Inositol 1,4,5-Trisphosphate (IP$_3$)-Mediated Ca$^{2+}$ Release Evoked by Metabotropic Agonists and Backpropagating Action Potentials in Hippocampal CA1 Pyramidal Neurons

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We examined the properties of [Ca$^{2+}$]i 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$^{2+}$ 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$^{2+}$ release from internal stores mediated by the synergistic effect of receptor-mobilized inositol 1,4,5-trisphosphate (IP$_3$) and spike-evoked Ca$^{2+}$. The amplitude of the changes reached several micromoles per liter when detected with the low-affinity indicators fura-6F, fura-2-FF, or furaptra. 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$^{2+}$ blocked release, but no single selective Ca$^{2+}$ channel blocker prevented release. These results suggest that IP$_3$ receptors are not associated closely with specific Ca$^{2+}$ channels and are not close to each other.

Key words: pyramidal neuron; dendrite; IP$_3$ receptor; metabotropic receptor; BAPTA; EGTA; carbachol; bis-fura-2; furaptra; endoplasmic reticulum

In addition to these mechanisms, we recently showed that action potential-associated [Ca$^{2+}$]i changes could be enhanced by pairing backpropagating spikes with repetitive synaptic activation (Nakamura et al., 1999a). The enhanced [Ca$^{2+}$]i increase results from the synergistic action of Ca$^{2+}$ entering through spike-opened Ca$^{2+}$ channels and metabotropic glutamate receptor (mGluR)-mobilized inositol 1,4,5-trisphosphate (IP$_3$) acting on IP$_3$ receptors (IP$_3$Rs) to release Ca$^{2+}$ 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$^{2+}$ by action potentials in pyramidal neurons (Nakamura et al., 1999a). Because other metabotropic agonists coupled to IP$_3$ 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$^{2+}$ 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$^{2+}$ release? Which Ca$^{2+}$ channels opened by action potentials promote release? What kinds of mechanisms are responsible for the preferred release of Ca$^{2+}$ in the proximal apical dendrites? How close in space are the Ca$^{2+}$ channels to the IP$_3$R channels and how close together are different IP$_3$Rs to each other? What levels of [Ca$^{2+}$]i, and IP$_3$ are required to cause regenerative release? These new experiments explore some of these questions.

Parts of this work have been published previously in abstract...
form (Ross and Sandler, 1998; Barbara et al., 1999; Nakamura et al., 1999b, 2000).

**MATERIALS AND METHODS**

Transverse hippocampal slices (300 μm thick) were prepared from 2- to 3-week-old Sprague Dawley rats as previously described (Tsubokawa and Ross, 1998; 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, 1.3 CaCl₂, 2 MgCl₂, 1.25 Na₂HPO₄, 26 NaHCO₃, and 10–20 glucose, bubbled with a mixture of 95% O₂/5% CO₂, 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 Stuarte, 1995; Nakamura et al., 1999b). 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 Na₂-phosphocreatine. This solution was supplemented with 0.1–1.0 mM of a selected calcium indicator (fura-2, bis-fura, furaptra, fura-6F, fura-2-FF), 13) in the first few minutes after the solution change. At later times osmolarity was 300 mOsm. The other compounds were added directly to the ACSF or experiments this solution was diluted 100 times with ACSF. Control experiments showed that 0.002% ascorbic acid in ACSF did not change the osmolarity. 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 changes in 

**RESULTS**

**Spike-evoked Ca²⁺ release in the presence of t-ACPD**

Backpropagating action potentials induce [Ca²⁺], increases at all pyramidal cell locations by opening voltage-sensitive Ca²⁺ channels in the dendrites (Jaffe et al., 1992). This is illustrated in the first column of Figure 1, which shows the [Ca²⁺] change that is associated with a pair of action potentials evoked by brief intrasomatic pulses. The rapid rise in [Ca²⁺] 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 μM CNQX and 100 μM AP-5 to the ACSF to block ionotropic glutamate receptors in all of the experiments described in this paper. When 30 μM t-ACPD was added to this solution and superfused into the bath, the cell depolarized 5.5 ± 0.3 mV (n = 21). The resting fluorescence of the bis-fura-2-filled cell, at dendritic locations within 30 μm from the soma, decreased by 3.8 ± 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 a single excitation wavelength. Assuming that the resting [Ca²⁺], in the cell was ~100 nM and that the KD for bis-fura-2 binding to Ca²⁺ is 370 nM (Molecular Probes catalogue), then this fluorescence decrease corresponds to an increase in [Ca²⁺], of <20 nM (Lev-Ram et al., 1992). This small increase could result from entry through voltage-sensitive Ca²⁺ 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 [Ca²⁺] increases are recorded. However, these increases were not observed in our experiments in which the bath composition was changed slowly by being superfused at ~1 ml/min.

