acid), 4-CPG (4-carboxyphenylglycine), MPEP [2-methyl-6-(phenylethynyl)-pyridine], and LY367385 [(+)-2-methyl-4-carboxyphenylglycine] were obtained from Tocris (Ballwin, MO).  $\omega$ -Agatoxin-IVA was obtained from Peptides International (Louisville, KY). Thapsigargin and 3-F-IP<sub>3</sub> (3-deoxy-3-fluoro-*D*-myo-inositol 1,4,5-trisphosphate) were obtained from Calbiochem (San Diego, CA). Caffeine was obtained from Wako Chemicals (Tokyo, Japan). Adenophostin A was a generous gift from Sankyo (Tokyo, Japan).

Nimodipine, nitrendipine, and nifedipine were prepared as stock solutions in ethanol or DMSO at 1000 $\times$  final concentration and diluted into ACSF. In separate experiments we found that this concentration of ethanol or DMSO had no measurable effect on the electrical or optical parameters that were measured in these experiments. Dopamine was prepared daily as a 30 mM stock solution containing 0.2% ascorbic acid. For experiments this solution was diluted 100 $\times$  in ACSF. Control experiments demonstrated that 0.002% ascorbic acid in ACSF did not change the electrical or optical properties of pyramidal neurons. For ACSF containing 10–20 mM caffeine, NaCl was reduced by a comparable amount to preserve osmolality. The other compounds were added directly to the ACSF or pipette solution at the indicated concentrations.

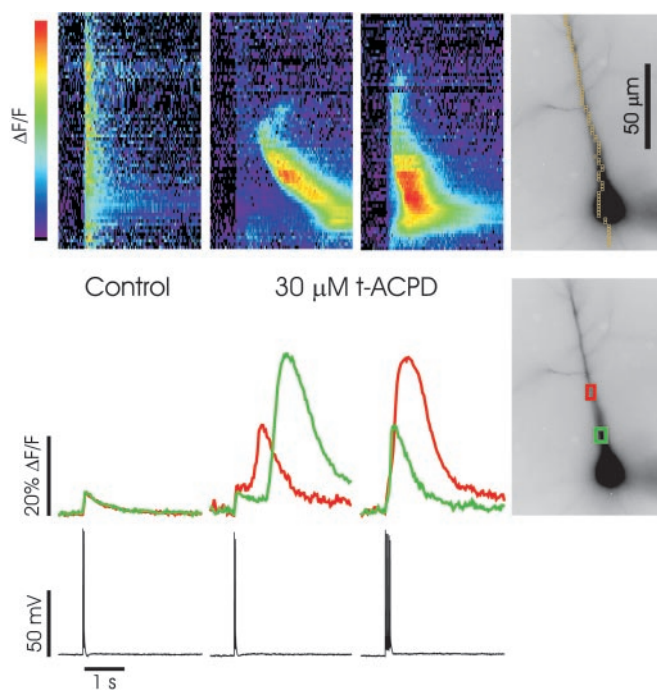
In a typical experiment a train of backpropagating action potentials was evoked with a train of 1 msec depolarizing pulses from the patch electrode. Time-dependent [Ca<sup>2+</sup>]<sub>i</sub> measurements from different regions of the pyramidal neuron were made as previously described (Lasser-Ross et al., 1991; Nakamura et al., 1999a). Electrical traces were recorded simultaneously and matched to the optical recordings. Data were taken and analyzed with Windows-based software written in our laboratory. Images were taken at 33 msec intervals. Electrical records were sampled at 200  $\mu$ sec intervals. During the experiments the solution changes were made by switching among different sources without changing the flow rate. In our system the chamber composition was changed by 90% in  $\sim$ 4 min.

## RESULTS

### Spike-evoked Ca<sup>2+</sup> release in the presence of *t*-ACPD

Backpropagating action potentials induce [Ca<sup>2+</sup>]<sub>i</sub> increases at all pyramidal cell locations by opening voltage-sensitive Ca<sup>2+</sup> channels in the dendrites (Jaffe et al., 1992). This is illustrated in the first column of Figure 1, which shows the [Ca<sup>2+</sup>]<sub>i</sub> change that is associated with a pair of action potentials evoked by brief intrasomatic pulses. The rapid rise in [Ca<sup>2+</sup>]<sub>i</sub> occurs at essentially the same time at all dendritic locations, reflecting the high velocity of spike backpropagation (Spruston et al., 1995).

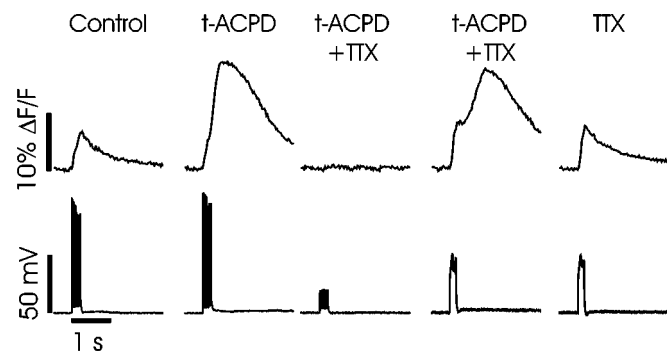
To isolate the effects of metabotropic agonists, we added 10  $\mu$ M CNQX and 100  $\mu$ M AP-5 to the ACSF to block ionotropic glutamate receptors in all of the experiments described in this paper. When 30  $\mu$ M *t*-ACPD was added to this solution and superfused into the bath, the cell depolarized 5.5  $\pm$  0.3 mV ( $n$  = 21). The resting fluorescence of the bis-fura-2-filled cell, at dendritic locations within 30  $\mu$ m from the soma, decreased by 3.8  $\pm$  0.4% ( $n$  = 13) in the first few minutes after the solution change. At later times it was difficult to assess the change because the indicator concentration was increasing slowly and these measurements were made at



**Figure 1.** Backpropagating action potentials evoke Ca<sup>2+</sup> waves in the presence of *t*-ACPD. In control ACSF (first column) a pair of spikes initiated with brief intrasomatic pulses caused rapid, almost simultaneous, increases in [Ca<sup>2+</sup>]<sub>i</sub> ( $\Delta F/F$ ) at all locations in the cell. This increase is shown in two ways. In the top panel the amplitude changes are indicated by a change in color. Positions along the ordinate correspond to the pixels overlaid on the cell image in the fourth column. Positions along the abscissa correspond to the same time scale as in the bottom panel. Below the pseudocolor image the same data are plotted as time-dependent changes for the two regions of interest (red and green boxes) indicated on the image of the cell. In ACSF containing 30  $\mu$ M *t*-ACPD, the same pair of spikes caused the same synchronous increases in [Ca<sup>2+</sup>]<sub>i</sub>, followed by larger increases at different times in different locations. The pseudocolor image shows that this secondary increase propagated as a wave that initiated at a location  $\sim$ 50  $\mu$ m from the soma. This wave did not propagate into the distal apical dendrites nor into the basal dendrites. Note that the pseudocolor scale has been changed to include the larger-amplitude secondary response. Five spikes (third column) caused a similar secondary response that initiated earlier and more synchronously at different dendritic locations.

a single excitation wavelength. Assuming that the resting [Ca<sup>2+</sup>]<sub>i</sub> in the cell was  $\sim$ 100 nM and that the  $K_D$  for bis-fura-2 binding to Ca<sup>2+</sup> is 370 nM (Molecular Probes catalogue), then this fluorescence decrease corresponds to an increase in [Ca<sup>2+</sup>]<sub>i</sub> of  $<$ 20 nM (Lev-Ram et al., 1992). This small increase could result from entry through voltage-sensitive Ca<sup>2+</sup> channels opened by the potential change near resting potential (Magee et al., 1996) or through a nonselective cation conductance activated by group I mGluRs (Congar et al., 1997). We did not investigate the mechanism of this increase in the present experiments. When *t*-ACPD is applied rapidly to the bath (Bianchi et al., 1999) or puffed onto the cell (Jaffe and Brown, 1994), larger [Ca<sup>2+</sup>]<sub>i</sub> increases are recorded. However, these increases were not observed in our experiments in which the bath composition was changed slowly by being superfused at  $\sim$ 1 ml/min.

In the presence of 30  $\mu$ M *t*-ACPD the backpropagating spikes evoked a secondary increase in [Ca<sup>2+</sup>]<sub>i</sub> that was much larger than in control ACSF (Nakamura et al., 1999a). With one or two action potentials the secondary increase propagated as a wave in the apical dendrites (Fig. 1, second column). The wave did not propagate into the distal dendrites although the sharp spike-evoked [Ca<sup>2+</sup>]<sub>i</sub> increase could be observed in this region. In this figure the wave had a clear initiation point  $\sim$ 50  $\mu$ m from the soma. The rapid rise in [Ca<sup>2+</sup>]<sub>i</sub> at the initiation point began  $\sim$ 0.5 sec after the spikes. In five cells in which release was activated by a single spike, the delays were 0.15, 0.35, 0.5, 0.8, and 2.1 sec. When there was a



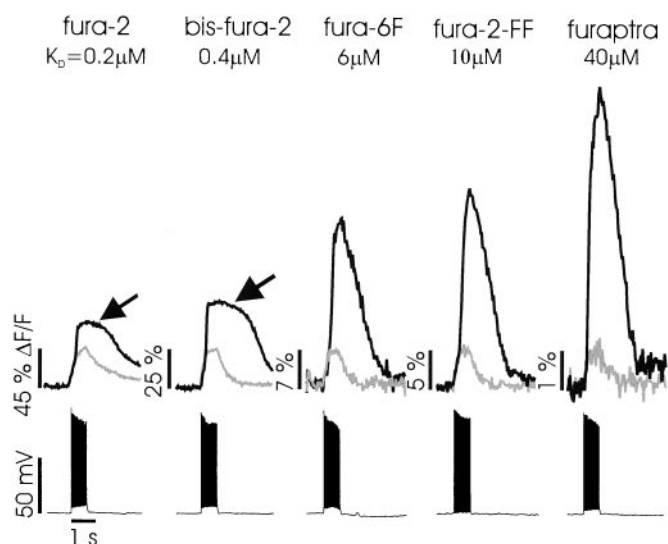
**Figure 2.** Membrane depolarization sufficient to cause a significant increase in  $[Ca^{2+}]_i$  is required to evoke  $Ca^{2+}$  release. In control ACSF a train of 10 backpropagating spikes initiated by 1 msec depolarizing pulses caused a rapid increase in  $[Ca^{2+}]_i$  at a dendritic location  $\sim 30 \mu m$  from the soma. When  $30 \mu M$  *t*-ACPD was added to the ACSF, the same 10 spikes caused a secondary  $[Ca^{2+}]_i$  increase. The addition of  $1.0 \mu M$  TTX blocked the spikes and eliminated both the primary and secondary  $[Ca^{2+}]_i$  increases. Substituting a single 200 msec pulse for the train of brief pulses restored both components of the  $[Ca^{2+}]_i$  transient. Finally, washing out *t*-ACPD left a rapid  $[Ca^{2+}]_i$  increase similar to that caused by spikes in the same solution.

single initiation site, this location was always in the proximal apical dendrites. In several cells two initiation points could be observed. In two of these cells the second initiation site was in the basal dendrites, within  $10 \mu m$  of the soma. However, these waves did not propagate basally  $>20 \mu m$  from the soma. When five action potentials were stimulated (Fig. 1, *third column*), the initial sharp  $[Ca^{2+}]_i$  increase was larger and the secondary response appeared more synchronously at all locations at which it was observed and with a much shorter delay. This was the consistent pattern in  $>100$  pyramidal neurons.

