Calcium From Internal Stores Triggers GABA Release From Retinal Amacrine Cells

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INTRODUCTION

The release of transmitter from neurons has long been known as an event triggered by an increase in cytoplasmic [Ca\(^{2+}\)] at the synaptic release site (Katz and Miledi 1965). Considerable evidence supports the idea that these Ca\(^{2+}\) ions are drawn from the supply of external Ca\(^{2+}\) admitted to the cytoplasm through voltage-gated Ca\(^{2+}\) channels (e.g., Roberts et al. 1990). In this respect, transmitter release, perhaps because of its requirement for high speed, apparently differs from the many other Ca\(^{2+}\)-triggered processes within cells now known to use Ca\(^{2+}\) derived from internal stores. In contrast to this well-accepted and longstanding idea, there is recent evidence suggesting that in some neurons, internal Ca\(^{2+}\) stores may participate in the supply of Ca\(^{2+}\) for Ca\(^{2+}\)-dependent facilitation and transmitter release. The significance of this mode of transmitter release, the circumstances under which it is used, and its exact mechanism are largely unknown.

Evidence in support of the involvement of internal Ca\(^{2+}\) stores in transmitter release can be drawn from several sources. There is good support for the idea that the release of the contents of large, dense core vesicles is promoted by internally derived Ca\(^{2+}\). Catecholamine release from adrenal chromaffin cells, a process similar to the release of fast transmitter molecules, is known to occur as a consequence of mobilization of Ca\(^{2+}\) through the inositol 1,4,5-trisphosphate (IP\(_3\)) pathway (Augustine and Neher 1992). Similarly, peptide release from the dendrites of hypothalamic oxytocin neurons is promoted by the experimentally induced liberation of Ca\(^{2+}\) from the endoplasmic reticulum (ER) (Ludwig et al. 2002).

Stimulation of the terminals of sympathetic neurons at a frequency known to release the peptide luteinizing hormone releasing hormone has been shown to result in calcium-induced calcium release (CICR), which is therefore surmised to be involved in the peptide release process (Peng 1996).

The evidence bearing on the release of transmitter from small clear vesicles, the predominant form of fast transmission in the nervous system, is more ambiguous but most persuasive with respect to spontaneous, rather than evoked, release. The frequency of spontaneous, autaptic minis seen in cultured retinal ganglion cells is reported to depend on Ca\(^{2+}\) mobilized by a pathway apparently involving the generation of IP\(_3\) (Han et al. 2001), and there is pharmacological evidence that spontaneous GABA release in the neonatal hippocampus (Savic and Scancarelli 1998) and onto cerebellar Purkinje neurons (Bardo et al. 2002) involves Ca\(^{2+}\) entering the cytoplasm through ryanodine receptors (RyRs). In rat barrel cortex, the frequency of miniature excitatory postsynaptic currents (mEPSCs) seems to be augmented by Ca\(^{2+}\) leaving internal stores through both the inositol 1,4,5-trisphosphate receptors (IP\(_3\)Rs) and RyRs (Simkus and Stricker 2002). A study of CA3 pyramidal neurons concluded that local, spontaneous CICR within presynaptic boutons is responsible for one-half of all spontaneous transmitter release events, and furthermore, triggered CICR underlies paired pulse facilitation in these terminals (Emptage et al. 2001). A subsequent study, however, using acute brain slices rather than organotypic slices, concluded that Ca\(^{2+}\) from internal stores had little effect on transmitter release (Carter et al. 2002).

A strong case has been made that the spontaneous release of GABA from basket cell terminals presynaptic to cerebellar Purkinje neurons is coordinated by suddenly arising [Ca\(^{2+}\)] transients originating from the opening of RyRs (Llano et al. 2000). In these terminals, the presence of RyRs was shown immunocytochemically, spontaneous [Ca\(^{2+}\)] transients were revealed with Ca\(^{2+}\) imaging, and treatments affecting the Ca\(^{2+}\) store were shown to have the expected effects on postsynaptic minis. A recent study has extended these findings to evoked release and shown that blockade of the RyR or emptying of internal Ca\(^{2+}\) stores reduces evoked inhibitory postsynaptic currents (IPSCs) to 70% of their control amplitude as a consequence of reduced transmitter release (Galante and Marty 2003).

In this study, we examine whether Ca\(^{2+}\) ions originating from the ER participate in evoked transmitter release from retinal amacrine cells. Amacrine cells are unusual neurons in that transmitter release occurs chiefly from dendrites and is largely accomplished through graded potentials rather than action potentials (Bieda and Copenhagen 1999; Gleason et al.)
A conjunction of facts suggests that in these neurons, internally derived Ca$^{2+}$ might be important in transmission. Transmitter release from these cells, as in some other types of neuron, both inhibitory (Lu and Trussell 2000) and excitatory (Atluri and Regehr 1998), can outlast a depolarization by hundreds of milliseconds or even seconds (Borges et al. 1995; Gleason et al. 1994). Moreover, dendritic [Ca$^{2+}$], both during a depolarization and for some time thereafter, is dependent on Ca$^{2+}$ release from the ER (Hurtado et al. 2002). Together with the finding that transmitter release from these neurons requires relatively low concentrations of Ca$^{2+}$ (Ferking et al. 1997), these observations strongly suggest that ER Ca$^{2+}$ stores contribute to transmitter release, although unlike the case of basket fibers in the cerebellum (Llano et al. 2000), internal Ca$^{2+}$ does not seem to coordinate the release of multiple vesicles (Ferking et al. 1997). We show here that, although Ca$^{2+}$ release from internal stores occurs through both RyRs and IP$_3$Rs in amacrine cell dendrites, it is only Ca$^{2+}$ from IP$_3$Rs that promotes both spontaneous and evoked transmitter release.