In the presence of 30 μM t-ACPD the backpropagating spikes evoked a secondary increase in [Ca²⁺], 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 that initiated at a location ~50 μm 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.
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 μm of the soma. However, these waves did not propagate basally >20 μm from the soma. When five action potentials were stimulated (Fig. 1, third column), the initial sharp [Ca^{2+}] 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+}] increases, we added TTX (1.0 μM) to the superfuse (Fig. 2). In this solution the action potentials were blocked, the rapid spike-evoked [Ca^{2+}] 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+}] 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+}] increases (often >50% ΔF/F, using 100–300 μM 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 (Schneegensburger et al., 1999) and furaptra (Naraghi, 1997). For each indicator the figure shows representative fluorescence changes recorded from a small region ~30 μm from the soma (fura-2, n = 9; bis-fura-2, n > 100; fura-6F, n = 68; fura-2-FF, n = 2; furaptra, 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+}] 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 the high-affinity fura-2 and the low-affinity furaptra. In the presence of 30 μM 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 furaptra. None of these responses appeared to saturate the indicators. The larger relative response with furaptra (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 furaptra corresponds to a [Ca^{2+}] increase of ~3 μM (Lev-Ram et al., 1992; Nakamura et al., 1999a). The 1% increase in normal ACSF corresponds to a change of ~0.4 μM. 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+}] changes of similar amplitude. Figure 4 shows representative experiments that used 300 μM 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 μM t-ACPD added to the bath. In normal ACSF 10 action potentials evoked a peak increase of 4% ΔF/F in a region in the proximal apical dendrites. In t-ACPD the spikes evoked...
Ca\textsuperscript{2+} release, and the peak fluorescence increase was 18–20%. After the baseline response was established, in each of the panels (Fig. 4A–D) the interval between trials was varied. When the interval was 10 sec (Fig. 4A), the magnitude of the fluorescence increase was reduced by 74.6 ± 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 μm 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 intertrial intervals showed less of a reduction. At 20 sec intervals the peak was reduced 53.5 ± 4.0% (n = 4); at 30 sec intervals the peak was reduced 23.5 ± 7.8% (n = 4); at 60 sec intervals the peak was reduced by 15.3 ± 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\textsuperscript{2+} in this interval or that the receptors on ER did not recover from inactivation (Hajnoczky and Thomas, 1994). Whichever the explanation, these experiments show that the recovery time constant after spike-evoked regenerative release is ~30 sec.

These experiments also suggest that the Ca\textsuperscript{2+} entry replenished Ca\textsuperscript{2+} 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 μM 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\textsuperscript{2+} release was attributable to mGluR-activated IP\textsubscript{3} synergistically acting with Ca\textsuperscript{2+} entry through spike-activated Ca\textsuperscript{2+} channels to open IP\textsubscript{3}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 5A shows that spike-evoked release was blocked by 20 μM CPA (n = 6), which blocks the ER Ca\textsuperscript{2+}-ATPase (Seidler et al., 1989). Release also was blocked by 10 μM ryanodine (Fig. 5B; 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 μM ryanodine (Fig. 5C; 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\textsubscript{3}R nonselectively 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\textsubscript{3}-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\textsuperscript{2+}-ATPase (Thastrup et al., 1990). Preincubation of the slice with 3 μM 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\textsubscript{3}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\textsuperscript{2+}] increase in normal ACSF without altering the time course of the [Ca\textsuperscript{2+}] 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...
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. 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 \(\Delta F/\Delta F\) 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.