To test whether the action potentials or just the brief stimulating pulses were responsible for both the rapid and secondary  $[Ca^{2+}]_i$  increases, we added TTX ( $1.0 \mu M$ ) to the superfusate (Fig. 2). In this solution the action potentials were blocked, the rapid spike-evoked  $[Ca^{2+}]_i$  increase was eliminated, and there was no secondary increase ( $n = 2$ ). However, when a strong 0.2 sec depolarizing pulse was given, both a rapid increase, time-locked to the pulse, and a secondary increase were recorded. Washing out the *t*-ACPD left only the rapid  $[Ca^{2+}]_i$  increase. These results suggest that it was not action potentials, specifically, that caused release but, rather, the membrane depolarization.

### Responses with different indicators and different stimulus intervals

The large magnitude of the secondary  $[Ca^{2+}]_i$  increases (often  $>50\% \Delta F/F$ , using  $100$ – $300 \mu M$  bis-fura-2 in the pipette) suggested that the released  $Ca^{2+}$  might be saturating the indicator. To examine this possibility, we tested different indicators resembling bis-fura-2. These indicators have similar spectral characteristics (Molecular Probes catalogue), and their properties could be assayed with the same fluorescence filter set. Figure 3 shows results with five indicators having different  $K_D$  values. The  $K_D$  values were obtained from the Molecular Probes catalogue except for fura-2-FF (Schneggenburger et al., 1999) and fura-2-FF (Naraghi, 1997). For each indicator the figure shows representative fluorescence changes recorded from a small region  $\sim 30 \mu m$  from the soma (fura-2,  $n = 9$ ; bis-fura-2,  $n > 100$ ; fura-6F,  $n = 68$ ; fura-2-FF,  $n = 2$ ; fura-2-FF,  $n = 2$ ). The smaller gray traces show the change that follows a train of 20 action potentials in normal ACSF. These traces all have been scaled to the same amplitude on the assumption that they all represent the same magnitude of  $[Ca^{2+}]_i$  change. This assumption is not exactly true, because the indicators differ in their buffering power and nonlinearity in response to  $Ca^{2+}$ . However, the similar time courses for all of the traces except that recorded with fura-2 suggest that the error is not large. Note that the percentage of change in fluorescence is very different between



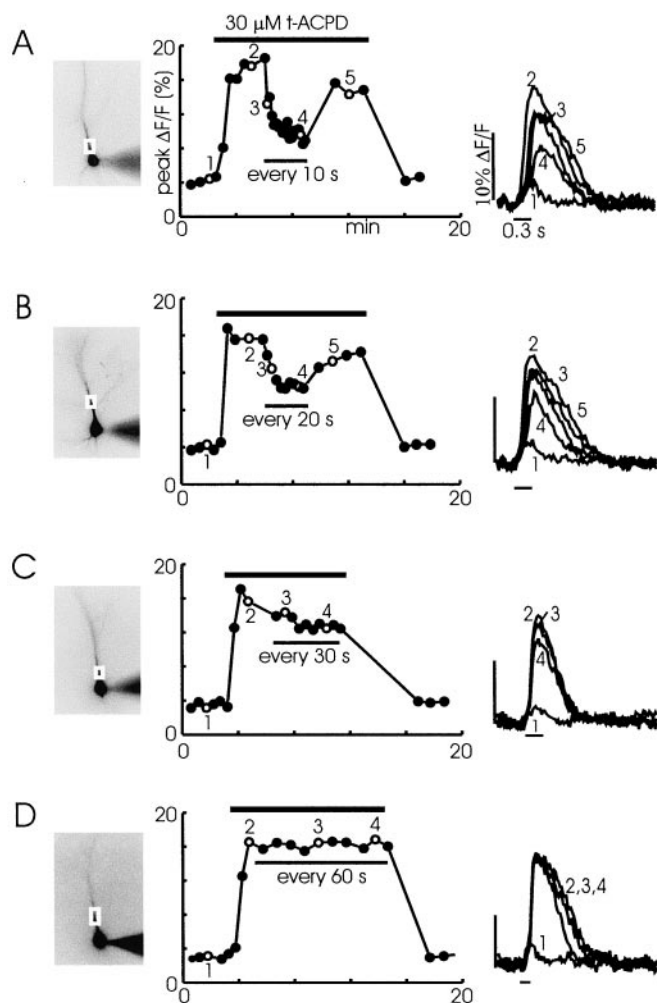
**Figure 3.** Fluorescence changes evoked by backpropagating action potentials, using indicators of different  $Ca^{2+}$  affinities. The different indicators and their approximate dissociation constants are shown at the top. The gray traces show the increases in normal ACSF that were observed at a location  $\sim 30 \mu m$  from the soma. All of these increases have been scaled to the same size, although the amplitudes ( $\Delta F/F$ ) varied from 45 to 1%. The black traces show the fluorescence increases that were observed in ACSF containing  $30 \mu M$  *t*-ACPD. The increases that were recorded with fura-2 and bis-fura-2 have flat tops (arrows) suggesting indicator saturation. The ratio of increases in *t*-ACPD to those in normal ACSF that were recorded with the lower-affinity indicators is much larger.

the high-affinity fura-2 and the low-affinity fura-2-FF. In the presence of  $30 \mu M$  *t*-ACPD the same train of action potentials evoked much larger fluorescence increases (Fig. 3, *darker traces*). The rounded tops of the traces recorded with fura-2 and bis-fura-2 (Fig. 3, *arrows*) suggests that these responses saturated the indicators. Much larger relative changes were recorded with the low-affinity indicators fura-6F, fura-2-FF, and fura-2-FF. None of these responses appeared to saturate the indicators. The larger relative response with fura-2-FF ( $n = 2$ ) may reflect the fact that this indicator interfered less with the regenerative release process as well as the fact that it was linear throughout the range of physiological fluorescence changes. The 7% fluorescence change detected with fura-2-FF corresponds to a  $[Ca^{2+}]_i$  increase of  $\sim 3 \mu M$  (Lev-Ram et al., 1992; Nakamura et al., 1999a). The 1% increase in normal ACSF corresponds to a change of  $\sim 0.4 \mu M$ . This is close to the magnitude reported by Helmchen et al. (1996) for a train of action potentials that used very low concentrations of fura-2.

For pharmacological experiments in the rest of the paper, in which measuring the relative magnitude of the fluorescence change under different conditions was important, we used fura-6F. This indicator is relatively linear in the physiological range, does not buffer the responses significantly, and gives signals with a satisfactory signal-to-noise ratio. For experiments designed to determine qualitatively whether release occurred, we used low concentrations of bis-fura-2. This indicator gives signals that can be recorded with a good signal-to-noise ratio over most of the cell, is not toxic to the cells, and bleaches  $<1\%$  in a typical trial.

To assess the effect of different pharmacological agents on the release process, we believed that it was important to have stable conditions in which experiments produced repeatable results. Therefore, we tested whether trains of spikes at different trial intervals consistently could evoke  $[Ca^{2+}]_i$  changes of similar amplitude. Figure 4 shows representative experiments that used  $300 \mu M$  fura-6F as the indicator. In each case a train of 5–10 action potentials at 30 msec intervals was evoked, first in normal ACSF and then with  $30 \mu M$  *t*-ACPD added to the bath. In normal ACSF, 10 action potentials evoked a peak increase of 4%  $\Delta F/F$  in a region in the proximal apical dendrites. In *t*-ACPD the spikes evoked





**Figure 4.**  $[Ca^{2+}]_i$  increases observed after trains were stimulated at different intervals. *A*, Trains evoked at 10 sec intervals. A train of 10 spikes at 30 msec intervals was evoked in normal ACSF. Trace 1 in the right panel shows the response at the location on the cell image (left panel). This pattern was repeated four times at an intertrain interval of 10 sec. The peak response amplitudes for each of these trials are plotted as the first four points in the middle graph. When  $30 \mu M$  *t*-ACPD was added to the bath (horizontal bar), the same spike train evoked a secondary  $[Ca^{2+}]_i$  increase of larger amplitude. This pattern was repeated several times. A representative response (2) is shown in the right panel. When the interval between trains was reduced to 10 sec, the amplitude decreased. At the end of several minutes (4) the amplitude was reduced  $\sim 60\%$ , and the response was delayed. When the intertrain interval was increased, the amplitude increased and the delay was shortened (5). Washing out the *t*-ACPD restored the initial response. *B*, A similar experiment that used 20 sec intertrain intervals. The amplitude reduction was less than that observed with 10 sec intervals. *C*, A similar experiment that used 30 sec intertrain intervals. There was almost no reduction in amplitude. *D*, A similar experiment that used 60 sec intervals. For these experiments there were only five spikes in the train. There was no observed amplitude reduction from trial to trial.

Ca<sup>2+</sup> release, and the peak fluorescence increase was 18–20%. After the baseline response was established, in each of the panels (Fig. 4*A–D*) the interval between trials was varied. When the interval was 10 sec (Fig. 4*A*), the magnitude of the fluorescence increase was reduced by  $74.6 \pm 5.5\%$  from the peak value ( $n = 7$ ). The percentage of reduction was not sensitive to the position of the selected region over the first 50  $\mu m$  of the apical dendrites. The selected examples to the right show that the release transient was delayed as well as reduced in size after repetitive stimulation at 10 sec intervals. After a 2 min wait the amplitude and delay recovered almost to their original values. Experiments at longer intertrain intervals showed less of a reduction. At 20 sec intervals the peak was reduced  $53.5 \pm 4.0\%$  ( $n = 4$ ); at 30 sec intervals the peak was reduced  $23.5 \pm 7.8\%$  ( $n = 4$ ); at 60 sec intervals the peak was

reduced by  $15.3 \pm 3.5\%$  ( $n = 6$ ). The reductions measured at 30 and 60 sec intervals were not significantly different. Therefore, most experiments were done at 30 or 60 sec intervals.