**METHODS**

**Immunocytochemistry**

In all immunostaining experiments performed, cells were fixed with 4% paraformaldehyde, washed in PBS, and permeabilized with 0.05 mg/ml saponin. Rabbit polyclonal anti-IP$_3$R antibody (Calbiochem, cat 407143, raised against the C-terminal domain of the type 1 IP$_3$R) and the mouse monoclonal anti-RyR antibody (34C, Developmental Studies Hybridoma Bank, University of Iowa, raised against a and b subunits from chick skeletal muscle) were applied at dilutions of 1:400 and 1:200, respectively. After washing off excess primary antibodies with PBS, Alexa 488 and 568 dye-conjugated secondary antibodies (Molecular Probes) were applied together at a dilution of 1:400. To yield images like those in Fig. 7, in which the relative positions of IP$_3$Rs and RyRs were determined accurately, cells were examined in a wide-field microscope and fluorescence images were improved using a constrained iterative deconvolution algorithm (API, Deltavision). Rabbit polyclonal antibodies against CABP1, CABP2, and CABP5 were kindly provided by F. Haeseleer, University of Washington, Seattle. Immunostained cells were examined using either a 488-nm Argon laser line or a 568-nm Krypton laser line of an Olympus FLUOVIEW FV300 microscope with TIEMPO software (Olympus). Images were further processed using custom code written in Matlab 6 (The Mathworks) and viewed in Photoshop (Adobe Systems).

As controls, we routinely omitted primary antibodies from the above procedures to check for secondary antibody staining. Control images did not show a significant level of fluorescence (data not shown), and subsequent imaging with primary antibodies were performed with excitation intensities and photomultiplier tube (PMT) parameters set to exclude control values of fluorescence.

**Cells and Ca$^{2+}$ imaging**

Chick retinase were dissociated on embryonic days 8–10, cultured on glass coverslips at low density as previously described (Gleason and Wilson 1989), and were used after 7–12 days in culture. Solitary, isolated neurons were identified as amacrine cells on the basis of their multipolar morphology (Gleason et al. 1993; Huba and Hofmann 1990). For Ca$^{2+}$ imaging, cells were loaded with 5μM acetoxymethoxy (AM) ester form of Oregon green BAPTA-1 (OGB-1, Molecular Probes, K$_{D}$ for Ca$^{2+}$, 170 nM) in control external solution with 0.05 mg/ml pluronid acid for 1 hr at room temperature. The use of a high affinity dye, like OGB-1, because it acts to buffer Ca$^{2+}$, inevitably makes [Ca$^{2+}$] transients rise and fall more slowly and reach lower peak values than control. Neither of these artifacts would substantially alter the conclusions of this study. Confocal imaging experiments used the 488-nm Argon laser line of an Olympus FLUOVIEW FV300 microscope with TIEMPO software (Olympus) to trigger acquisition starts under the control of Clampex 8.0 (Axon Instruments). Continuous flow of solution over the cells was maintained during imaging, and in those experiments requiring rapid application of solutions, Clampex protocols allowed the puff pipette to be positioned close to the cell during the puff and to be withdrawn at other times.

XY imaging was performed at a resolution of 800 $\times$ 600 pixels. In line scan mode, a line drawn on the monitor screen tracing a dendrite was scanned (Fig. 1C) $\sim$430 times with a period of 115.3 ms. Control experiments showed that the maximum deviation of the line drawn on the screen from the actual scan path was never more than 3 pixel widths (0.17 μm), and the drift of the scan line relative to the dendrite was less than a pixel width per 50 s. Although amacrine cells readily form autapases and synapses in culture, unlike hippocampal cultures, for example (Murphy et al. 1995), there are no spines or boutons that identify these sites.

Raw FLUOVIEW tif images were analyzed using software written in Matlab. An average baseline fluorescence value, determined just before depolarization, was used to compute $(\Delta F/F_0)$, and care was taken to ensure that baseline fluorescence values did not increase during the course of an experiment. To derive plots like those in Fig. 1B, fluorescence changes were integrated over the entire measured length of the dendrite. In these plots, a small decline in fluorescence caused by bleaching was corrected by fitting a polynomial to the baseline using TableCurve-2D (SPSS) and subtracting this from the entire record.

**Electrophysiology**

Cells were voltage clamped at $-70$ mV using the whole cell patch-clamp configuration with a Cs$^+$-based internal solution capable of ATP regeneration (Hoffspaur and Gleason 2002). For deriving I-V relations, cells were either stepped from $-80$ to $+30$ mV in 5-mV increments or ramped from $-70$ to $+40$ mV at 0.55 V/s. In both cases, leak currents were subtracted, but no correction was made for junction potential. Results obtained from the 2 protocols were similar. While looking at the effects of 2-aminoethoxydiphenyl borate (2-APB), ryanodine (Ry) and heparin on autaptic transmission, we measured the rate of transmission as the frequency of minis elicited during a 5-s depolarizing step to 0 mV ($E_{Cl-} = -60$ mV). The first second of response was omitted from measurements because individual minis in this period were very numerous and rode on the inactivating inward Ca$^{2+}$ current, making them difficult to count accurately.

In experiments where we applied (RS)-2-chloro-5-hydroxyphenylglycine (CHPG) and caffeine, we used solutions with an $E_{Cl-}$ of $0$ mV (see Drugs and solutions) to enable measurement of mini frequency at the holding potential. In these experiments, “evoked minis” refer to events that occur during a $10$ s hyperpolarization to $-80$ mV after 1-s depolarization to 0 mV. “Spontaneous minis” refer to events seen in the absence of depolarization. In experiments where we imaged [Ca$^{2+}$] transients induced by weaker stimuli, we performed nystatin perforated patch clamp on OGB-loaded cells. The average $R_i$ value for patch-clamp experiments, before compensation, was $\sim 15 – 25$ MOhm, whereas perforated patch experiments were performed at $\sim 40 – 50$ MOhm values with $\sim 50$% series resistance correction. All records were low-pass Gaussian filtered using Clampfit 8.0 (Axon Instruments), and minis with amplitude $\geq 5$ pA were identified by their fast rising phase and exponential decline (Gleason et al. 1993) and counted manually.
Drugs and solutions

Thapsigargin, 2-APB, and caffeine were obtained from Calbiochem (La Jolla, CA), Ry and GABA were obtained from Sigma (St. Louis, MO), CHPG was obtained from Tocris (Ellisville, MO), and TEACl was obtained from ICN (Aurora, OH). The sodium salt of heparin, derived from bovine intestinal mucosa, was obtained from Sigma (Cat H0777, 140 U/mg, average molecular weight 12,500). All other reagents were obtained from Sigma. Unless otherwise specified, the following solutions were used for patch clamp and imaging studies.

