Na\(^+\)-Ca\(^{2+}\) Exchanger Controls the Gain of the Ca\(^{2+}\) Amplifier in the Dendrites of Amacrine Cells

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INTRODUCTION

Calcium entering neurons can be temporarily sequestered within Ca\(^{2+}\) stores or chelated by the many native Ca\(^{2+}\) buffer molecules. Eventually, however, almost all excess Ca\(^{2+}\) is expelled via the plasmalemmal Ca\(^{2+}\) ATPase (PMCA) or the plasmalemmal Na\(^+\)-Ca\(^{2+}\) exchanger. The relative importance of these mechanisms and their interactions are not well understood, particularly at synapses where Ca\(^{2+}\) plays several roles, including the triggering of transmitter release. To the extent that it has been looked at, it appears that the relative importance of different mechanisms of Ca\(^{2+}\) clearance varies from preparation to preparation. In the retina, the ribbon synapses at the photoreceptor and bipolar cell terminals are thought to chiefly use the PMCA (Krizaj and Copenhagen 1998; Morgans et al. 1998; Zenisek and Matthews 2000). In cultured retinal amacrine cells, however, the plasmalemmal Na\(^+\)-Ca\(^{2+}\) exchanger seems to play a dominant role in clearing Ca\(^{2+}\) loads because preventing the normal operation of this exchanger greatly prolongs transmission following Ca\(^{2+}\) influx through voltage-gated channels (Gleason et al. 1994). Similar results have been found with cultured hippocampal neurons (Reuter and Porzig 1995).

Many types of retinal neuron, including many types of amacrine cell, signal with graded potentials and release transmitter asynchronously without the need for action potentials (Bieda and Copenhagen 1999; Gleason et al. 1993). An important characteristic of asynchronous transmission is that, unlike fast synchronous transmission, it requires only a modest increase in cytoplasmic [Ca\(^{2+}\)]. Retinal amacrine cells from the chick release transmitter even at resting [Ca\(^{2+}\)], (Frerking et al. 1997), and in both retinal photoreceptors (Rieke and Schwartz 1996) and bipolar cells (Lagnado et al. 1996; Rouze and Schwartz 1998) continuous transmitter release requires only low-micromolar [Ca\(^{2+}\)]. Because of this, the mechanism of Ca\(^{2+}\) clearance is likely to be of particular significance at these synapses.

The mechanisms controlling asynchronous transmission are not well known, but there is some evidence suggesting that internal Ca\(^{2+}\) stores in the terminal boutons of hippocampal pyramidal neurons play a significant role in providing the Ca\(^{2+}\) required for this form of transmission (Emptage et al. 2001). A similar result has been found in cultured rat ganglion cells where the continuous release of quanta is made possible by Ca\(^{2+}\) release from the endoplasmic reticulum (ER) through IP3Rs (Han et al. 2001). Interestingly, there is some evidence in neurons and other preparations, suggesting a close association between the Na\(^+\)-Ca\(^{2+}\) exchanger and internal Ca\(^{2+}\) stores. This association is well established in the case of smooth muscle where the Na\(^+\)-Ca\(^{2+}\) exchanger has an intimate and perhaps molecular linkage to the superficial sarcoplasmic reticulum such that Ca\(^{2+}\) release from the SR through IP3Rs or ryanodine receptors (RYs) is closely coupled to its removal to the extracellular space by the exchanger (Nazer and van Bremen 1998). In neurons, there is evidence for a spatial association of the exchanger with ER stores (Blaustein et al. 1996), but the functional significance of this is largely unknown. Among retinal neurons, catfish horizontal cells have been examined with respect to the interaction between the exchanger...
and the caffeine-sensitive Ca\textsuperscript{2+} store. In this preparation, reverse mode operation of the exchange refills the ER once it is depleted of Ca\textsuperscript{2+} (Micci and Christensen 1998).