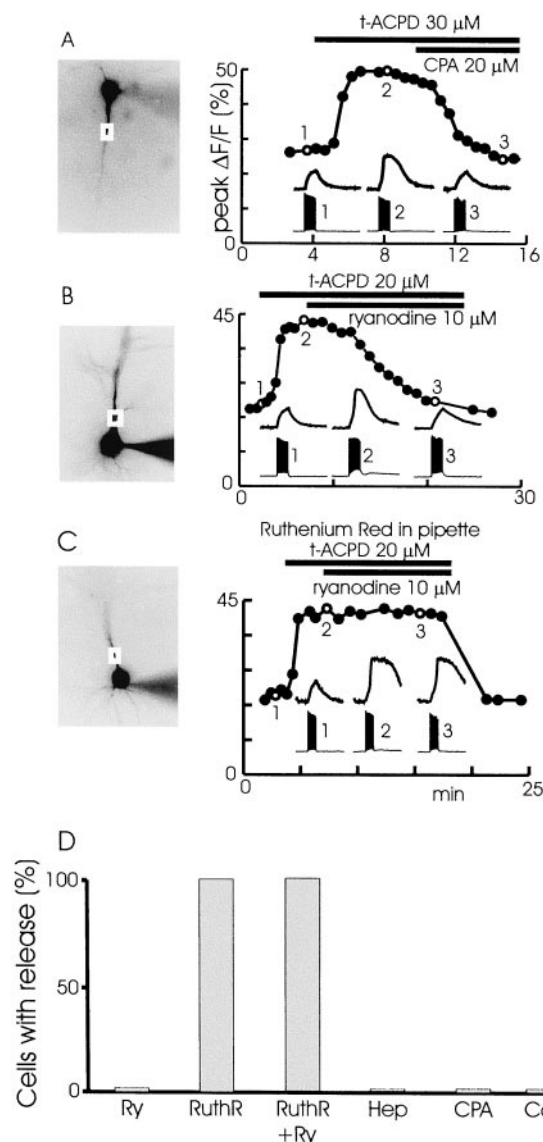
The strong peak reduction at 10 and 20 sec intervals suggests that the stores did not replenish their Ca<sup>2+</sup> in this interval or that the receptors on ER did not recover from inactivation (Hajnóczky and Thomas, 1994). Whichever the explanation, these experiments show that the recovery time constant after spike-evoked regenerative release is  $\sim 30$  sec.

These experiments also suggest that the Ca<sup>2+</sup> entering the cytoplasm with the spikes did not make a significant contribution to replenishing the stores. The magnitude of the  $[Ca^{2+}]_i$  increase after five spikes in control conditions was five times less than the magnitude in  $30 \mu M$  *t*-ACPD when the trials were conducted at 1 min intervals (Fig. 4*D*). Nevertheless, this large-amplitude  $[Ca^{2+}]_i$  increase could be evoked repeatedly over a 15 min interval. It seems unlikely that the small  $[Ca^{2+}]_i$  increase attributable to the spikes could supply enough Ca<sup>2+</sup> to refill the stores. Rather, the stores probably were refilled from the Ca<sup>2+</sup> that was released in the previous trial. However, we cannot rule out the possibility that some of the spike-evoked Ca<sup>2+</sup> entry replenished Ca<sup>2+</sup> that leaked out or was pumped from the cell. We also found that release could be evoked with only a few spikes in the first trial in experiments in which the slice was preincubated with  $30 \mu M$  *t*-ACPD (data not shown). In these experiments no action potentials were given to load the stores. This result supports the idea that spikes are not needed to fill the stores. This conclusion differs from that of some previous investigators (Jaffe and Brown, 1994) who emphasized the importance of filling stores before evoking release.

### Mechanism of release

In experiments that used synaptic stimulation, we showed that regenerative Ca<sup>2+</sup> release was attributable to mGluR-activated IP<sub>3</sub> synergistically acting with Ca<sup>2+</sup> entering through spike-opened Ca<sup>2+</sup> channels to open IP<sub>3</sub>Rs (Nakamura et al., 1999a). The similar shape of the release transients evoked in *t*-ACPD suggests that the same mechanism was at work in these new experiments. To test this hypothesis, we repeated the pharmacological experiments of Nakamura et al. (1999a) by using bath-applied *t*-ACPD. Figure 5*A* shows that spike-evoked release was blocked by  $20 \mu M$  CPA ( $n = 6$ ), which blocks the ER Ca<sup>2+</sup>-ATPase (Seidler et al., 1989). Release also was blocked by  $10 \mu M$  ryanodine (Fig. 5*B*;  $n = 5$ ), which depletes the ER by keeping the ryanodine receptor channel open (Rousseau et al., 1987). Including ruthenium red, which blocks the ryanodine receptor in the closed state (Smith et al., 1988), in the pipette did not block release but did prevent the action of  $10 \mu M$  ryanodine (Fig. 5*C*;  $n = 5$ ). In addition, release was prevented by including low-molecular-weight heparin (1 mg/ml,  $n = 5$ ; data not shown) in the pipette. Heparin blocks the IP<sub>3</sub>Rs nonspecifically in a variety of cell types (Ghosh et al., 1988; Kobayashi et al., 1988). Heparin did not block the slow depolarization induced by *t*-ACPD, suggesting that a non-IP<sub>3</sub>-dependent pathway requiring mGluR activation mediated this voltage change.

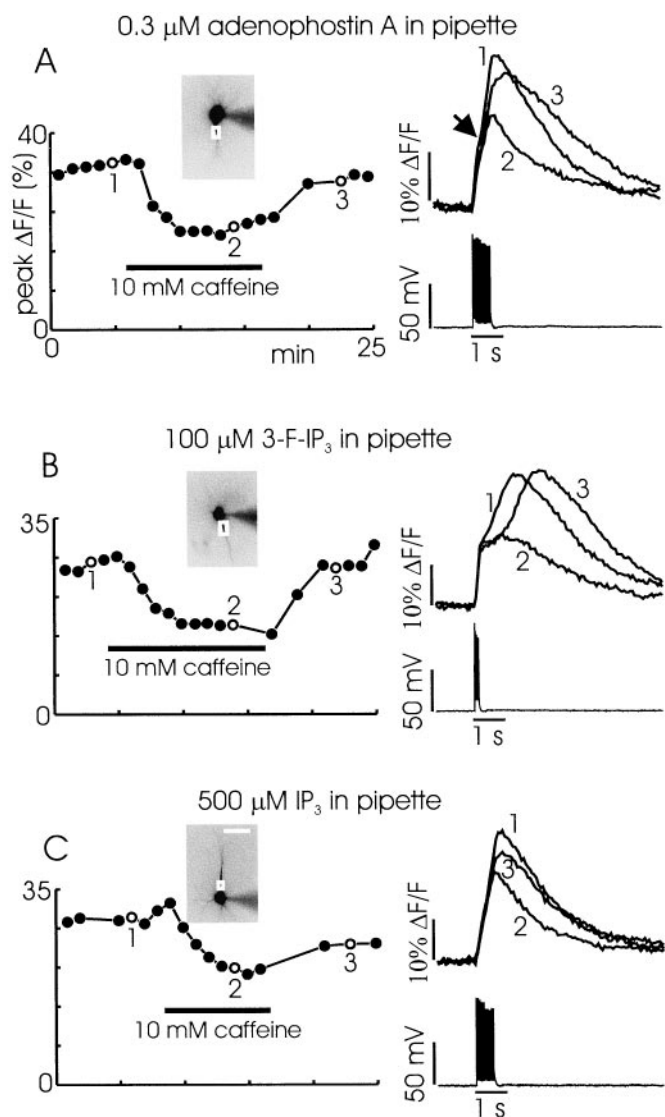
These results parallel those we found when mGluRs were activated by synaptic transmission (Nakamura et al., 1999a). We also tested several additional compounds that are known to affect intracellular stores. Thapsigargin, like CPA, blocks the ER Ca<sup>2+</sup>-ATPase (Thastrup et al., 1990). Preincubation of the slice with  $3 \mu M$  thapsigargin ( $n = 3$  of 4) prevented spike-evoked release (data not shown). Caffeine sensitizes the ryanodine receptor and, at high concentrations, opens the receptor at rest, depleting the stores (Zucchi and Ronca-Testoni, 1997). Caffeine also blocks IP<sub>3</sub>Rs in some preparations (Parker and Ivorra, 1991). Caffeine (2–10 mM,  $n = 7$ ; data not shown) blocked *t*-ACPD-mediated release in all experiments, an effect compatible with either mechanism. Caffeine by itself caused only a 10–20% increase in the spike-evoked  $[Ca^{2+}]_i$  change in normal ACSF without altering the time course of the  $[Ca^{2+}]_i$  transient (Sandler and Barbara, 1999). This small increase may be attributable to the effect of caffeine on the indicator (Muschol et al., 1999). However, we have no direct evidence



**Figure 5.** Effect of different pharmacological agents on spike-evoked release in the presence of 30  $\mu$ M *t*-ACPD. *A*, CPA blocks release. Cell image, peak  $\Delta F/F$  graph, and insets are similar to those shown in Figure 4. In normal ACSF a train of 20 backpropagating spikes evoked at 30 msec intervals caused a small  $[Ca^{2+}]_i$  increase linked in time to the spikes (1). When 30  $\mu$ M *t*-ACPD was added to the bath, a larger increase was observed (2). Adding 20  $\mu$ M CPA to the solution reduced the  $[Ca^{2+}]_i$  increase to the level that was observed in control conditions (3). Each point on the graph represents a separate trial evoked at 30 sec intervals. Some points have been deleted because other protocols were tested at those times. *B*, A similar experiment showing that 10  $\mu$ M ryanodine added to the ACSF blocked the secondary  $[Ca^{2+}]_i$  increase. *C*, A similar experiment showing that 10  $\mu$ M ryanodine did not block the secondary  $[Ca^{2+}]_i$  increase when 120  $\mu$ M ruthenium red was included in the patch pipette. *D*, Summary histogram showing the effects of the different agents. In addition to showing the results of parts *A–C* of this figure, the histogram shows that 1 mg/ml of heparin in the pipette blocked release, 20 mM caffeine that was added to the ACSF blocked release, and preincubation with 3  $\mu$ M thapsigargin blocked release.

supporting this explanation. The absence of regenerative spike-evoked release in the presence of 2–5 mM caffeine is interesting because this protocol has been reported to cause all-or-none  $Ca^{2+}$ -induced  $Ca^{2+}$  release (CICR) transients in rat sensory neurons (Usachev and Thayer, 1997). That mechanism, mediated by ryanodine receptors, does not appear to be significant in pyramidal neurons. Figure 5*D* summarizes the results by using agents that modulate intracellular stores.