CONTROL EXTERNAL. KCl (5.3 mM), NaCl (116.9 mM), TEACl (20 mM), CaCl2 (3 mM), MgCl2 (0.41 mM), HEPES (3 mM), and glucose (5.6 mM).

CA. KCl (5.3 mM), NaCl (116.9 mM), TEACl (20 mM), MgCl2 (0.41 mM), HEPES (3 mM), and glucose (5.6 mM).

HIGH K. KCl (100 mM), NaCl (22.2 mM), TEACl (20 mM), CaCl2 (3 mM), MgCl2 (0.41 mM), HEPES (3 mM), and glucose (5.6 mM).

PIPETTE SOLUTION. Cs Acetate (100 mM), CsCl (10 mM), MgCl2 (2 mM), CaCl2 (0.1 mM), EGTA (1.1 mM), and HEPES (10 mM). The pipette solution also had the ATP regenerating system [phosphocreatine (20 mM), ATP dipotassium (3 mM), ATP disodium (1 mM), GTP sodium (2 mM), creatine phosphokinase (50 IU/ml)] added fresh each day (Hoffpaur and Gleason 2002).

In experiments involving CHPG and caffeine, the following solutions were used.

EXTERNAL. NMG (50 mM), Na methanesulfonate (72.2 mM), TEACl (20 mM), CaCl2 (3 mM), MgCl2 (0.41 mM), HEPES (3 mM), and glucose (5.6 mM).

PIPETTE SOLUTION. Cs Acetate (38.7 mM), CsCl (71.3 mM), MgCl2 (2 mM), CaCl2 (0.1 mM), EGTA (1.1 mM), and HEPES (10 mM).

In all experiments examining Ca2+ currents, biczuculline methiodide (3 μM) was added to suppress autaptic minis. All external solutions included 300 nM TTX and were adjusted to pH 7.4. In perforated-patch experiments, freshly prepared nystatin was added to the pipette solution at a concentration of 100 μg/ml along with pluronic acid at 200 μg/ml (Gleason et al. 1995).

In this study, we used 2-APB, heparin, caffeine, and Ry in our investigations of the IP3 and RyRs. Our choices for concentrations of these drugs were made as follows. The IC50 value of 2-APB on IP3Rs is reported as 42 mM (Maruyama et al. 1997), and lowest effective concentration used by other authors is 50 μM (Braun et al. 2003; Peppiatt et al. 2003; Soulsby and Wojcikiewicz 2002; White and McGeown 2003). Hence, we used this concentration in most of our experiments.
experiments. However, in Ca^{2+} imaging experiments, we observed that a lower concentration of 20 μM works quite well. The alkaloid, Ry, inhibits RyRs at low micromolar concentrations, whereas at high concentrations (>100 μM), it locks the receptors in a closed state (Fill and Copello 2002). Based on this and the experience of other authors (Mitra and Slaughter 2002b; Sheehan and Blatter 2003; Simkus and Stricker 2002), we used 20 μM Ry to block RyRs. For complete and irreversible block of RyRs, we used 200 μM. The optimal concentration of caffeine used to activate RyRs varies between preparations (Gomez-Viquez et al. 2003; Sitsapesan and Williams 1990; Solovyova and Verkhratsky 2003). We used a concentration of 20 mM, which we have found empirically activates RyRs reliably. Heparin blocks IP_{3}Rs in permeabilized cells (Nilsson et al. 1988) with an IC_{50} of 0.6 mg/ml (Ghosh et al. 2001). However, the high molecular weight and impermeant nature of heparin necessitate the use of high concentrations, especially in neuronal cells with long diffusion paths to target sites (Caillard et al. 2000; Larkum et al. 2003; Otani et al. 2002). Heparin was in pipette solutions containing either 1 or 5 mg/ml.

RESULTS

**IP_{3}Rs and RyRs both contribute to depolarization-induced [Ca^{2+}] transients**

We have previously shown that depletion of ER stores with thapsigargin, an irreversible inhibitor of the sarcoendoplasmic reticulum Ca^{2+} ATPases (SERCA) pump, greatly reduces the [Ca^{2+}] transient seen on depolarization of dendrites, implying that most of the cytoplasmic [Ca^{2+}] increase is contributed by Ca^{2+} efflux from the ER (Hurtado et al. 2002). To examine whether Ca^{2+} liberated from cytoplasmic stores participates in triggering transmitter release, we looked more closely at the pharmacology and characteristics of Ca^{2+} release before using these findings to study transmitter release.

To discover whether Ca^{2+} efflux from the ER occurs through IP_{3}Rs or RyRs, we used the drugs 2-APB and Ry as blockers for these two receptors, respectively. Segments of dendrites were examined in line scan mode and depolarized with a rapid local perfusion of 100 mM K^{+} solution for 2 s. Transient increases in [Ca^{2+}] in control external solution lasted ~10–15 s and frequently showed longer lasting hotspots. A few perforated-patch experiments indicated that brief depolarizations also produced [Ca^{2+}] transients that considerably outlasted the stimulus. Depolarizations to 0 mV in voltage clamp, lasting 500 ms, produced [Ca^{2+}] transients of 2.4 ± 0.3 s (n = 13 cells, data not shown). Both Ry and 2-APB reduced the [Ca^{2+}] transient, obtained by integrating the OGB fluorescence change (Fig. 1, A and B), although Ry often had a more pronounced effect in abbreviating the response than did 2-APB (Fig. 1, D and E). Ry (4 μM) reduced the [Ca^{2+}] transient in 3 of 10 cells, but 20 μM Ry was more effective with 8 of 9 cells showing a reduction in the [Ca^{2+}] transient.