To reinforce the conclusion that release was mediated by the activation of IP\(_{3}\)Rs, we tested several compounds that are relatively specific activators of this receptor. Adenophostin A at low concentrations is a potent IP\(_{3}\)R agonist (Takahashi et al., 1994). When 0.3 \(\mu\)M adenophostin A was included in the 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\(_{3}\) 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\(_{3}\) was included in the pipette. IP\(_{3}\) also caused a secondary [Ca\(^{2+}\)]\(_{i}\), increase that was blocked by 10 mM caffeine. Scale in cell image, 50 \(\mu\)m.
evoked release (Fig. 6A, trace 3). Figure 6B shows similar experiments that used 100 μM 3-F-IP3 in the pipette instead of adenosphin A (n = 3). This analog of IP3, resistant to 3-kinase, is equipotent to IP3 in releasing Ca2+ from stores in other preparations (Kozikowski and Fauq, 1990). Finally, IP3 itself, at a concentration of 500 μM in the pipette, also caused Ca2+ release when it was paired with a train of action potentials (Fig. 6C; n = 6). These concentrations of agonists were close to the threshold concentrations for this protocol because 0.1 μM adenosphin A (n = 3), 30 μM 3-F-IP3 (n = 5), and 300 μM IP3 (n = 5) did not cause spike-associated Ca2+ release. Together, these results strongly support the conclusion that release is mediated via the action of IP3 on the IP3R.

One difference between release mediated by tonic activation of mGluRs and release mediated by intracellular injection of IP3R agonists is that the amplitude of spike-associated Ca2+ release in the presence of intracellular IP3R agonists was smaller than the amplitude in the presence of t-ACPD. Increasing the concentration of IP3R agonists did not increase the amplitude of the [Ca2+]i increase. This result might suggest that the IP3R agonists are not activating the same release process as t-ACPD. However, injection of IP3R 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 μM adenosphin A, n = 5; 100 μM 3-F-IP3, n = 3; 500 μM IP3, n = 2; data not shown). Lower concentrations of IP3R agonists (0.1 μM adenosphin A, n = 2; 30 μM 3-F-IP3, n = 3; 300 μM IP3, 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 IP3R agonists is not clear. The IP3R 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 [Ca2+]i in the cells to act cooperatively with IP3 to open the IP3R channels. First, blocking Ca2+ entry with 200 μM Cd2+ strongly reduced the immediate [Ca2+]i increase and prevented the secondary [Ca2+]i 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 Ca2+ entry through voltage-dependent Ca2+ channels. This figure also shows that Cd2+ had no effect on the resting bis-fura-2 fluorescence intensity, indicating that Cd2+ 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 [Ca2+]i level reached in a train. In contrast, when furaptra was used, only one or two spikes were necessary. This low-affinity indicator does not buffer Ca2+ significantly and allows the peak free [Ca2+]i to reach higher levels. These experiments suggest that spikes produce a threshold level of [Ca2+]i that cooperatively acts with IP3 to trigger release.

Because the almost complete block of Ca2+ entry by Cd2+ was effective in preventing release, it was of interest to see whether entry through any of the major kinds of Ca2+ channels was particularly effective in inducing release. Figure 8 shows experiments in which we tested several antagonists that are relatively specific for different Ca2+ channel types. At 100 μM, Ni2+ preferentially blocks low-threshold T-type channels (Ozawa et al., 1989; Mogul and Fox, 1991). When superfused over the preparation, Ni2+-containing ACSF reduced the spike-evoked [Ca2+]i increase by 25.3 ± 1.1% (n = 3) as previously reported (Christie et al., 1995). However, with the addition of 30 μM t-ACPD the spikes still were able to evoke a large secondary [Ca2+]i increase (Fig. 8A; n = 4 of 5). Similar results were obtained with 1–5 μM omega-conotoxin-GVIA, a blocker of high-threshold N-type Ca2+ channels (McCleskey et al., 1987). This toxin reduced the sharp spike-evoked [Ca2+]i, increase by 26.7 ± 1.4% (n = 7) but did not prevent release (Fig. 8B).