To reinforce the conclusion that release was mediated by the activation of IP<sub>3</sub>Rs, we tested several compounds that are relatively



**Figure 6.** Effect of IP<sub>3</sub> and analogs in evoking spike-associated  $Ca^{2+}$  release. *A*, Adenophostin A (0.3  $\mu$ M) was included in the patch pipette. A train of 10 backpropagating spikes evoked at 30 msec intervals caused a  $[Ca^{2+}]_i$  increase that had an inflection (arrow) on the rising phase (1). The addition of 10 mM caffeine to the ACSF reduced the amplitude of the  $[Ca^{2+}]_i$  increase and removed the inflection (2). Washing out the caffeine restored the larger amplitude and rounded time course of the  $[Ca^{2+}]_i$  increase (3). *B*, A similar experiment in which 100  $\mu$ M 3-F-IP<sub>3</sub> was included in the pipette. This analog also caused a spike-associated secondary  $[Ca^{2+}]_i$  increase that was blocked by 10 mM caffeine. *C*, A similar experiment in which 500  $\mu$ M IP<sub>3</sub> was included in the pipette. IP<sub>3</sub> also caused a secondary  $[Ca^{2+}]_i$  increase that was blocked by 10 mM caffeine. Scale in cell image, 50  $\mu$ m.

specific activators of this receptor. Adenophostin A at low concentrations is a potent IP<sub>3</sub>R agonist (Takahashi et al., 1994). When 0.3  $\mu$ M adenophostin A was included in the pipette, a train of action potentials evoked an increase in dendritic  $[Ca^{2+}]_i$  that outlasted the train of spikes ( $n = 5$ ; Fig. 6*A*, trace 1). The time course of the  $[Ca^{2+}]_i$  change resembled that evoked by *t*-ACPD and spikes (see, for example, Fig. 1). To confirm that this increase was attributable to release of  $Ca^{2+}$  and not some nonspecific effect, we added 10 mM caffeine. Caffeine, as indicated above, empties the stores and blocks IP<sub>3</sub>Rs. In this condition the same train of spikes evoked a smaller  $[Ca^{2+}]_i$  increase that was linked in time to the action potentials (Fig. 6*A*, trace 2). In control experiments (data not shown) we found that caffeine did not reduce the spike-evoked  $[Ca^{2+}]_i$  increase. Washing out the caffeine allowed the stores to refill. Because adenophostin A was still in the cell, spikes again

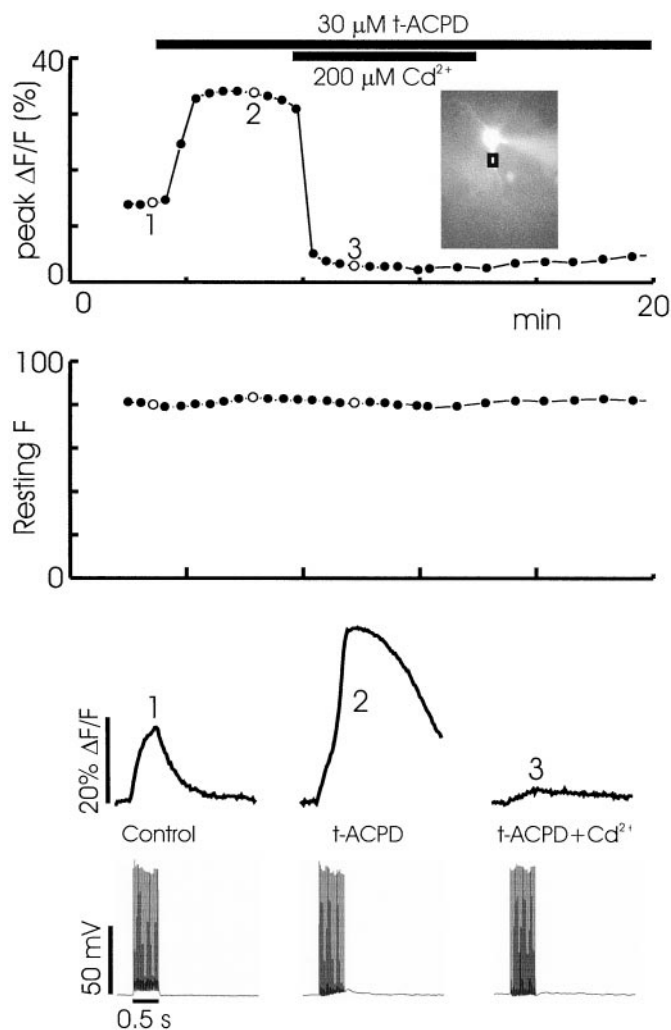
evoked release (Fig. 6*A*, trace 3). Figure 6*B* shows similar experiments that used 100  $\mu$ M 3-F-IP<sub>3</sub> in the pipette instead of adenophostin A ( $n = 3$ ). This analog of IP<sub>3</sub>, resistant to 3-kinase, is equipotent to IP<sub>3</sub> in releasing Ca<sup>2+</sup> from stores in other preparations (Kozikowski and Fauq, 1990). Finally, IP<sub>3</sub> itself, at a concentration of 500  $\mu$ M in the pipette, also caused Ca<sup>2+</sup> release when it was paired with a train of action potentials (Fig. 6*C*;  $n = 6$ ). These concentrations of agonists were close to the threshold concentrations for this protocol because 0.1  $\mu$ M adenophostin A ( $n = 3$ ), 30  $\mu$ M 3-F-IP<sub>3</sub> ( $n = 5$ ), and 300  $\mu$ M IP<sub>3</sub> ( $n = 5$ ) did not cause spike-associated Ca<sup>2+</sup> release. Together, these results strongly support the conclusion that release is mediated via the action of IP<sub>3</sub> on the IP<sub>3</sub>R.

One difference between release mediated by tonic activation of mGluRs and release mediated by intracellular injection of IP<sub>3</sub>R agonists is that the amplitude of spike-associated Ca<sup>2+</sup> release in the presence of intracellular IP<sub>3</sub>R agonists was smaller than the amplitude in the presence of *t*-ACPD. Increasing the concentration of IP<sub>3</sub>R agonists did not increase the amplitude of the [Ca<sup>2+</sup>]<sub>i</sub> change. This result might suggest that the IP<sub>3</sub> agonists are not activating the same release process as *t*-ACPD. However, injection of IP<sub>3</sub>R activators at concentrations high enough to cause release occluded the effect of *t*-ACPD; i.e., when *t*-ACPD was added to the bath, large amplitude spike-evoked release did not occur in the presence of intracellular agonists (1.0  $\mu$ M adenophostin A,  $n = 5$ ; 100  $\mu$ M 3-F-IP<sub>3</sub>,  $n = 3$ ; 500  $\mu$ M IP<sub>3</sub>,  $n = 2$ ; data not shown). Lower concentrations of IP<sub>3</sub>R agonists (0.1  $\mu$ M adenophostin A,  $n = 2$ ; 30  $\mu$ M 3-F-IP<sub>3</sub>,  $n = 3$ ; 300  $\mu$ M IP<sub>3</sub>,  $n = 3$ ), which did not cause even a low level of release, did not occlude the effect of *t*-ACPD. The reason for the small amplitude of release with injected IP<sub>3</sub>R agonists is not clear. The IP<sub>3</sub>R agonists did not occlude the resting membrane depolarization caused by *t*-ACPD, an observation that parallels the results with injected heparin.

### Effects of calcium channel blockers

Two kinds of experiments indicate that the critical role of spikes in the release process is to increase transiently the [Ca<sup>2+</sup>]<sub>i</sub> in the cells to act cooperatively with IP<sub>3</sub> to open the IP<sub>3</sub>R channels. First, blocking Ca<sup>2+</sup> entry with 200  $\mu$ M Cd<sup>2+</sup> strongly reduced the immediate [Ca<sup>2+</sup>]<sub>i</sub> increase and prevented the secondary [Ca<sup>2+</sup>]<sub>i</sub> increase attributable to release (Fig. 7). Because the immediate increase was time-locked to the spikes and its amplitude was insensitive to CPA and ryanodine, it reflects Ca<sup>2+</sup> entry through voltage-dependent Ca<sup>2+</sup> channels. This figure also shows that Cd<sup>2+</sup> had no effect on the resting bis-fura-2 fluorescence, indicating that Cd<sup>2+</sup> did not enter the cell to quench the indicator fluorescence. Second, in most experiments that used bis-fura-2 as the indicator, one or two spikes did not cause release, but a larger number of spikes were effective. More spikes increase the peak [Ca<sup>2+</sup>]<sub>i</sub> level reached in a train. In contrast, when fura-2 was used, only one or two spikes were necessary. This low-affinity indicator does not buffer Ca<sup>2+</sup> significantly and allows the peak free [Ca<sup>2+</sup>]<sub>i</sub> to reach higher levels. These experiments suggest that spikes produce a threshold level of [Ca<sup>2+</sup>]<sub>i</sub> that cooperatively acts with IP<sub>3</sub> to trigger release.