In the work described here, we investigate the relationship between Na\textsuperscript{+}-Ca\textsuperscript{2+} exchange and Ca\textsuperscript{2+} release from internal stores with the aim of understanding how Na\textsuperscript{+}-Ca\textsuperscript{2+} exchange might influence synaptic transmission from amacrine cell dendrites. Unexpectedly, we find that the increase in [Ca\textsuperscript{2+}] produced by depolarization is chiefly due to Ca\textsuperscript{2+} released internally, triggered by a relatively small influx through voltage-gated Ca\textsuperscript{2+} channels. The role of Na\textsuperscript{+}-Ca\textsuperscript{2+} exchange appears to be that of moderating the gain of this Ca\textsuperscript{2+} amplifier mechanism.

**METHODS**

**Cells**

Chick retinae were dissociated on embryonic day 8 and cultured on coverslips at low density as previously described (Gleason et al. 1993). Solitary, isolated neurons identified as amacrine cells were used after 7–12 days in culture.

**Dye loading**

Cells were loaded for \( \sim 1 \) h at room temperature with 10 \( \mu \)M Fura-2 AM in normal external solution (see **Solutions**) with 0.02% wt/vol pluronic. A second set of experiments employed confocal imaging, chiefly in linescan mode, and for these, the AM salt of Oregon Green 1,1000 (Molecular Probes, Eugene, OR) in 0.02% wt/vol pluronic. A second set of experiments employed confocal imaging, chiefly in linescan mode, and for these, the AM salt of Oregon Green 1,1000 (Molecular Probes, Eugene, OR) in 0.02% wt/vol pluronic. A second set of experiments employed confocal imaging, chiefly in linescan mode, and for these, the AM salt of Oregon Green 1,1000 (Molecular Probes, Eugene, OR) in 0.02% wt/vol pluronic. A second set of experiments employed confocal imaging, chiefly in linescan mode, and for these, the AM salt of Oregon Green 1,1000 (Molecular Probes, Eugene, OR) in 0.02% wt/vol pluronic.

**Electrophysiology**

Fura-2-AM-loaded cells were voltage clamped using the nystatin perforated-patch technique previously described (Gleason et al. 1995). Voltage-clamped cells were stimulated with 500 ms depolarizations from \(-60\) or \(-70\) to 0 mV, which is sufficient to activate the Ca\textsuperscript{2+} currents in these cells close to maximally (Gleason et al. 1995). Unclamped cells were stimulated with a 2-s puff of 100 mM K\textsuperscript{+} external solution, applied from a pipette with a tip diameter of \( \sim 1 \) \( \mu \)m positioned over the cell body. For both the confocal and the video imaging experiments, coverslips with cells were mounted in a 40-\( \mu \)L Plexiglas chamber (model RC-24, Warner Instruments, Hampden, CT) in which gravity fed solution changes could be made within \( \sim 4 \) s. In puff experiments, leakage from the pipette tip was unavoidable; so to minimize its effects, the puff pipette was parked a long way from the cell just before the 2- to 6-kPa pressure step and withdrawn immediately after its application. Puffette movement was automated by programming two positions into a modified Eppendorf 7171 micromanipulator (Eppendorf, Westbury, NY) and triggering movement with a TTL pulse. In some experiments such as that shown in Fig. 28, two solutions had to be applied rapidly one after the other. In those experiments, two manipulators were employed, each carrying its own puff pipette.

**Solutions**

Pipette solution was initially made up as follows (in mM) 10 CsCl, 150 CsAc, 3 NaCl, 2 MgCl\textsubscript{2}, 0.1 CaCl\textsubscript{2}, 10 HEPES, 1.1 EGTA, and 5 TEA\textsubscript{Ac}. pH was adjusted to 7.4 with CsOH. Empirically we found that an osmolarity of 230 mosM, \( \sim 20\% \) hypotonic to the external solution, produced the longest recordings from neurons without any swelling or shrinking. External solutions used in patch-clamp and unclamped experiments were as follows (in mM): Normal: 5.3 KCl, 116.9 NaCl, 20 TEACl, 3 CaCl\textsubscript{2}, 0.41 MgCl\textsubscript{2}, 3 HEPES, 5.6 glucose, 0 Ca: 5.3 KCl, 116.9 NaCl, 20 TEACl, 3.41 MgCl\textsubscript{2}, 3 HEPES, 5.6 glucose. High K: 100 KCl, 22.2 NaCl, 20 TEACl, 3 CaCl\textsubscript{2}, 0.41 MgCl\textsubscript{2}, 3 HEPES, 5.6 glucose. 0 Na: 5.3 KCl, 116.9 LiCl, 20 TEACl, 3 CaCl\textsubscript{2}, 0.41 MgCl\textsubscript{2}, 3 HEPES, 5.6 glucose. Mtr: 1 mM in high-K solution.