Almost similar results were obtained with dihydropyridine antagonists of L-type Ca2+ channels. Nimodipine (Fig. 8C; 10–30 μM; n = 15), nifedipine (10 μM; n = 6), and nitrendipine (10 μM; n = 1) did not prevent spike-evoked Ca2+ release. Interestingly, none of these antagonists had a significant effect on the sharp spike-evoked [Ca2+]i increases in these slice experiments. This result differs from previous sharp electrode experiments that found an L-type channel component in spike-associated [Ca2+]i increases (Christie et al., 1995). Two control experiments confirmed that dihydropyridine-containing ACSF was capable of blocking L-type Ca2+ channels. In one, 10 μM nimodipine reduced the [Ca2+]i increase caused by a 1 sec depolarizing pulse (n = 3; data not shown). This rules out the possibility that many of the L-type Ca2+ 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
Ca$^{2+}$ channel blockers do not prevent spike-associated Ca$^{2+}$ release in the presence of 30 μM t-ACPD. A train of 10 back-propagating spikes evoked at 30 msec intervals caused a rapid [Ca$^{2+}$]i 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 (Fig. 8). The addition of 100 μM Ni$^{2+}$ 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$^{2+}$]i increase was observed (3). B–D. Similar experiments are shown with 1 μM ω-CTX-GVIA, 10 μM nimodipine, and 400 nM ω-Aga-IVA. None of these agents prevented release by t-ACPD (B, D) or CCh (C). ω-CTX-GVIA reduced the spike-associated [Ca$^{2+}$]i in normal ACSF, whereas nimodipine and ω-Aga-IVA did not. For the experiment with ω-Aga-IVA, fura-6F was the Ca$^{2+}$ indicator, resulting in faster transients.

Similar results were found with the P-type Ca$^{2+}$ channel blocker, ω-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$^{2+}$]i increase. To test the potency of our sample, we confirmed that ω-agatoxin-IVA could block the voltage-dependent [Ca$^{2+}$]i increase in rat cerebellar Purkinje cells (n = 2; data not shown). We did not test the possibility that R-type Ca$^{2+}$ channels could be linked specifically to the release process.

**Effect of calcium buffers**

The observation that several action potentials were effective in causing Ca$^{2+}$ release when one or two were not suggests that a threshold [Ca$^{2+}$]i level might be needed to trigger release. If this hypothesis is correct, then buffering the peak [Ca$^{2+}$]i 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 bis-fura-2 prevented release. Different concentrations of EGTA and BAPTA in the pipette were tested with electrodes also containing 100–200 μM 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.

**Pharmacology of mGluRs**

In previous experiments (Nakamura et al., 1999a) we showed that synaptically activated Ca$^{2+}$ 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 μM t-ACPD (n = 28), 5 μM DHPG (n = 6), and 500 μM CHPG (n = 9) were effective (Table 1). Of
these, CHPG is reportedly the most selective agonist, acting preferentially on mGluR5 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 μM t-ACPD preferentially evoked release only in the dendrites (n = 16 of 29), whereas 30 μM 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 μM t-ACPD. As with the agonists, we found that the sensitivity to the antagonists was not spatially uniform. Figure 10A shows that 1 μM MPEP blocked release in the soma but did not block release in the dendrites (n = 3 of 3). However, 3–10 μM MPEP blocked release at both locations (Fig. 10B; 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 μM; 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 mGluR5 antagonist (Gasparini et al., 1999) and LY367385 is reportedly an mGluR5 antagonist (Clark et al., 1997), these results suggest that both mGluR5 and mGluR3 receptors are coupled to IP3 mobilization on CA1 pyramidal neurons.