A complicating factor in quantifying these effects is that, even in the absence of drugs, responses become progressively smaller at each trial, because of progressive emptying of the Ca^{2+} stores. Allowing for the roughly linear decline in response with trial number (Fig. 2B), we estimated that 20 μM Ry reduced the [Ca^{2+}] transient, measured as the fluorescence increase integrated over time and distance along the dendrite, by 21% (mean, n = 8 cells). 2-APB (50 μM) reduced the [Ca^{2+}] transient by 55% (mean, n = 23 cells). These results argue that both IP_{3}Rs and RyRs mediate Ca^{2+} efflux from the endoplasmic reticulum to the cytoplasm.

To verify that Ry was exerting its effect by diminishing Ca^{2+} efflux through RyRs, we examined its ability to prevent the [Ca^{2+}] increase induced by the RyR agonist, caffeine. In the absence of extracellular Ca^{2+}, a first 2-s puff of 20 mM caffeine produced a sharp [Ca^{2+}] increase, generally lasting ~2–5 s (Fig. 2A) but often followed several seconds later by smaller and slower increases. Repeated applications of caffeine produced much diminished responses, suggesting that a single application released a majority of available Ca^{2+}. Ry at 4 μM partially antagonized caffeine’s effect (3 of 5 cells), but higher concentrations (20 μM) almost completely abolished the response to caffeine (5 of 6 cells). At 200 μM, Ry irreversibly and completely blocked the caffeine response of cells (n = 5) on the same coverslip as cells that had previously shown robust responses to caffeine (data not shown). In the absence of caffeine, Ry was without effect on resting [Ca^{2+}].

We have previously shown (Gleason et al. 1994) that the majority of Ca^{2+} current in these cells is conducted through L-type Ca^{2+} channels and that these channels are chiefly implicated in synaptic transmission. Consistent with the established picture of Ca^{2+} release from the endoplasmic reticulum, we have shown that Ca^{2+} entering the cytoplasm through voltage-gated Ca^{2+} channels is required to trigger internal Ca^{2+} release (Hurtado et al. 2002). A possible explanation for the results seen in Fig. 1 might therefore be that 2-APB and Ry act directly to inhibit voltage-gated Ca^{2+} channels. To examine this, we compared whole cell patch-clamp Ca^{2+} currents in nine cells, in which Ry affected Ca^{2+} currents, the peak current was immediately and reversibly reduced by ~40 ± 10% on application of Ry. The mechanism by which this occurred is unknown and was not studied further.

2-APB (50 μM) was without effect on Ca^{2+} currents in nine of nine cells (Fig. 3B). Besides its action as an antagonist at IP_{3}Rs, 2-APB was able to block TRP channels in some preparations (Gregory et al. 2001), and in others, it was thought to be an inhibitor of the SERCA pump (Bilmen et al. 2002; Bootman et al. 2002). Cultured chick amacrine cells may have functional transient receptor potential (TRP) channels in the plasma membrane as suggested by the presence of TRPC-1, TRPC-3, and TRPC-6 subunits (Croussillac et al. 2003; Sosa et al. 2002) and our finding of a small cation current suppressed with 25 μM La^{3+} (E. Gleason and M. Wilson, unpublished data). Several pieces of evidence, however, argue that 2-APB action is not on Ca^{2+} entry through TRP channels in these cells, nor is its effect chiefly at the SERCA pump. First, unlike 25 μM La^{3+}, 2-APB does not suppress an inward current at ~70 mV or cause a drop in resting [Ca^{2+}], as is often the case with La^{3+} (data not shown). Second, 2-APB, in experiments like that shown in Fig. 2B, prevents the emptying of ER Ca^{2+} stores, a result inconsistent with the expectation if the drug were either preventing Ca^{2+} influx from the external medium or inhibiting the SERCA pump from accumulating Ca^{2+} into the ER.

**Ry and 2-APB conserve internally stored Ca^{2+}**

If, as we suggest from the foregoing results, Ca^{2+} from the ER stores contributes to the [Ca^{2+}] transient elicited by depo-
polarization, and Ry and 2-APB act as antagonists for ER \([Ca^{2+}]\) release, we would expect these antagonists not only to diminish the size of \([Ca^{2+}]\) transients seen with repeated depolarizations but also to protect against store depletion. To see if this was the case, we used the integral of fluorescence increase in a segment of dendrite as a measure of the total \([Ca^{2+}]\) released in a series of 2-s depolarizations, separated by rest periods of 3 min. As a control against run-down unrelated to \([Ca^{2+}]\) stores, such as dye bleaching or \([Ca^{2+}]\) channel inactivation, we examined a comparable group of cells differing only in that, between the second and fourth trials, both Ry and 2-APB were present continuously to inhibit \([Ca^{2+}]\) efflux from ER stores. As shown in Fig. 2B, the decline in \([Ca^{2+}]\) response in control external solution was progressive and roughly linear, so that on average, the fifth trial produced only 10\% of the \([Ca^{2+}]\) transient seen in the first trial. In the presence of the two drugs, \([Ca^{2+}]\) transients were much diminished. However, transients were not entirely abolished, partly because the drugs are incompletely effective and partly because some of the \([Ca^{2+}]\) transient is the result of \([Ca^{2+}]\) entry through voltage-gated \([Ca^{2+}]\) channels. On the fifth trial of the drug-treated group, the drugs were washed out of the chamber, resulting in a bigger response than seen in the untreated group \((P < 0.05)\). A similar, although slightly less pronounced, effect was seen when 2-APB alone was used (Fig. 2B, triangles). These results lend strong support to the idea that the drugs conserve the \([Ca^{2+}]\) store and are consistent with the drugs acting as antagonists for \([Ca^{2+}]\) release. As described later, a similar kind of experiment using CHPG to challenge ER stores also gave results confirming the emptying of stores by repeated depolarizations.