Because the almost complete block of Ca<sup>2+</sup> entry by Cd<sup>2+</sup> was effective in preventing release, it was of interest to see whether entry through any of the major kinds of Ca<sup>2+</sup> channels was particularly effective in inducing release. Figure 8 shows experiments in which we tested several antagonists that are relatively specific for different Ca<sup>2+</sup> channel types. At 100  $\mu$ M, Ni<sup>2+</sup> preferentially blocks low-threshold T-type channels (Ozawa et al., 1989; Mogul and Fox, 1991). When superfused over the preparation, Ni<sup>2+</sup>-containing ACSF reduced the spike-evoked [Ca<sup>2+</sup>]<sub>i</sub> increase by  $25.3 \pm 1.1\%$  ( $n = 3$ ) as previously reported (Christie et al., 1995). However, with the addition of 30  $\mu$ M *t*-ACPD the spikes still were able to evoke a large secondary [Ca<sup>2+</sup>]<sub>i</sub> increase (Fig. 8*A*;  $n = 4$  of 5). Similar results were obtained with 1–5  $\mu$ M  $\omega$ -conotoxin-GVIA, a blocker of high-threshold N-type Ca<sup>2+</sup> channels (McCleskey et al., 1987). This toxin reduced the sharp spike-evoked [Ca<sup>2+</sup>]<sub>i</sub>

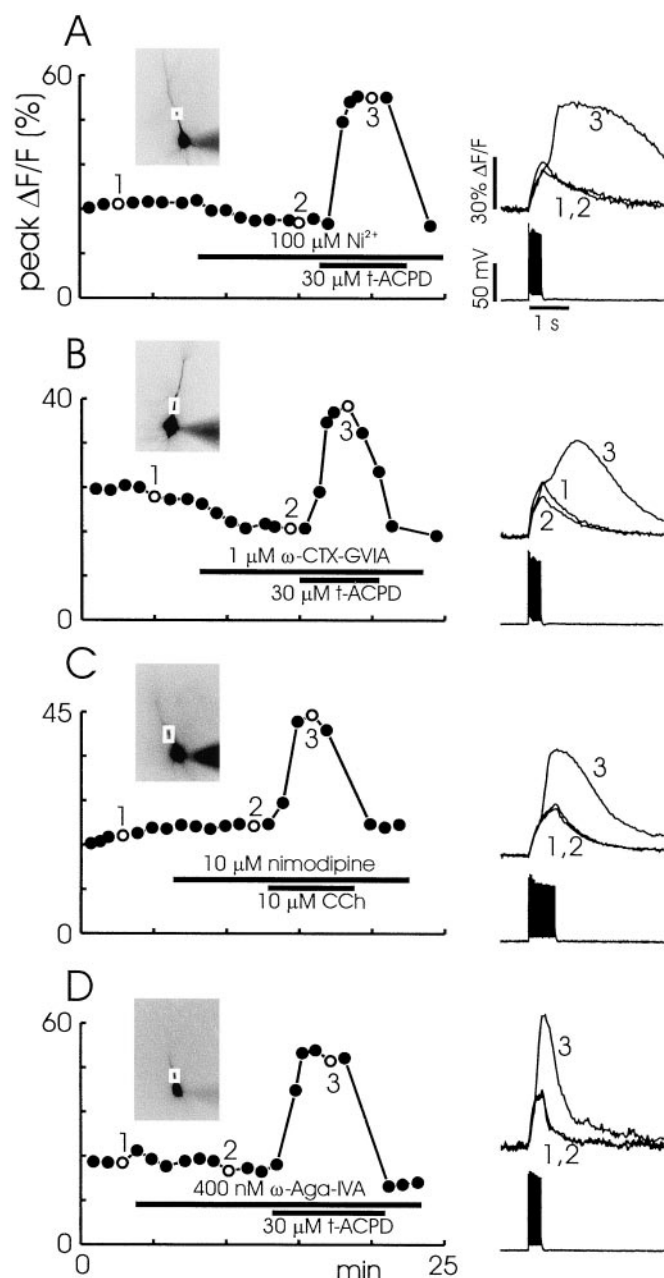


**Figure 7.** Cd<sup>2+</sup> (200  $\mu$ M) blocks both the primary and secondary spike-associated [Ca<sup>2+</sup>]<sub>i</sub> increase in the presence of 30  $\mu$ M *t*-ACPD. The middle panel shows that Cd<sup>2+</sup> did not affect the resting fluorescence level in the cell, indicating that Cd<sup>2+</sup> did not enter the cell to quench the indicator fluorescence. Scale is in arbitrary units. The bottom panels show representative responses. A small spike-associated ACSF response, insufficient to evoke release, remained in Cd<sup>2+</sup>-containing ACSF.

increase by  $26.7 \pm 1.4\%$  ( $n = 7$ ) but did not prevent release (Fig. 8*B*).

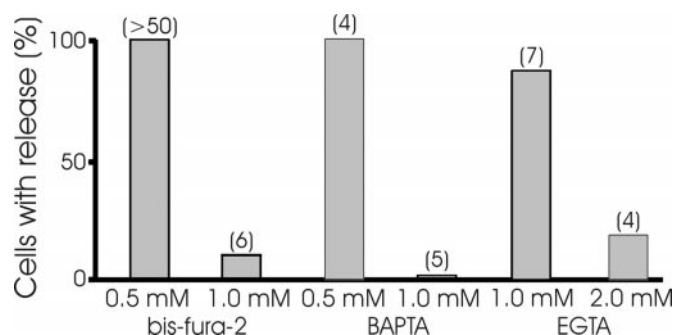
Almost similar results were obtained with dihydropyridine antagonists of L-type Ca<sup>2+</sup> channels. Nimodipine (Fig. 8*C*; 10–30  $\mu$ M;  $n = 15$ ), nifedipine (10  $\mu$ M;  $n = 6$ ), and nitrendipine (10  $\mu$ M;  $n = 1$ ) did not prevent spike-evoked Ca<sup>2+</sup> release. Interestingly, none of these antagonists had a significant effect on the sharp spike-evoked [Ca<sup>2+</sup>]<sub>i</sub> increases in these slice experiments. This result differs from previous sharp electrode experiments that found an L-type channel component in spike-associated [Ca<sup>2+</sup>]<sub>i</sub> increases (Christie et al., 1995). Two control experiments confirmed that dihydropyridine-containing ACSF was capable of blocking L-type Ca<sup>2+</sup> channels. In one, 10  $\mu$ M nimodipine reduced the [Ca<sup>2+</sup>]<sub>i</sub> increase caused by a 1 sec depolarizing pulse ( $n = 3$ ; data not shown). This rules out the possibility that many of the L-type Ca<sup>2+</sup> channels were washed out (Tombaugh and Somjen, 1997). In the second, the same solution diluted isolated rat blood vessels (Ungvari et al., 2000), confirming the effectiveness of the nimodipine we purchased. Thus, it is likely that few L-type channels are opened by backpropagating action potentials, although these channels are found in CA1 pyramidal neurons (Westenbroek et al., 1990; Magee and Johnston, 1995). One possible explanation for these results is that the activation kinetics of the L-type channels





**Figure 8.** Selective Ca<sup>2+</sup> channel blockers do not prevent spike-associated Ca<sup>2+</sup> release in the presence of 30  $\mu$ M t-ACPD. *A*, A train of 10 back-propagating spikes evoked at 30 msec intervals caused a rapid [Ca<sup>2+</sup>]<sub>i</sub> increase at a location close to the soma in the apical dendrites. The amplitude of this change was approximately constant when these trains were evoked at 30 sec intervals (1). The addition of 100  $\mu$ M NiCl<sub>2</sub> to the ACSF reduced the amplitude of this increase without changing the shape of the transient (2). When t-ACPD was added to this solution, a secondary [Ca<sup>2+</sup>]<sub>i</sub> increase was observed (3). *B–D*, Similar experiments are shown with 1  $\mu$ M  $\omega$ -CTX-GVIA, 10  $\mu$ M nimodipine, and 400 nM  $\omega$ -Aga-IVA. None of these agents prevented release by t-ACPD (*B*, *D*) or CCh (*C*).  $\omega$ -CTX-GVIA reduced the spike-associated [Ca<sup>2+</sup>]<sub>i</sub> in normal ACSF, whereas nimodipine and  $\omega$ -Aga-IVA did not. For the experiment with  $\omega$ -Aga-IVA, fura-6F was the Ca<sup>2+</sup> indicator, resulting in faster transients.

are slow and the channels are not opened by the action potential (Mermelstein et al., 2000). The important result, however, is that L-type channels are not connected specifically to the IP<sub>3</sub>R-mediated release mechanism in pyramidal neurons. This conclusion applies even if L-type Ca<sup>2+</sup> channels are washed out, because in that case there can be no connection between these Ca<sup>2+</sup> channels and any intracellular receptor. A strong connection has been implicated between L-type channels and ryanodine receptor-mediated release in other neurons (Chavis et al., 1996).



**Figure 9.** Effect of different Ca<sup>2+</sup> buffers on the ability of spikes to evoke Ca<sup>2+</sup> release in the presence of 30  $\mu$ M t-ACPD. When the pipette contained 0.5 mM bis-fura-2, release was always observed. Increasing the concentration to 1.0 mM bis-fura-2 prevented release. Different concentrations of EGTA and BAPTA in the pipette were tested with electrodes also containing 100–200  $\mu$ M of the low-affinity indicator fura-6F. BAPTA (1.0 mM) and EGTA (2.0 mM) prevented release. Numbers over the bars indicate the cells that were tested.

Similar results were found with the P-type Ca<sup>2+</sup> channel blocker,  $\omega$ -agatoxin-IVA (Mintz et al., 1992). In five experiments 200–400 nM of this toxin did not prevent release and had no significant effect on the sharp spike-evoked [Ca<sup>2+</sup>]<sub>i</sub> increase. To test the potency of our sample, we confirmed that  $\omega$ -agatoxin-IVA could block the voltage-dependent [Ca<sup>2+</sup>]<sub>i</sub> increase in rat cerebellar Purkinje cells ( $n = 2$ ; data not shown). We did not test the possibility that R-type Ca<sup>2+</sup> channels could be linked specifically to the release process.

### Effect of calcium buffers

The observation that several action potentials were effective in causing Ca<sup>2+</sup> release when one or two were not suggests that a threshold [Ca<sup>2+</sup>]<sub>i</sub> level might be needed to trigger release. If this hypothesis is correct, then buffering the peak [Ca<sup>2+</sup>]<sub>i</sub> level with high concentrations of indicator (Helmchen et al., 1996) will make it more difficult to evoke release. Indeed, we found that 1.0 mM bis-fura-2 prevented synaptically evoked release, whereas 0.3 mM did not (Nakamura et al., 1999a). In new experiments we found that the same conclusion applied to spike-evoked release in the presence of t-ACPD (Fig. 9).

Release measured in the presence of different buffers also can reveal information about whether Ca<sup>2+</sup> ions acting on particular IP<sub>3</sub>Rs diffuse far from their source (either Ca<sup>2+</sup> channels or other IP<sub>3</sub>Rs) or whether the receptors and channels are close to each other. Because the rate constants for Ca<sup>2+</sup> binding to EGTA are much slower than the rate constants for binding to BAPTA, a large difference in the effectiveness of these two buffers has been taken to indicate that Ca<sup>2+</sup> does not go far before activating its target (Adler et al., 1991). To test this possibility, we included EGTA and BAPTA in the recording pipette at different concentrations. No Ca<sup>2+</sup> was added except for trace amounts. We also included a low concentration of fura-6F to monitor the [Ca<sup>2+</sup>]<sub>i</sub> change. This low-affinity indicator ( $K_D \sim 6 \mu$ M) has only a weak buffering effect on the spike-evoked [Ca<sup>2+</sup>]<sub>i</sub> increase. Figure 9 shows that the effective concentration of BAPTA and EGTA differed by only a factor of two. This result suggests that the source and target of Ca<sup>2+</sup> in the release process are not very close to each other.