Some agonists and antagonists, like MPEP (Gasparini et al., 1999), are reported to be very selective in distinguishing between mGluR1 and mGluR5 receptors. Others (Schopp 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 agonist CHPG. Table 1 shows that AIDA, MPEP, and LY367385 were as effective against 0.5 μM CHPG-induced release as they were against 30 μM t-ACPD-induced release. Because LY367385 is considered a mGluR5 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 Ca2+ from internal stores is mediated by IP3Rs produced by activation of mGluR. Because other neurotransmitters are known to activate receptors that mobilize IP3s, we examined whether these transmitters could participate in spike-evoked Ca2+ release in pyramidal neurons. Figure 11 shows the effect of carbacol (CCh), a nonhydrolyzable analog of acetylcholine. In this cell 3 μM CCh evoked release with approximately the same amplitude and spatial distribution as 20 μM t-ACPD (n = 3). The effect of 3 μM (n = 10) or 10 μM (n = 11) CCh was very reliable, producing spike-evoked release in 20 of 21 cells. However, 3 μM was close to threshold, because 1 μM did not cause release (n = 6). CCh probably acts via M1 receptors (Hammer and Giachetti, 1984) because release was blocked by atropine (1 μM, n = 3; 3 μM, n = 4; 10 μM, n = 2) and pirenzepine (0.5 μM, n = 3; data not shown). M1 receptors couple to IP3Rs (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 μM CCh (n = 3). In addition, release mediated by including 3-F-IP3 in the pipette occluded the release mediated by 3 μM CCh (n = 2), similar to the results with t-ACPD. Therefore, it is likely that both t-ACPD and CCh release Ca2+ from the same compartment in the same IP3-dependent manner. Some evidence for the coupling of voltage-dependent Ca2+ 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 IP3 mobilization in other preparations. Figure 12 shows typical experiments in which we tested 30 or 100 μM phenylephrine (Phe, an α-adrenergic agonist; n = 5), 100 or 300 μM dopamine (DA; n = 7), and 10 μM 5-HT. In each case the agonists did not

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Table 1. Effective concentrations of mGluR agonists and antagonists in controlling Ca2+ release

<table>
<thead>
<tr>
<th>Compound</th>
<th>Putative selectivity</th>
<th>Effective concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Agonists</td>
<td></td>
<td></td>
</tr>
<tr>
<td>t-ACPD</td>
<td>Groups I and II</td>
<td>20 μM</td>
</tr>
<tr>
<td>DHPG</td>
<td>Group I</td>
<td>5 μM</td>
</tr>
<tr>
<td>CHPG</td>
<td>mGluR5</td>
<td>500 μM</td>
</tr>
<tr>
<td>Antagonistsa</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MCPG</td>
<td>Groups I and II</td>
<td>1 mM</td>
</tr>
<tr>
<td>AIDA</td>
<td>Group I</td>
<td>1 mM</td>
</tr>
<tr>
<td>4-CPG</td>
<td>Group I</td>
<td>500 μM</td>
</tr>
<tr>
<td>MPEP</td>
<td>mGluR5</td>
<td>3 μM</td>
</tr>
<tr>
<td>LY367385</td>
<td>mGluR1</td>
<td>100 μM</td>
</tr>
<tr>
<td>Antagonistsb</td>
<td></td>
<td></td>
</tr>
<tr>
<td>AIDA</td>
<td>Group I</td>
<td>1 mM</td>
</tr>
<tr>
<td>LY367385</td>
<td>mGluR1</td>
<td>100 μM</td>
</tr>
<tr>
<td>MPEP</td>
<td>mGluR5</td>
<td>5 μM</td>
</tr>
</tbody>
</table>

aAgainst release evoked by 30 μM t-ACPD.
bAgainst release evoked by 500 μM CHPG.