**Mini frequency and \([Ca^{2+}]\) sources**

Autaptic transmission was elicited by stepping from –70 to 0 mV using 5-s steps in whole cell patch clamp. As shown in Fig. 4A, responses, all elicited in the presence of TTX, consisted of discrete currents previously shown to be generated by single quanta of GABA acting at \(\text{GABA}_A\)Rs (Frerking et al. 1997; Gleason et al. 1993) and referred to here as minis. The relatively small number of autapses made it possible to identify and count individual minis occurring during the voltage step, but we excluded the first second of the depolarization from...
quantitative analysis because the relatively high frequency of minis during this period, superimposed on the inactivating Ca\(^{2+}\) current, made it harder to identify individual minis with certainty. Effects of 2-APB and Ry were assessed with a protocol that alternated voltage steps in control solution with steps in drug solution, allowing 45 s between steps. 2-APB had a strong and immediate effect on the number of minis elicited during a step (Fig. 4, A and B), often resulting in trials in which no minis were seen. A significant statistical difference between control and 2-APB-elicited mini frequencies is apparent from our data when pairwise comparisons were made between drug and control response in the same cell (P < 0.01, n = 8 cells, Wilcoxon signed rank test). The error bars in Fig. 4B are large but refer to the population of sampled cells, within which there is substantial variability. Within individual cells there is less variability, but part of the variability observed stems from the temporal distribution of minis during the 5-s voltage step sometimes showed bursting. We hypothesize that these bursts of minis might be caused by the sudden appearance of [Ca\(^{2+}\)] hotspots as seen in Ca\(^{2+}\) imaging (Fig. 1C) (Hurtado et al. 2002).

In contrast to 2-APB, Ry at concentrations of 20 \(\mu\)M had no significant effect on mini frequency during the voltage steps (P > 0.2, n = 10 cells, Fig. 4C).

Although mini frequency was much reduced in 2-APB, comparison of the mean mini amplitudes in control, 2-APB, and Ry solutions showed them to be not significantly different (Mann-Whitney U statistic, 2-tailed test, P = 0.1; Fig. 4E), supporting the conclusion that 2-APB acts on a presynaptic mechanism rather than on postsynaptic receptors. Consistent with this, we also found that neither 2-APB (50 \(\mu\)M, 10 trials from 7 cells) nor Ry (20 \(\mu\)M, 8 trials from 4 cells) had any effect on the GABA currents elicited in patch-clamped cells held at −70 mV and subjected to 2-s puffs of GABA (300 \(\mu\)M; Fig. 3, C and D; Wilcoxon signed rank test for paired data, P = 0.05).

As we have shown elsewhere (Frerking et al. 1997), resting levels of [Ca\(^{2+}\)] are sufficient to promote a sustained low frequency of “spontaneous” transmitter release, so it might be thought that some of the minis suppressed in 2-APB represented spontaneous, rather than evoked, release. Our estimate of the spontaneous mini rate is about 0.6 Hz in the cells examined, consistent with a previous estimate (Frerking et al. 1997), and about an order of magnitude less than the evoked rate, implying that the majority of minis suppressed by 2-APB are evoked.

2-APB is thought, in other preparations, to act at sites other than the IP\(_3\)R (Bootman et al. 2002). Results like those shown in Fig. 2B are most consistent with 2-APB acting at the IP\(_3\)R in our cells, but because this point is critical, we conducted some experiments using heparin, a drug thought to be more specific for IP\(_3\)R. Because heparin is not membrane permeant, these experiments were conducted using heparin dissolved in the pipette solution. Heparin was used at two concentrations, 1 and 5 mg/ml, and its effects were compared with a control group of cells treated identically except that heparin was omitted from the pipette solution. Both heparin concentrations reduced the frequency of evoked minis, but because heparin takes tens of seconds to diffuse into the furthest dendrites, the effect of the drug was more pronounced at later times and at the higher concentration. A confounding factor in these experiments was that, even in the control group of cells, mini frequency elicited by our protocol declined with time. Nevertheless, at the three time-points chosen, 140, 270, and 350 s, the higher concentration of heparin reduced mini frequency significantly (P < 0.01; Fig. 4D).
Store emptying with thapsigargin reduces evoked transmission

Calcium stores in the ER can be depleted by treating cells with thapsigargin, a SERCA pump blocker. We have previously shown that incubating amacrine cells in 1 μM thapsigargin for 1 h completely emptied the stores, as tested by responsiveness to thimerosal (Hurtado et al. 2002) and ionomycin (unpublished results). To determine whether emptying ER stores with thapsigargin affects mini frequencies, we performed experiments on two groups of cells. One group was incubated in 1 μM thapsigargin for 1 h, and the other was incubated in control solution. Cells were subsequently whole cell patch clamped, and currents were recorded during 5-s depolarizing steps to 0 mV. Minis were manually counted as before, and mean mini frequency was calculated. The evoked mini frequency was much reduced in thapsigargin-treated cells (2.13 ± 1.35 Hz) compared with controls (9.88 ± 3.15 Hz, P < 0.001; Fig. 4F). When we examined the effects of 2-APB on mini frequency in thapsigargin-treated cells, we found that frequencies were no different, with or without 2-APB (n = 10, P > 0.5, data not shown). These results provide additional evidence that Ca^{2+} stored in the ER plays an important role in synaptic transmission.

Because our Ca^{2+} imaging experiments were conducted with high K⁺ puffs rather than patch clamp depolarizations to 0 mV, as used in the examination of transmission, we conducted a few experiments to establish that a reduction in both OGB fluorescence and mini frequency could be simultaneously observed in the same cell. To do this, we modified our protocol by setting $E_{Cl}$ to be 0 mV and included 100 μM cell impermeant OGB in the pipette, as well as loading cells with OGB-AM as usual. This arrangement minimized changes in OGB concentration on access to the cell and allowed minis to be readily seen at 80 mV, after a voltage step. An abbreviated voltage protocol employed 1-s depolarizing steps to 0 mV followed by 10-s hyperpolarizations to −80 mV, thereby permitting us to conduct experiments more quickly. Minis were counted after the step in either 2-APB or control solution for 1 h. Minis were counted during this step and mini frequencies calculated for both control (n = 6) and thapsigargin-treated (n = 7) cells. Error bars in F represent SE. Error bars in B–E show SD.
Caffeine has multiple actions on transmission

The absence of any effect of Ry on evoked mini frequency suggests that caffeine, a well-known agonist for the RyR, ought also to have no effect on mini frequency. This proved not to be true; rather than increase transmission, caffeine substantially reduced both spontaneous and evoked mini frequency (spontaneous transmission by 77 ± 14%, n = 11 and evoked transmission by 79 ± 18%, n = 7; Fig. 5, B and C). In part, these effects might be attributed to caffeine’s action as an inhibitor of IP3Rs (Bezprozvanny et al. 1994; Parker and Ivorra 1991), but in addition, we have evidence that caffeine inhibits both voltage-gated Ca2+ currents and GABA currents.