### Pharmacology of mGluRs

In previous experiments (Nakamura et al., 1999a) we showed that synaptically activated Ca<sup>2+</sup> release could be blocked by 1 mM MCPG, a group I and II mGluR antagonist (Schoepp et al., 1999). To determine more precisely the receptor types participating in the release process, we tested a series of known mGluR agonists and antagonists. For the agonists we determined the minimum concentration that reliably evoked release after a train of 10–20 action potentials. We found that 20  $\mu$ M t-ACPD ( $n = 28$ ), 5  $\mu$ M DHPG ( $n = 6$ ), and 500  $\mu$ M CHPG ( $n = 9$ ) were effective (Table 1). Of

**Table 1. Effective concentrations of mGluR agonists and antagonists in controlling Ca<sup>2+</sup> release**

	Compound	Putative selectivity	Effective concentration
Agonists	t-ACPD	Groups I and II	20 $\mu$ M
	DHPG	Group I	5 $\mu$ M
	CHPG	mGluR <sub>5</sub>	500 $\mu$ M
Antagonists <sup>a</sup>	MCPG	Groups I and II	1 mM
	AIDA	Group I	1 mM
	4-CPG	Group I	500 $\mu$ M
	MPEP	mGluR <sub>5</sub>	3 $\mu$ M
	LY367385	mGluR <sub>1</sub>	100 $\mu$ M
Antagonists <sup>b</sup>	AIDA	Group I	1 mM
	LY367385	mGluR <sub>1</sub>	100 $\mu$ M
	MPEP	mGluR <sub>5</sub>	3 $\mu$ M

<sup>a</sup>Against release evoked by 30  $\mu$ M t-ACPD.<sup>b</sup>Against release evoked by 500  $\mu$ M CHPG.

these, CHPG is reportedly the most selective agonist, acting preferentially on mGluR<sub>5</sub> receptors (Doherty et al., 1997).

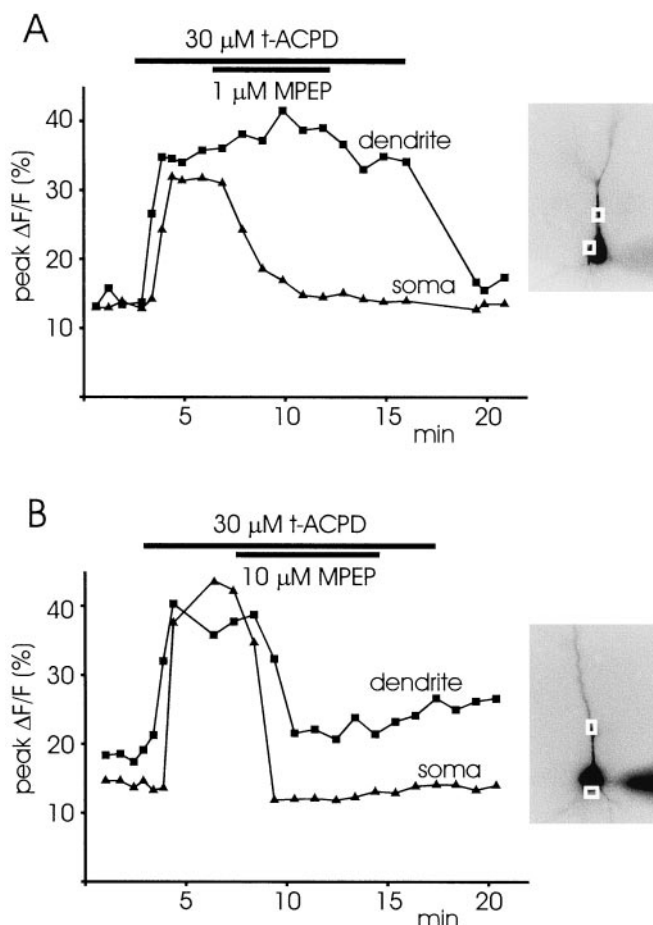
We found that the threshold agonist concentration capable of evoking spike-associated release was not uniform in all regions of the cell. When we used 0.5 mM CHPG, release was detected only in the dendrites ( $n = 6$  of 7). However, 1.0 mM CHPG evoked release in both the dendrites and soma ( $n = 7$  of 8; data not shown). Similarly, 20  $\mu$ M t-ACPD preferentially evoked release only in the dendrites ( $n = 16$  of 29), whereas 30  $\mu$ M t-ACPD consistently evoked release in both locations ( $n = 29$  of 30).

We next tested the ability of various mGluR antagonists to block spike-evoked release in the presence of 30  $\mu$ M t-ACPD. As with the agonists, we found that the sensitivity to the antagonists was not spatially uniform. Figure 10*A* shows that 1  $\mu$ M MPEP blocked release in the soma but did not block release in the dendrites ( $n = 3$  of 3). However, 3–10  $\mu$ M MPEP blocked release at both locations (Fig. 10*B*;  $n = 6$  of 6). This differential sensitivity to antagonists in the soma and dendrites, measured at threshold concentrations, also was observed with AIDA ( $n = 5$  of 7 blocked only in the soma at 1 mM;  $n = 4$  of 4 blocked at both locations at 2 mM) and 4-CPG ( $n = 1$  of 1 blocked only in the soma at 500  $\mu$ M;  $n = 2$  of 2 blocked at both locations at 1 mM). Table 1 summarizes the results for MCPG, AIDA, 4-CPG, MPEP, and LY367385. Because MPEP is reportedly an mGluR<sub>5</sub> antagonist (Gasparini et al., 1999) and LY367385 is reportedly an mGluR<sub>1</sub> antagonist (Clark et al., 1997), these results suggest that both mGluR<sub>1</sub> and mGluR<sub>5</sub> receptors are coupled to IP<sub>3</sub> mobilization on CA1 pyramidal neurons.

Some agonists and antagonists, like MPEP (Gasparini et al., 1999), are reported to be very selective in distinguishing between mGluR<sub>1</sub> and mGluR<sub>5</sub> receptors. Others (Schoepp et al., 1999) are less selective. To examine this selectivity, we tested several antagonists for their ability to block the release induced by the putative mGluR<sub>5</sub> agonist CHPG. Table 1 shows that AIDA, MPEP, and LY367385 were as effective against 0.5  $\mu$ M CHPG-induced release as they were against 30  $\mu$ M t-ACPD-induced release. Because LY367385 is considered a mGluR<sub>1</sub> antagonist, this result implies that either or both LY367385 and CHPG cannot be completely selective for their putative receptor subtypes. Consequently, these pharmacological experiments, by themselves, cannot establish which of the two group I receptors are most prominent on CA1 pyramidal neurons.

### Other metabotropic agonists

The experiments with t-ACPD established that spike-evoked release of Ca<sup>2+</sup> from internal stores is mediated by IP<sub>3</sub> produced by activation of mGluR. Because other neurotransmitters are known to activate receptors that mobilize IP<sub>3</sub>, we examined whether these transmitters could participate in spike-evoked Ca<sup>2+</sup> release in pyramidal neurons. Figure 11 shows the effect of carbachol (CCh),

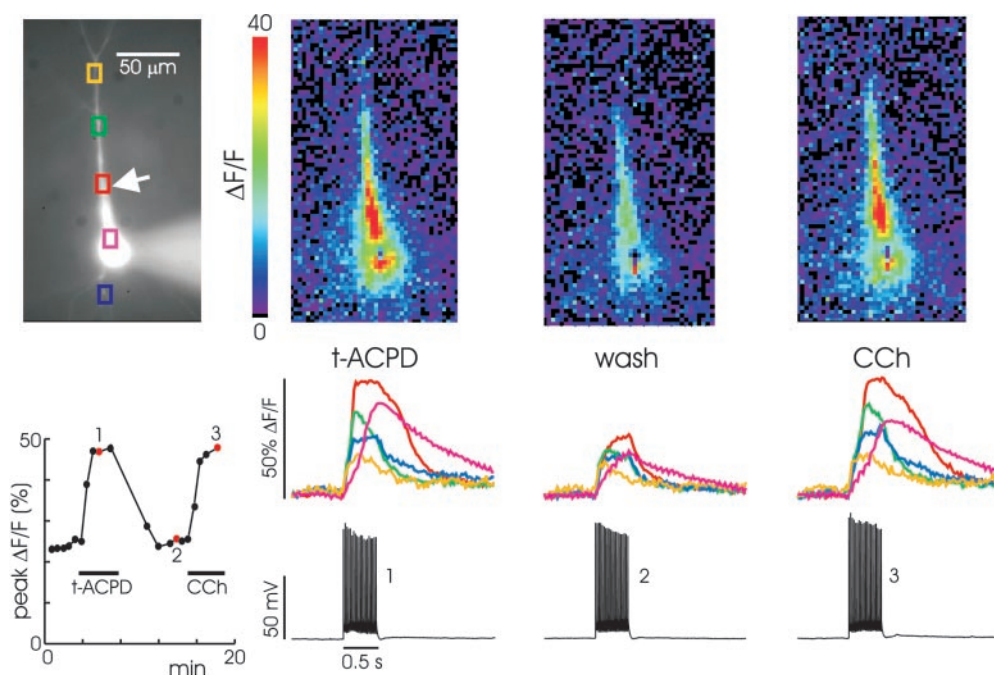


**Figure 10.** MPEP has a differential effect in blocking release in the soma and dendrites when it is applied at low concentration. *A*, [Ca<sup>2+</sup>]<sub>i</sub> increases in response to a train of backpropagating spikes are shown at two locations, one at the soma and one in the dendrites. When 30  $\mu$ M t-ACPD was added to the ACSF, a secondary increase of larger amplitude was observed at both locations. When 1  $\mu$ M MPEP was added to this solution, the secondary increase in the soma was blocked, but the increase in the dendrites remained. *B*, A similar experiment in which 10  $\mu$ M MPEP was applied. In this case the secondary increase was blocked at both locations.

a nonhydrolyzable analog of acetylcholine. In this cell 3  $\mu$ M CCh evoked release with approximately the same amplitude and spatial distribution as 20  $\mu$ M t-ACPD ( $n = 3$ ). The effect of 3  $\mu$ M ( $n = 10$ ) or 10  $\mu$ M ( $n = 11$ ) CCh was very reliable, producing spike-evoked release in 20 of 21 cells. However, 3  $\mu$ M was close to threshold, because 1  $\mu$ M did not cause release ( $n = 6$ ). CCh probably acts via M<sub>1</sub> receptors (Hammer and Giachetti, 1984) because release was blocked by atropine (1  $\mu$ M,  $n = 3$ ; 3  $\mu$ M,  $n = 4$ ; 10  $\mu$ M,  $n = 2$ ) and pirenzepine (0.5  $\mu$ M,  $n = 3$ ; data not shown). M<sub>1</sub> receptors couple to IP<sub>3</sub>Rs (Hulme et al., 1990). Consistent with this conclusion and with the similarity to t-ACPD-induced release, we found that including low-molecular-weight heparin in the pipette (1 mg/ml) blocked release in the presence of 10  $\mu$ M CCh ( $n = 3$ ). In addition, release mediated by including 3-F-IP<sub>3</sub> in the pipette occluded the release mediated by 3  $\mu$ M CCh ( $n = 2$ ), similar to the results with t-ACPD. Therefore, it is likely that both t-ACPD and CCh release Ca<sup>2+</sup> from the same compartment in the same IP<sub>3</sub>-dependent manner. Some evidence for the coupling of voltage-dependent Ca<sup>2+</sup> entry and muscarinic activation in pyramidal neurons has been reported by other investigators (Irving and Collingridge, 1998; Sah, 1999; Yamamoto et al., 2000).