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Figure 10. MPEP has a differential effect in blocking release in the soma and dendrites when it is applied at low concentration. A, [Ca2+]i 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 μM t-ACPD was added to the ACSF, a secondary increase of larger amplitude was observed at both locations. When 1 μM 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 μM MPEP was applied. In this case the secondary increase was blocked at both locations.
cause $\text{Ca}^{2+}$ 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 $\text{Ca}^{2+}$ release in ACSF containing 10 $\mu$M 5-HT (Fig. 13; $n = 6$ of 35). This $[\text{Ca}^{2+}]_i$ 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$_2$ receptor agonist known to mobilize IP$_3$ (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 $\text{Ca}^{2+}$ 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 synthaptically 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 (Parpura 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 $[\text{Ca}^{2+}]_i$ increase are consistent with our analysis of synthaptically activated $\text{Ca}^{2+}$ 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 $\text{Ca}^{2+}$. In new experiments we showed that the injection of adenophostin A, 3-F-IP$_3$, and IP$_3$ could promote spike-associated release without the need for metabotropic agonists. The effectiveness of these specific IP$_{3R}$ agonists is more direct evidence for the involvement of the IP$_{3R}$. In addition, pharmacological experiments indicate that release was promoted by group I mGluRs, M$_1$ muscarinic receptors, and 5-HT$_2$ receptors. All of these receptors are coupled to the mobilization of IP$_3$ (McKinney, 1993; Conn and Pin, 1997; Hoyer and Martin, 1997). One caution is that high concentrations of IP$_{3R}$ agonists were required to promote release (100 $\mu$M 3-F-IP$_3$ and 500 $\mu$M IP$_3$). These levels are higher than are needed to evoke release in other preparations (Hirotá et al., 1995; Missiaen et al., 1996). This difference could mean that the IP$_{3R}$s in pyramidal neurons are different from receptors in other preparations or that intracellular kinases or other degradative enzymes rapidly metabolize the injected IP$_{3R}$ agonists. However, all of the injected IP$_{3R}$ agonists occluded the large-amplitude spike-associated release mediated by bath-applied metabotropic agonists. This result suggests that the exogenously applied IP$_{3R}$ 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 $[\text{Ca}^{2+}]_i$ 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 $[\text{Ca}^{2+}]_i$ 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$_{3R}$s are inactivated or desensitized for brief periods after stimulation (Hajnoczy 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
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, M1 muscarinic, and 5-HT2. 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 Ca2+ channels is also unlikely to be responsible because (1) selective block of different Ca2+ 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 IP3Rs are distributed more densely on the ER in the proximal apical dendrites or that the IP3Rs are more sensitive in this region of the cell (Thomas et al., 2000).

Receptors contributing to spike-evoked Ca2+ release
Using specific agonists and antagonists, we analyzed the receptors contributing to the regenerative [Ca2+]i increase. Previously (Nakamura 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 mGluR5 agonist, also evoked release. These results are expected because pyramidal neurons express mGluR2 receptors (Shigemoto et al., 1995; 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 mGluR2 and mGluR5 varies (Schoepp et al., 1999). We were not able to determine whether only one of these subtypes releases Ca2+ 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 IP3 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 act on receptors coupled to IP3 mobilization. The effect of CCh was reliable, consistent with the
strong expression of M1 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 IP3 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, Ca2+ channels, and IP3Rs can affect the sensitivity and specificity of the signaling process underlying the release of Ca2+ 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 Ca2+ channels and ryanodine receptors are thought to be close to each other, allowing Ca2+ 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 IP3Rs are linked by Homer, a protein enriched at the postsynaptic density (Tu et al., 1998), suggesting that these IP3Rs are positioned appropriately to detect the IP3 mobilized by mGluR activation. In addition, there is conflicting evidence from different preparations that IP3Rs 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 Ca2+ channel types were linked closely to IP3Rs. Ca2+, a general Ca2+ channel blocker, prevented almost all Ca2+ entry and prevented Ca2+ release. No specifically tested channel blocker (nimodipine, Ni2+, ω-conotoxin-GVIA, or α-agatoxin-IVA) by itself prevented release. However, the Ca2+ 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 IP3Rs was not examined.

Most models of neuronal Ca2+ waves assume that Ca2+ released through IP3Rs contributes to the activation of nearby IP3Rs (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 IP3R to the next must be local, with receptors separated from each other by 1 μm or less. In our experiments in which these two buffers were loaded through the pipette, we found that the threshold for suppressing Ca2+ release was only two times higher for EGTA than for BAPTA. This approximately equal value, despite the difference in rate constants for Ca2+ binding (Naraghi, 1997), suggests that in pyramidal neurons the IP3Rs are further apart. This more widespread separation of IP3Rs is consistent with results in Xenopus oocytes. In those cells Marchant et al. (1999) found that Ca2+ was released in “puffs” from clusters of IP3Rs that were at least several micrometers apart. It is also possible that EGTA is buffering Ca2+ as it diffuses from the Ca2+ channels to the IP3Rs. 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 Ca2+ 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 IP3Rs 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 IP3 is mobilized instantaneously, this result suggests that IP3 is metabolized rapidly in pyramidal cells. Therefore, IP3 does not diffuse far from its source before activating IP3Rs. From this perspective the close association of mGluRs and IP3Rs mediated by Homer ensures the effectiveness of IP3 before it is metabolized. We do not know whether there is a parallel mechanism associating muscarinic receptors and IP3Rs.

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