All external solutions had 300 nM TTX, and were adjusted for pH to 7.4 with NaOH or LiOH (for 0 Na\textsuperscript{+} solution). In some experiments, bicuculline methiodide (3 \( \mu \)M) was added to suppress autaptic currents. Drugs were used at the following concentrations: thapsigargin, 1–1.6 \( \mu \)M (Calbiochem); sodium orthovanadate, 1 mM (Sigma); FCCP, 1 \( \mu \)M (Sigma); ionomycin, 10 \( \mu \)M (Calbiochem); thimerosal; 10 \( \mu \)M (Sigma); Digitonin, 20 \( \mu \)M (in 0 Ca).

**Imaging**

For video imaging of Fura-2-loaded cells, mounted coverslips were placed on an inverted Nikon Diaphot microscope and visualized with an oil-immersion objective (1.3 NA, \( \times 100 \)). UV excitation was generated with a short arc Xenon lamp (XBO 150W/CR, Osram), filtered through 40-nm band pass UV interference filters (peak, 340 and 380 nm, Chroma Technology) and introduced into the microscope by a liquid light guide. Switching between the two wavelengths was achieved rapidly (\( \sim 2 \) ms) by means of a galvanometer mirror (DX-1000, Solamere Technology Group, Salt Lake City, UT). The intensity of excitation light at the two wavelengths could be set separately. This was a useful feature when imaging thin dendrites, as it was often necessary to adjust the excitation intensities to obtain acceptable signal-to-noise ratios without leaving the linear range of the imaging setup. As a consequence, fluorescence ratios had to be corrected for excitation intensities to be consistent with the settings used for calibration. To measure the relative intensities at the two excitation wavelengths, a photodiode (UV-360BQ, EG&G Canada) was introduced into the excitation path following every experiment.

Fluorescence images (\( \sim 510 \) nm) were projected to a GenIII intensified CCD camera (Stanford Photonics) and digitized to 12 bits at video rate using the Axon Imaging Workbench 2.0 and 4.0 software and Image Lightning 2000 Frame Grabber (Axon Instruments, Union City, CA). In most experiments, ratio imaging used frame averaging at alternating wavelengths to give a rate of one ratio image every 0.429 s. Because the volume of a dendrite is much smaller than the volume of a cell body, the light scattered from a cell body could swamp the signal in a nearby dendrite. To avoid this, the field of the excitation light was restricted to a circle of 32 \( \mu \)m diam by placing a pinhole on the end of the light guide, conjugate with the object plane.

Confocal experiments were run on an Olympus FLUOVIEW confocal microscope using the 488-nm line of an Argon Laser. Line scanning utilized a mode whereby the user could draw a single line on the image of a dendrite that could then be repeatedly scanned. Control experiments confirmed that there was no movement of the line relative to the dendrite and the deviation of the drawn line from the scanned line was nowhere \( \geq 3 \) pixel widths (0.17 \( \mu \)m).