Bath-applied caffeine (20 mM) inhibited voltage-gated Ca2+ currents elicited by ramp protocols from −70 to +40 mV by about 25% (25 ± 10%, n = 7, data not shown). This reduction could be seen even when 10 mM BAPTA was included in the internal solution (reduction of 31 ± 16%, n = 8, data not shown) and when cells were treated with 1 μM thapsigargin for 1 h (n = 14, data not shown), suggesting that, unlike the situation in rods (Krizaj et al. 1999), this effect is not dependent on the release of stored Ca2+. Caffeine (20 mM) also inhibited GABA currents elicited with 2-s puffs of 300 μM GABA. Nine cells examined at −70 mV showed a 43 ± 5% reduction in GABA current during caffeine application (Fig. 5A). Unlike the situation in teleost amacrine cells and turtle retinal ganglion cells (Akopian et al. 1998; Vigh and Lasater 2003), this effect was only partly dependent on Ca2+ released from stores because, after preincubating cells with 1 μM thapsigargin, caffeine still produced a 34 ± 10% reduction (n = 6, Fig. 5A).

Metabotropic glutamate receptors promote transmitter release through elevated [Ca2+]

Group I metabotropic glutamate receptors (mGluRs) are known to be located on rat amacrine cell dendrites (Koulen et al. 1997), postsynaptic to bipolar terminals, and are also expressed in chick retinal amacrine cells in culture (Kreimborg et al. 2001), where agonists for mGluR5 produce an elevation of cytoplasmic [Ca2+] through the activation of IP3Rs (Sosa et al. 2002) and also changes in GABA conductance (Hoffpaurier and Gleason 2002). To see if mGluR5 was capable of promoting and enhancing transmitter release, we applied the agonist CHPG at a concentration of 150 or 300 μM. As in previous experiments, we counted the number of minis after a depolarizing step using a protocol that alternated between control external solution and one containing the drug. In the presence of CHPG, there was a clear enhancement of the number of minis seen (n = 9, P < 0.05, Wilcoxon signed rank test for paired data; Fig. 6, A and B). Furthermore, application of CHPG was seen to increase the frequency of spontaneous minis (n = 9, P < 0.05, Wilcoxon signed rank test for paired data; Fig. 6C). This effect typically took 20–30 s to appear and a similar time to reverse on return to control external solution.

To establish whether CHPG was acting through the IP3R pathway, we applied 2-APB and CHPG simultaneously and found that the effect of CHPG on evoked mini frequency was abolished (n = 11, P < 0.01, Wilcoxon signed rank test for paired data; Fig. 6D and E).

Because CHPG releases Ca2+ from IP3R-gated ER stores, we also used it in Ca2+ imaging experiments to confirm the conclusions drawn from the experiments shown in Fig. 2B, where we determined the state of ER stores after repeated depolarizations. The advantage of using CHPG is that it can be used to challenge ER stores in a way that bypasses plasma-membrane Ca2+ channels. In an experimental group of OGB-loaded cells, we applied 2-s puffs of 100 mM K+ at 3-min intervals and, consistent with the results in Fig. 2B, we ob-
served a progressive decrease in depolarization-induced \( \delta[Ca^{2+}] \) during repeated depolarizations. When these cells were challenged with 300 \( \mu M \) CHPG after this treatment, cytosolic \( [Ca^{2+}] \) showed only a very slight increase, suggesting that repeated depolarizations had indeed depleted the stores. We followed the same procedure in the control group, only in this case, we replaced 100 mM K\( ^+ \) with control external solution and hence, repeated puffs did not depolarize the cell. Mean OGB fluorescence change after the CHPG challenge was more than six times higher in control cells (\( n = 6 \)) than in the experimental group (\( n = 6 \); \( t \)-test for independent samples, \( P < 0.03 \)), supporting our interpretation that the progressively smaller \( [Ca^{2+}] \) transients elicited by repeated depolarizations results chiefly from ER Ca\(^{2+} \) store depletion.

### Distribution of IP\(_3\)Rs and RyRs

Our finding that both RyRs and IP\(_3\)Rs permit the release of Ca\(^{2+} \) into the dendritic cytoplasm requires, at a minimum, that both IP\(_3\)Rs and RyRs be present on the endoplasmic reticulum within dendrites. The fact that IP\(_3\)Rs but not RyRs promote the release of transmitter makes it likely that these receptors are not co-located. To examine this prediction, we used antibody staining. Both IP\(_3\)Rs and RyRs show a highly punctate distribution in the cell body and dendrites of amacrine cells (Fig. 7A). For both kinds of receptors, fluorescent spots were very small, and with deconvolution methods, appeared no larger than the point spread function of the imaging system. In the thinnest dendrites, immunoreactive spots were laid out in single file, allowing us to form an estimate of their spacing (Fig. 7B). Measurements from three cells showed a mean spacing of 2.1 \( \pm \) 0.97 \( \mu m \) for IP\(_3\)Rs and 3.8 \( \pm \) 2.8 \( \mu m \) for RyRs. Both antigens had a skewed unimodal distribution with fewer close neighbors than consistent with a completely random (Poisson) placement. Distributions of intervals for both types of receptor closely followed log-normal functions, implying that the placement of receptor clusters involves several random variables whose effects are multiplicative. Both IP\(_3\)R and RyR distributions were found to be stationary (no dependence of local mean interval on distance along the dendrite) and independent (each interval was uncorrelated with neighboring intervals). The relatively scarce coincidence of an IP\(_3\)R and a RyR in the same location (Fig. 7A) implies that there is no obligatory co-location of these two receptors. Nevertheless, the placement of one kind of receptor might not be entirely independent of the other, and consequently, they might be observed together either more or less often than consistent with chance.