We tested several other neurotransmitters that are known to couple to IP<sub>3</sub> mobilization in other preparations. Figure 12 shows typical experiments in which we tested 30 or 100  $\mu$ M phenylephrine (Phe, an  $\alpha$ -adrenergic agonist;  $n = 5$ ), 100 or 300  $\mu$ M dopamine (DA;  $n = 7$ ), and 10  $\mu$ M 5-HT. In each case the agonists did not





**Figure 11.** CCh and *t*-ACPD evoke spike-associated Ca<sup>2+</sup> release of similar amplitude and spatial distribution in pyramidal cells. In the presence of 30  $\mu$ M *t*-ACPD a train of 20 spikes at 30 msec intervals evoked a secondary increase in the soma and proximal apical dendrites (*panel 2*). The colored traces show the time-dependent increases at the indicated locations on the cell image (*panel 1*). The pseudocolor image shows the spatial distribution of the [Ca<sup>2+</sup>]<sub>i</sub> increase at the end of the spike train. *Panel 3* shows the traces and spatial distribution of the [Ca<sup>2+</sup>]<sub>i</sub> increase after *t*-ACPD was washed from the bath. *Panel 4* shows the traces and distribution after 3  $\mu$ M CCh was added. The traces and spatial distribution are similar to those observed in *t*-ACPD. The graph on the left shows the peak amplitude of the [Ca<sup>2+</sup>]<sub>i</sub> change recorded at the location of the white arrow during the entire course of the experiment. The events at times 1, 2, and 3 are shown in the three panels.

cause Ca<sup>2+</sup> release from intracellular stores. Subsequent application of either 10  $\mu$ M CCh or 30  $\mu$ M *t*-ACPD did evoke release, demonstrating that the ER was intact and capable of release.

The experiments with 5-HT were interesting. Although most cells did not respond to 5-HT, in six experiments we found that spikes evoked Ca<sup>2+</sup> release in ACSF containing 10  $\mu$ M 5-HT (Fig. 13; *n* = 6 of 35). This [Ca<sup>2+</sup>]<sub>i</sub> increase initiated and propagated as a wave in the proximal apical dendrites, similar to the release transients observed with *t*-ACPD and CCh. Increasing the concentration of 5-HT to 30  $\mu$ M did not increase the probability of observing release (*n* = 0 of 3). Tests with 100  $\mu$ M  $\alpha$ -methyl-5-HT, a selective 5-HT<sub>2</sub> receptor agonist known to mobilize IP<sub>3</sub> (Richardson et al., 1985), also caused release in only a fraction of the tested cells (*n* = 1 of 5). Because the effect of 5-HT was unreliable, we could not analyze its pharmacology nor the spatial distribution of release in more detail.

## DISCUSSION

These experiments explore the effects of bath-applied and pipette-injected pharmacological agents on the spike-associated release of Ca<sup>2+</sup> from intracellular stores. Although the bath-applied agents affected the entire hippocampal slice, several arguments suggest that the major effects were directly on the tested pyramidal neurons and not mediated via other cells. First, all of the measured properties of release evoked by metabotropic agonists matched those of synaptically activated release (Nakamura et al., 1999a). In those experiments the close correspondence of the site of release to the site of synaptic activation indicated that the effect was mediated directly by glutamate released from stimulated Schaffer axon collaterals and not via intermediate cells. Second, astrocytes primarily release glutamate when activated (Parrup et al., 1994). If these cells or other glutamatergic neurons were stimulated in our experiments, their effects on pyramidal neurons would have been overwhelmed by the bath-applied glutamate agonists. Third, the pharmacological profile of effective agonists (CHPG and CCh, but not Phe and DA) matches the profile of known receptor types on CA1 pyramidal neurons (see below).

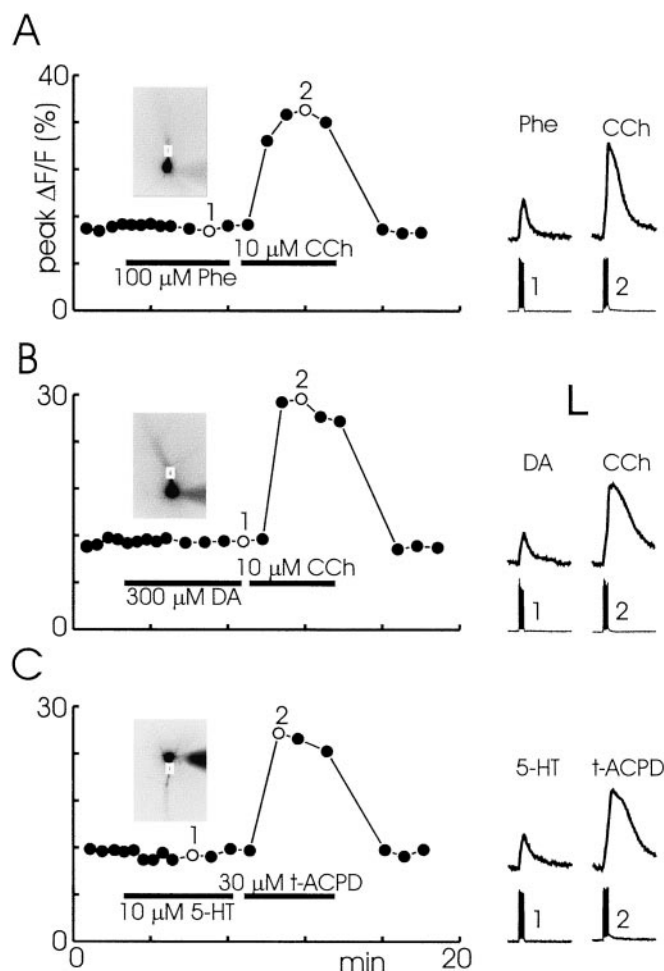
## Mechanism of release

The properties of agonist-induced regenerative [Ca<sup>2+</sup>]<sub>i</sub> increase are consistent with our analysis of synaptically activated Ca<sup>2+</sup> waves (Nakamura et al., 1999a). These results indicate that the activation of ryanodine-sensitive stores is not necessary to evoke

release, although ryanodine receptors are present on the compartment that releases Ca<sup>2+</sup>. In new experiments we showed that the injection of adenophostin A, 3-F-IP<sub>3</sub>, and IP<sub>3</sub> could promote spike-associated release without the need for metabotropic agonists. The effectiveness of these specific IP<sub>3</sub>R agonists is more direct evidence for the involvement of the IP<sub>3</sub>R. In addition, pharmacological experiments indicate that release was promoted by group I mGluRs, M<sub>1</sub> muscarinic receptors, and 5-HT<sub>2</sub> receptors. All of these receptors are coupled to the mobilization of IP<sub>3</sub> (McKinney, 1993; Conn and Pin, 1997; Hoyer and Martin, 1997). One caution is that high concentrations of IP<sub>3</sub>R agonists were required to promote release (100  $\mu$ M 3-F-IP<sub>3</sub> and 500  $\mu$ M IP<sub>3</sub>). These levels are higher than are needed to evoke release in other preparations (Hirota et al., 1995; Missiaen et al., 1996). This difference could mean that the IP<sub>3</sub>R in pyramidal neurons are different from receptors in other preparations or that intracellular kinases or other degradative enzymes rapidly metabolize the injected IP<sub>3</sub>R agonists. However, all of the injected IP<sub>3</sub>R agonists occluded the large-amplitude spike-associated release mediated by bath-applied metabotropic agonists. This result suggests that the exogenously applied IP<sub>3</sub>R agonists also might be exerting some inhibitory effect on the release mechanism. We have no additional information concerning this possibility.

Spike-evoked release reached levels of several micromoles per liter when measured with low-affinity indicators. This suggests that resting [Ca<sup>2+</sup>]<sub>i</sub> levels in the stores are in the micromolar per liter range, consistent with indicator measurements in other preparations (Miyawaki et al., 1997). We found that large [Ca<sup>2+</sup>]<sub>i</sub> increases were observed consistently when short trains of spikes were evoked at 30–60 sec intervals, but lower amplitudes were recorded when they were stimulated at 10–20 sec intervals. The simplest explanation of this result is that the stores require ~20 sec to refill after regenerative release. This time is comparable to the recovery time that is observed in these cells after store depletion by caffeine puffs (Garaschuk et al., 1997). However, we cannot rule out the possibility that the IP<sub>3</sub>Rs are inactivated or desensitized for brief periods after stimulation (Hajnoczky and Thomas, 1994).