**Calculation of free [Ca\textsuperscript{2+}] and correction of imaging artifacts**

The steps in the calculation of free [Ca\textsuperscript{2+}] from ratio images were performed in Matlab using in-house software summarized here. Analysis of images was restricted to the region within the boundaries of the dendrite as drawn by hand but guided by the Laplacian transform of the average of 40 consecutive phase images, saved prior to the fluorescence imaging. An exact registration between the phase and fluorescence images assured us that the dendrite had not moved during the experiment. The processing steps outlined below result in a time
series of ratio images. From this, \([\text{Ca}^{2+}]\) was calculated pixel by pixel using the following equation (Grynkiewicz et al. 1985; Poeini 1990)

\[
[\text{Ca}^{2+}] = K_{\text{eff}} \frac{R - R_{\text{min}}}{R_{\text{max}} - R}
\]

Where \(R\) is the ratio between corrected images at the two wavelengths, \(K_{\text{eff}}\) is the effective dissociation constant

\[
K_{\text{eff}} = K_D \frac{I_{\text{eff}}}{I_{\text{max}}}
\]

and \(K_D\) is the dissociation constant of the dye, \(I^e\) and \(I^b\) are the background corrected fluorescences of the bound and free forms of Fura-2 with 380 nm excitation. The parameters \(R_{\text{min}}, R_{\text{max}},\) and \(I^e/I^b\) were obtained from in vitro calibrations.

In calculating \([\text{Ca}^{2+}]\), several corrections were applied to raw images. Raw images were first smoothed with a Gaussian having a 2 pixel SD before application of a shading correction (also smoothed). An adjustment was applied to correct a small amount of bleeding between the two channels due to imperfect synchrony between the switching of excitation wavelengths and frame initiation in the camera. Backgrounds, chiefly due to thermal noise in the intensified CCD, were determined for every cell and subtracted from raw images. Autofluorescence in dendrites was found to be very small, 5–10% of the total background, and was not routinely measured.

We performed in vitro calibrations on a routine basis. Microcapillaries (100-μm thick) were filled with solutions containing 50 μM Fura-2 and either 5 mM CaCl₂ (Max \([\text{Ca}^{2+}]\) solution) or 0 \([\text{Ca}^{2+}]\) 5 mM EGTA (Min \([\text{Ca}^{2+}]\) solution), pH 7.2. From background subtracted ratios we obtained \(R_{\text{max}}, R_{\text{min}},\) and \(S_p/S_f\). For \(K_D\), we used a typical value reported in the literature, \(K_D = 252 \text{ nM}\), and we applied a viscosity correction factor of 0.7 to \(R_{\text{max}}\) and \(R_{\text{min}}\) (Poeini 1990).

Are Ca gradients real?

One of the conclusions of the work described here is that local differences in \([\text{Ca}^{2+}]\) may exist within a dendrite over distances on the order of 1 μm. Numerous errors and artifacts are associated with the use of \([\text{Ca}^{2+}]\) indicator dyes, especially those loaded as membranepermeant AM esters, and we briefly consider whether these might have artifactualy generated the observed gradients.

The use of a high-affinity dye, because it acts to buffer \([\text{Ca}^{2+}]\), inevitably distorts the kinetics of \([\text{Ca}^{2+}]\) transients in three significant ways. \([\text{Ca}^{2+}]\) is expected to rise and fall more slowly, reach lower peak values, and diffuse more quickly than normal. This last effect follows from the likely fact that native buffers within the cell are mostly immobile, whereas the indicator dye is able to carry \([\text{Ca}^{2+}]\) from high to low concentration regions. None of these three consequences, however, is expected to accentuate spatial gradients for \([\text{Ca}^{2+}]\); rather they would all tend to lessen gradients, leading us to suppose that gradients might be more common and larger in unperturbed cells than those measured here. Two pieces of evidence stemming directly from our data indicate that \([\text{Ca}^{2+}]\) gradients are not the effect of imaging or dye artifacts. The first is that in a given cell, gradients could change sign over time, a hot spot becoming cool and vice versa. Second, resting \([\text{Ca}^{2+}]\), was uniform and gradients appeared only after stimulation (see Fig. 7A), yet gradients should be present in both situations if the cause was an optical or dye artifact. For instance, if dye was compartmentalized, the effect should be more evident in the resting state, because \([\text{Ca}^{2+}]\) in intracellular compartments is high enough to saturate the dye and would contrast sharply with the low resting cytosolic \([\text{Ca}^{2+}]\).