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**FIG. 6.** (RS)-2-chloro-5-hydroxyphenylglycine (CHPG) increases spontaneous and evoked mini frequency through an inositol 1,4,5-trisphosphate receptors (IP\(_3\)R)-mediated mechanism. Evoked minis and spontaneous minis were recorded as in Fig. 5. CHPG (300 \( \mu M \)) increased both evoked (A and B) and spontaneous (B and C) minis in comparison with controls. Evoked mini frequency was increased ~4 times in comparison with controls. Spontaneous mini frequency increased after a period of 20–30 s and washing off the drug reversed the process after a similar time period. D and E: application of 50 \( \mu M \) 2-APB along with 150 \( \mu M \) CHPG produced no increase in minis, but mini frequency increased significantly when 2-APB was washed off with 150 \( \mu M \) CHPG solution (\( n = 11, P < 0.01 \), Wilcoxon signed rank test for paired data).
To assess this critically, we performed a nonparametric statistical test in which the relative locations of the two kinds of receptors were compared with the expectations of chance, generated by averaging the nearest neighbor measurements from 200 trials in which the two distributions were shuffled. Unexpectedly, the distribution of actual relative positions was indistinguishable from chance in nine analyzed dendrites from three cells (Kolmogorov-Smirnoff test, $P < 0.05$). This result is somewhat surprising because a minimum expectation would be that some correlation would exist between the placement of the two kinds of receptors, if only because the ER membranes on which both types of receptor reside are unlikely to be uniformly distributed along a dendrite.

We report that Ca$^{2+}$ released from the ER through IP$_3$Rs, but not RyRs, promotes synaptic transmission. The mechanism behind this differential effect of stored Ca$^{2+}$ is unclear. One possibility is that there are more IP$_3$Rs than RyRs in the ER of amacrine cell dendrites, resulting in [Ca$^{2+}$] increases over a wider area during IP$_3$R activation. Accordingly, we observed that IP$_3$R puncta are more numerous than RyR puncta in double staining experiments.

It is presently unclear how the influx of calcium through voltage-gated calcium channels triggers the opening of IP$_3$Rs. Several alternatives are considered in the discussion section, but one possibility is that a calcium-binding protein (CABP) from the caldendrin family might be involved in gating the IP$_3$R, as has been suggested in another system (Yang et al. 2002). As shown in Fig. 8, antibodies against CABP1 and CABP2 show heavy labeling in both cell body and dendrites of amacrine cells. Antibodies against CABP5 also label amacrine cells, although less heavily.

**DISCUSSION**

**IP$_3$Rs provide calcium for transmitter release**

In this study, we showed that depolarization-evoked transmitter release from amacrine cells has a significant component
that uses Ca\(^{2+}\) entering the cytoplasm from the ER, specifically, through IP\(_3\)Rs. Four findings support this conclusion. First, immunocytochemistry clearly shows that IP\(_3\)Rs are present in the dendrites of our cultured amacrine cells. Second, pharmacological treatments thought to inhibit IP\(_3\)Rs reduce the amount of Ca\(^{2+}\) seen in a dendrite after depolarization. Third, antagonists to the IP\(_3\)R reduce the amount of transmitter released by depolarization. Fourth, application of a metabotropic glutamate receptor agonist, known to activate IP\(_3\)Rs, also increases evoked transmitter release.

Our finding that IP\(_3\)Rs are localized in the dendrites of amacrine cells agrees with the work of Peng et al. (1991), whose study, like this one, used a polyclonal antibody that, in their case, was raised against cerebellar IP\(_3\)Rs. Nevertheless, another study using an antibody recognizing only type 1 IP\(_3\)Rs, which are also present in the cerebellum (Wang et al. 1999), failed to see IP\(_3\)Rs in amacrine dendrites but did find heavy labeling of cone outer segments, something not seen in either this study or that of Peng et al. (1991). It is not clear how this discrepancy can be explained, although it should be pointed out that the other studies looked at mature, rather than late embryonic, retinas as examined here. RyRs are also clearly present in our amacrine cell dendrites, and other physiological and pharmacological studies are consistent with the presence of RyRs in other amacrine cells. Mitra and Slaughter (2002a,b) have shown that spontaneous outward currents in salamander amacrine cells are the consequence of CICRs and are sensitive to both ryanodine and caffeine-induced depletion of internal Ca\(^{2+}\) stores. A recent study of teleost amacrine cells (Vigh and Lasater 2003) using pharmacological methods, has also shown the presence of both RyRs and IP\(_3\)Rs.

The functional role of RyRs in the dendrites of chick amacrine cells is unknown. While Ca\(^{2+}\) is liberated by CICRs, this Ca\(^{2+}\) seems to play no part in triggering transmitter release, because Ry, unlike 2-APB and heparin, has no effect on evoked mini frequency. In rod photoreceptors, Ca\(^{2+}\) released from RyRs suppresses transmitter release as a consequence of a reduction in voltage-gated Ca\(^{2+}\) current (Krizaj et al. 1999). A similar effect may occur in amacrine cells, but as we have shown, caffeine, the agonist of choice, has effects unrelated to Ca\(^{2+}\) stores that would tend to obscure any such result.

The question of whether the RyRs play a subtle and indirect role in transmitter release remains to be determined, but it is not unprecedented for the two kinds of receptors to play different, even antagonistic roles in cellular processes. For example, in smooth muscle, IP\(_3\)Rs promote contraction (Iino et al. 1994), whereas Ca\(^{2+}\) efflux through RyRs activates K\(^+\) channels and thereby promotes relaxation (Nelson et al. 1995). Similarly, it is well established at postsynaptic locations that small spatial separations of IP\(_3\)Rs and RyRs permit these receptors to serve different, potentially antagonistic, functions (Delmas and Brown 2002; Nishiyama et al. 2000). Whatever the function of RyRs in amacrine cell dendrites, it is apparent that Ca\(^{2+}\) liberated from the two kinds of ER Ca\(^{2+}\) channels is not equivalent, presumably because IP\(_3\)Rs have a privileged spatial relationship with transmitter release sites not shared by RyRs. In a previous study we showed that very local calcium domains comprising several hundred nanomolar differences in dendritic [Ca\(^{2+}\)] can exist over distances of about 1 \(\mu m\) (Hurtado et al. 2002), some of which we presume are generated by both IP3Rs and overlap with presynaptic sites.