Spike-evoked release in the presence of *t*-ACPD was most prominent in the proximal apical dendrites (Nakamura et al., 1999a). Two new kinds of measurements reinforce the conclusion that this part of the cell is particularly sensitive. First, we found that threshold levels of mGluR agonists caused release only in the proximal

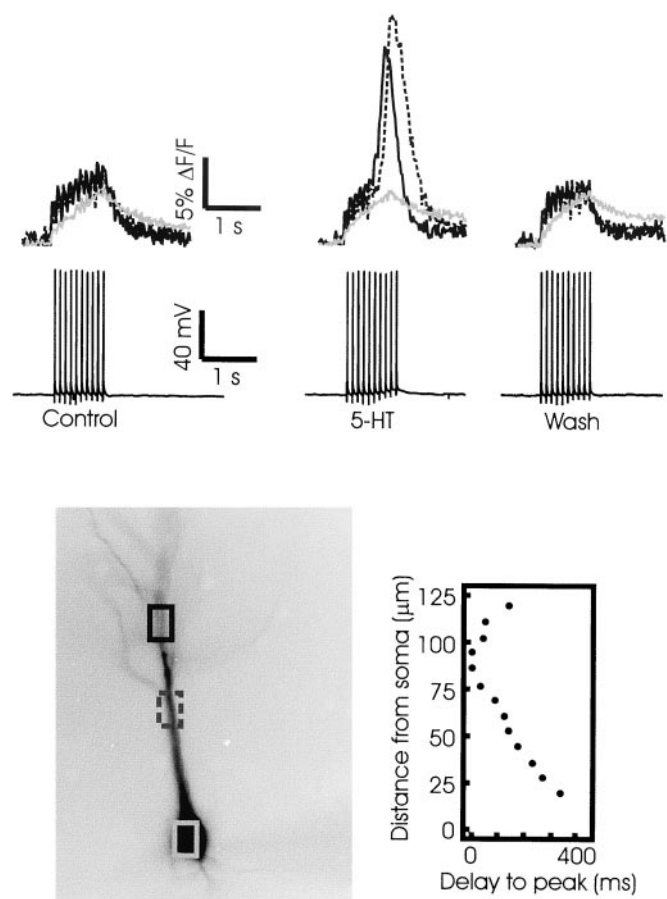


**Figure 12.** The metabotropic agonists Phe, DA, and 5-HT do not evoke spike-associated Ca<sup>2+</sup> release in pyramidal neurons. *A*, In the presence of 100  $\mu\text{M}$  Phe a train of 10 backpropagating action potentials at 30 msec intervals evoked only a small spike-linked  $[\text{Ca}^{2+}]_i$  increase. Trials were repeated at 30 sec intervals. One example (1) is shown at the right. When Phe was washed out and replaced by 10  $\mu\text{M}$  CCh, the same train evoked Ca<sup>2+</sup> release (2). Scale under the traces: Ordinate, 10%  $\Delta F/F$ , 50 mV; abscissa, 1 sec. *B*, A similar experiment showing that 300  $\mu\text{M}$  DA did not cause Ca<sup>2+</sup> release. *C*, A similar experiment showing that in most cases 10  $\mu\text{M}$  5-HT did not cause release.

apical dendrites, whereas higher concentrations also caused release in the soma. Mirroring this finding, we found that threshold levels of antagonists selectively blocked release in the soma, sparing the dendrites, whereas higher concentrations blocked release everywhere. In addition, when a single initiation site was detected (see, for example, Fig. 1), it was always in the proximal apical dendrites. Second, we found that the same spatial distribution was determined with all of the agonists that evoked release—group I mGluR, M<sub>1</sub> muscarinic, and 5-HT<sub>2</sub>. This similarity suggests that the distribution of metabotropic receptors on the surface of the pyramidal neuron is not responsible for the spatial distribution of regenerative release, because this would require the same nonuniform distribution for all receptors. The distribution of Ca<sup>2+</sup> channels is also unlikely to be responsible because (1) selective block of different Ca<sup>2+</sup> channel types did not affect the pattern of release and (2) the same pattern was observed without spikes when the release was evoked synaptically (Nakamura et al., 1999a). The more likely explanations are either that IP<sub>3</sub>Rs are distributed more densely on the ER in the proximal apical dendrites or that the IP<sub>3</sub>Rs are more sensitive in this region of the cell (Thomas et al., 2000).

#### Receptors contributing to spike-evoked Ca<sup>2+</sup> release

Using specific agonists and antagonists, we analyzed the receptors contributing to the regenerative  $[\text{Ca}^{2+}]_i$  increase. Previously (Na-



**Figure 13.** 5-HT sometimes evokes spike-associated Ca<sup>2+</sup> release in pyramidal cell dendrites. In control conditions a train of 10 spikes at 100 msec intervals evoked spike-linked  $[\text{Ca}^{2+}]_i$  increases in the soma and along the dendrite. When 10  $\mu\text{M}$  5-HT was added to the ACSF, the spikes evoked a large secondary increase in the dendrites that peaked at different times at the two marked locations. The graph to the right of the cell image shows the delay-to-peak of the secondary release along the cell axis. Time 0 is the peak time at the initiation site. The length scale (ordinate) also applies to the cell image. The increase occurred earliest at a point  $\sim 90 \mu\text{m}$  from the soma and then spread as a wave in both directions from that point. Washing out the 5-HT restored the transients to the control response.

kamura et al., 1999a), we found that t-ACPD, a relatively nonselective mGluR agonist, reliably evoked release. In these new experiments we found that DHPG, a group I agonist, and CHPG, a mGluR<sub>5</sub> agonist, also evoked release. These results are expected because pyramidal neurons express mGluR<sub>5</sub> receptors (Shigemoto et al., 1993; Romano et al., 1995; Lujan et al., 1996). Consistent with these observations, we found that the phenylglycine-related antagonists MCPG, AIDA, and 4-CPG blocked release evoked by t-ACPD. These compounds block both group I receptors, although their relative affinity for mGluR<sub>1</sub> and mGluR<sub>5</sub> varies (Schoepp et al., 1999). We were not able to determine whether only one of these subtypes releases Ca<sup>2+</sup> in pyramidal neurons, because some of the pharmacological agents were not as specific as previously reported. Nevertheless, the presence of either receptor on pyramidal neurons is consistent with our other results because both receptor subtypes are known to couple to IP<sub>3</sub> mobilization (Schoepp et al., 1999).

Experiments with other neurotransmitters are consistent with these results and with the known distribution of receptors on pyramidal neurons. CCh and 5-HT evoked spike-associated release with the same spatial distribution as was evoked with mGluR agonists, suggesting that the spatial distribution of release was not determined by the distribution of the transmitter receptors on the surface of pyramidal neurons. Pharmacological experiments indicated that both of these agonists acted on receptors coupled to IP<sub>3</sub> mobilization. The effect of CCh was reliable, consistent with the



strong expression of M<sub>1</sub> receptors on pyramidal neurons (Rouse et al., 1999). In contrast, release evoked by 5-HT was observed in only a fraction of cells. Neither DA nor Phe evoked release in pyramidal neurons, although these transmitters are known to mobilize IP<sub>3</sub> in other systems. The weak effect of these three agents may be related to the low expression level of the matching receptors or mRNAs in the CA1 region (Wright et al., 1995; Day et al., 1997; Khan et al., 1998). However, the density of these receptors on pyramidal neurons has not been determined carefully at the electron microscope level.

### Relative localization of critical molecules

The configuration of mGluR receptors, Ca<sup>2+</sup> channels, and IP<sub>3</sub>Rs can affect the sensitivity and specificity of the signaling process underlying the release of Ca<sup>2+</sup> from intracellular stores. In other cell types a close relationship among some of these components has been described. In muscle and some neurons, L-type Ca<sup>2+</sup> channels and ryanodine receptors are thought to be close to each other, allowing Ca<sup>2+</sup> entry through the L-type channels to activate the ryanodine receptors preferentially (Chavis et al., 1996). In CA1 pyramidal cells, group I mGluR receptors and IP<sub>3</sub>Rs are linked by Homer, a protein enriched at the postsynaptic density (Tu et al., 1998), suggesting that these IP<sub>3</sub>Rs are positioned appropriately to detect the IP<sub>3</sub> mobilized by mGluR activation. In addition, there is conflicting evidence from different preparations that IP<sub>3</sub>Rs may be very close to each other in certain regions of the ER. Several of our experiments provide data that can be used to explore these issues.

We found no evidence that specific Ca<sup>2+</sup> channel types were linked closely to IP<sub>3</sub>Rs. Cd<sup>2+</sup>, a general Ca<sup>2+</sup> channel blocker, prevented almost all Ca<sup>2+</sup> entry and prevented Ca<sup>2+</sup> release. No specifically tested channel blocker (nimodipine, Ni<sup>2+</sup>,  $\omega$ -conotoxin-GVIA, or  $\omega$ -agatoxin-IVA) by itself prevented release. However, the Cd<sup>2+</sup> results suggest that a cocktail of blockers might have been effective. We did not test this hypothesis. In addition, the possibility that R-type channels might be linked to IP<sub>3</sub>Rs was not examined.

Most models of IP<sub>3</sub>-mediated Ca<sup>2+</sup> waves postulate that Ca<sup>2+</sup> released through IP<sub>3</sub>Rs contributes to the activation of nearby IP<sub>3</sub>Rs (Lechleiter and Clapham, 1992; Wang and Thompson, 1995). In their analysis of waves in neuroblastoma cells, Wang and Thompson (1995) found that BAPTA was much more effective than EGTA in suppressing the waves. Consequently, they concluded that the feedback from one IP<sub>3</sub>R to the next must be local, with receptors separated from each other by 1  $\mu$ m or less. In our experiments in which these two buffers were loaded through the pipette, we found that the threshold for suppressing Ca<sup>2+</sup> release was only two times higher for EGTA than for BAPTA. This approximately equal value, despite the difference in rate constants for Ca<sup>2+</sup> binding (Naraghi, 1997), suggests that in pyramidal neurons the IP<sub>3</sub>Rs are further apart. This more widespread separation of IP<sub>3</sub>Rs is consistent with results in *Xenopus* oocytes. In those cells Marchant et al. (1999) found that Ca<sup>2+</sup> was released in "puffs" from clusters of IP<sub>3</sub>Rs that were at least several micrometers apart. It is also possible that EGTA is buffering Ca<sup>2+</sup> as it diffuses from the Ca<sup>2+</sup> channels to the IP<sub>3</sub>Rs. These experiments do not distinguish between these two possibilities.

It is well established that both EGTA (Lynch et al., 1983) and BAPTA (Huang and Malenka, 1993), injected postsynaptically, can prevent the induction of LTP by tetanic stimulation, whereas only BAPTA can block some other postsynaptic processes (Deisseroth et al., 1996). If regenerative release of Ca<sup>2+</sup> from intracellular stores is an important step in the induction of LTP, then our experiments may explain the effectiveness of both of these buffers in blocking it.

Physiological evidence concerning the spatial relationship between mGluRs and IP<sub>3</sub>Rs is much weaker. In our previous experiments (Nakamura et al., 1999a) we found that spikes had to occur within ~0.5 sec from the time of synaptic activation of mGluR. Even if IP<sub>3</sub> is mobilized instantaneously, this result suggests that IP<sub>3</sub> is metabolized rapidly in pyramidal cells. Therefore, IP<sub>3</sub> cannot diffuse far from its source before activating IP<sub>3</sub>Rs. From this perspective the close association of mGluRs and IP<sub>3</sub>Rs mediated

by Homer ensures the effectiveness of IP<sub>3</sub> before it is metabolized. We do not know whether there is a parallel mechanism associating muscarinic receptors and IP<sub>3</sub>Rs.

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