Aside from these observations, an experimental test was designed to validate the non-artifactual origin of gradients. The experiment is based on the observation that repeated high-K⁺ stimuli can, in some cells, induce long-lasting (>10 min) \([\text{Ca}^{2+}]\) gradients in uncapped dendrites (data not shown). A similar phenomenon has been reported in pyramidal neurons (Connor et al. 1988). If the gradients are due to \([\text{Ca}^{2+}]\) release and uptake from intracellular sources and sinks, then disabling the \([\text{Ca}^{2+}]\) handling mechanisms of the cell should bring the cell to a uniform \([\text{Ca}^{2+}]\). For this purpose, we used an external solution with 1 mM vanadate (an inhibitor of plasma membrane ATPases), 1 μM thapsigargin (an specific inhibitor of SERCA pumps), 0 Na⁺ (Li⁺ replacement) to disable the Na⁺-\([\text{Ca}^{2+}]\) exchanger, 1 μM FCCP (a protonophore that collapses the mitochondrial membrane potential), 10 μM mononycin, and 800 nM \([\text{Ca}^{2+}]\) EGTA. In two cells where we were able to induce gradients, bathing the cell in this external solution collapsed \([\text{Ca}^{2+}]\) gradients.

A second test consisted in loading cells (n = 5) with the high-molecular-mass dextran conjugate of Fura-2 from a pipette in the whole cell configuration. The pipette was then retracted after partial filling of the cell, allowing the plasma membrane to seal and the cell to recover for ~15 min before imaging. The dextran conjugate of Fura-2 does not cross intracellular membranes neither does it bind to immobile proteins or suffer from deesterification problems. A series of high-K⁺ puffs was then applied from a pipette near the cell body as described in the preceding text. In one cell, we established a clear gradient that lasted for >10 min and was abolished by 0 Ca²⁺ external solution (data not shown).

We also performed quantitative tests to assess the importance of the known imaging artifacts including dye compartmentalization in intracellular organelles, incomplete deesterification of the AM form (Oakes et al. 1988), and binding of dye to immobile proteins without loss of fluorescence. We performed the following tests to examine these effects.

To examine compartmentalization, we adapted the method described in Kao (1994) in which digitonin and Triton X-100 are applied sequentially. Over the majority of a dendrite, compartmentalization was negligible, and averaged over entire dendrites the percentage of fluorescence compartmentalized was typically 3%, in certain locations; however, it could reach 15%.

As a test for deesterification, we used the Mn²⁺-quenching method (McCarthy et al. 1994). Cells were loaded with Fura-2 AM as described previously and Ca-independent fluorescence was assessed using the isocoefficient method (Neher 2000; Zhou and Neher 1993). A measurement was done after 1 h AM loading, with the cell bathed in 0 Ca²⁺ external solution, and a second one after quenching by bubbling the cell for 5 min in a 0 Ca²⁺ solution containing 1 mM Mn²⁺ and high (100 mM) K⁺. Comparing the fluorescence between the two we found that residual fluorescence after quenching amounted to 11 ± 2% (mean ± SD within a dendrite) of the initial fluorescence. Because this residual value includes not only unquenched Fura-2 but also autofluorescence, and probably compartmentalized dye, the small variability within a dendrite indicates that quenching was uniform. This experiment was repeated in four cells with similar results.

RESULTS

Preventing forward mode exchange prolongs \([\text{Ca}^{2+}]\), recovery after depolarization

Synapses between cultured amacrine cells are mostly dendro-dendritic. For this reason, we have looked closely at \([\text{Ca}^{2+}]\) handling within dendrites to see if Na⁺-\([\text{Ca}^{2+}]\) exchange is present there and if so, how it controls \([\text{Ca}^{2+}]\). In other neurons, there is evidence that dendrites and cell bodies have significantly different \([\text{Ca}^{2+}]\)-handling mechanisms (Thayer and Miller 1990), consistent with their different functions as well as their different surface to volume ratios.