Our results leave unresolved the mechanism by which depolarization activates the IP\(_3\)R. Because the opening of these channels usually requires both Ca\(^{2+}\) and IP3, it is possible that IP3 is constitutively present at concentrations sufficient that IP3Rs are effectively gated by a rise in [Ca\(^{2+}\)] alone during the opening of voltage-gated Ca\(^{2+}\) channels. Two other alternatives need to be considered, however. Production of IP3 in Purkinje cells is known to be stimulated by Ca\(^{2+}\) influx, leading to the suggestion that some isoforms of phospholipase-C are activated by Ca\(^{2+}\) alone (Okubo et al. 2001). A more unorthodox possibility is suggested by the finding that members of a family of Ca\(^{2+}\) binding proteins, with homologies to calmodulin (Haeseleer et al. 2000), whose best known member is caldendrin (Menger et al. 1999), are heavily expressed in the retina. As we show here, three members of that family are found in chick retinal amacrine cells. It has been shown that binding of Ca\(^{2+}\) to these proteins can allow them to act as ligands for the IP3R in the absence of IP3, at least when expressed in Xenopus oocytes (Yang et al. 2002).

A major underlying question in this context, and one of functional importance, concerns the relative contributions of store-derived and external Ca\(^{2+}\) in triggering transmitter release. Simply removing external Ca\(^{2+}\) abolishes all transmission (Hurtado et al. 2002), but the complementary experiment of disabling internal stores with 2-APB, heparin (Fig. 4D), or thapsigargin shows that transmission is reduced by as much as fivefold. These results emphasize the importance of internal stores, although undoubtedly the relative store contribution is a function of stimulus parameters, which in our experiments has been strong depolarization for several seconds. Presuming that the majority of asynchronous transmitter release, release occurring during presynaptic repolarization, is driven by stored Ca\(^{2+}\), it seems that steps as short as 50 ms are effective at mobilizing this Ca\(^{2+}\), because these steps evoke asynchronous release (Gleason et al. 1994).

**Nature of the Ca\(^{2+}\) store in amacrine cell dendrites**

The exact nature of the Ca\(^{2+}\) store within dendrites is yet to be determined. If the store is a continuous endoplasmic reticulum, for the most part, this membrane would be free of both RyRs or IP3Rs because these are found only at discrete spots. An alternative possibility is that, as in Purkinje cell dendrites, the ER is elaborated into cisternae lying just below the plasmalemma (Peters et al. 1991), some of which would bear RyRs and other IP3Rs. In any event, our results indicate that the Ca\(^{2+}\) store is readily exhausted, and on these grounds, likely to comprise very small volumes. Refilling of the store is, in contrast, an apparently slow process taking many minutes, as evidenced by the almost complete emptying of the store by five depolarizations within a period of 12 min (Fig. 2B). It is generally believed that refilling of stores in excitable cells is achieved through voltage-gated Ca\(^{2+}\) channels; because we found that repeated depolarization leads not to filling but to depletion of the stores, some other mechanism must be responsible for refilling.
Functional significance of internal Ca\(^{2+}\) stores for transmitter release

What function is served by involving internal Ca\(^{2+}\) stores in the process of transmitter release? We suggest that the answer to this question is connected with the unusual structure of amacrine cells. Stereotyped neurons have separate structures associated with synaptic input and synaptic output, dendritic branches, and axon terminals, respectively. The original defining feature of amacrine cells, in contrast, was that they have no axon (Ramon y Cajal 1972) but instead accommodate both pre- and postsynaptic structures in more or less close proximity along a dendrite. It was proposed some time ago that amacrine cell dendrites could perform local integration by having local electrical spread (Ellias and Stevens 1980), but subsequent examination of the cable properties of amacrine cells ruled out this possibility (Bloomfield 1992).

We suggest that amacrine cell dendrites can indeed function as multiple local integrators but that, rather than the local spread of voltage, it is by means of the local cytoplasmic Ca\(^{2+}\) concentration that integration is performed. Several pieces of evidence support this idea. Calcium imaging of the dendrites of starburst amacrine cells in the intact retina (Euler et al. 2002) has shown that, while no directional selectivity was apparent when membrane voltage was monitored, individual branches and sub-branches displayed pronounced and different directional selectivities when cytoplasmic Ca\(^{2+}\) concentration was examined. Support also derives from experiments showing that depolarization of a single Mb-1 bipolar cell of the goldfish retina (Vigh et al. 2005) or a single rod bipolar cell of the mammalian retina (Hartveit 1999) is sufficient to drive GABAergic feedback. In the case of the rod bipolar cell, feedback very likely comes from an A17 amacrine cell that makes contact with about 1,000 bipolar terminals (Nelson and Kolb 1985) and would therefore be unlikely to show much voltage change with only a single input, but might mediate reciprocal feedback through local [Ca\(^{2+}\)] changes.

Also consistent with the general notion that local Ca\(^{2+}\) provides the currency for the integration of inputs is our result that stimulation of metabotropic glutamate receptors stimulates and augments transmitter release. This may well be an important property of amacrine cells in the intact retina, and in fact, Vigh et al. (2005) have shown that strong depolarization of an Mb-1 bipolar cell in a slice, or the application of an agonist for mGluR1, enhances feedback from amacrine cells. We note, however, that in our experiments, the agonist for mGluR5 took several tens of seconds to have an effect. It remains to be seen whether this is an artifact of cultured cells or our procedures.

Allowing that local synaptic input to an amacrine cell dendrite may permit local output does not directly address the issue of the Ca\(^{2+}\) release seen in this study, which is triggered by depolarization, a global rather than local signal. We speculate that local release of transmitter is governed both by a local signal, synaptic input to a dendrite, and a global signal, the membrane voltage of the cell.

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