Isolated, solitary amacrine cells were held at ~60 or ~70 mV in perforated whole cell patch-clamp and stepped to 0 mV for 500 ms. A change in Fura-2 absorption ratio indicated a sharp increase in \([\text{Ca}^{2+}]\) in dendrites accompanying an inward
current carried by Ca\(^{2+}\), mostly through L-type Ca\(^{2+}\) channels (Gleason et al. 1994, 1995). The magnitude of this [Ca\(^{2+}\)] increase varied considerably between cells, and even within a single dendrite was often very nonuniform. Typically peak [Ca\(^{2+}\)] values, as illustrated in Fig. 1, ranged from 200 nM to 1 \(\mu\)M. Recovery back to baseline occurred with several time constants but was >80% complete within 10 s. In a typical experiment (Fig. 1), normal external solution was rapidly replaced with one in which either Li\(^+\) or N-methylglucamine (NMG), neither of which support Na\(^{+}\)-Ca\(^{2+}\) exchange, was substituted for Na\(^{+}\). These two agents gave indistinguishable results. Transition to Na\(^{+}\)-free solution produced no change in resting dendritic [Ca\(^{2+}\)]; but when the voltage step was repeated in the Na\(^{+}\)-free solution, the peak [Ca\(^{2+}\)], was usually a little higher than in normal solution and the return of [Ca\(^{2+}\)] to baseline was always prolonged.

As illustrated in Fig. 1, considerable variability between cells was seen with respect to the kinetics of [Ca\(^{2+}\)] recovery. In an initial survey of 46 cells, 50% showed a prolongation of the decline but no change to its monotonic nature. In 33% of cells, [Ca\(^{2+}\)] fell, or in some cases rose, slowly, to reach a plateau value. In these cases, as shown in Fig. 1, B and C, washing with normal solution permitted recovery of dendritic [Ca\(^{2+}\)] with a time course similar to that following the original voltage step in normal solution. The remaining 17% of cells showed oscillatory or chaotic behavior of [Ca\(^{2+}\)], that also quickly returned to baseline in normal external solution. A second voltage step in normal solution was always applied to verify that changes in [Ca\(^{2+}\)] kinetics were reversible.

Although we have here classified the observed dendritic [Ca\(^{2+}\)] as falling into three kinetic patterns, these are not natural classes and there is, in reality, a full range of intermediate conditions. A commonly observed feature in many cells was the sudden increase in [Ca\(^{2+}\)], by, typically, one to a few hundred nanomolar. Often these events could be seen to occur on the falling phases of [Ca\(^{2+}\)] (Fig. 1E), but sometimes they occurred “spontaneously” without any obvious timelocking to the stimulus (Fig. 1, D and E). Because these cells were voltage-clamped, the origin of these events does not lie with voltage-gated Ca\(^{2+}\) channels.

Is reverse mode exchange causing Ca\(^{2+}\) influx?

In catfish horizontal cells (Micci and Christensen 1998) and other neurons (Hoyt et al. 1998), it has been shown that the Na\(^{+}\)-Ca\(^{2+}\) exchanger can effect a rapid Ca\(^{2+}\) influx through

![FIG. 1. Disabling Na\(^{+}\)-Ca\(^{2+}\) exchange prolongs Ca\(^{2+}\) clearance following depolarization. A–F: dendritic [Ca\(^{2+}\)] transients from 6 representative Fura-2-loaded cells in response to 500-ms depolarizations from −60-mV holding potential to 0 mV (†). •••, time of external solution exchange from normal to 0 Na\(^{+}\) and back to normal. With the possible exception of E, removal of Na\(^{+}\) produces no increase in resting [Ca\(^{2+}\)]. A shows a cell in which removal of Na\(^{+}\) has only a moderate effect on [Ca\(^{2+}\)] dynamics after depolarization. In B and C, the effect is more dramatic, and [Ca\(^{2+}\)] only returns to rest when Na\(^{+}\) is returned. D and E show “spontaneous” local increases in [Ca\(^{2+}\)] (arrows); and in F, depolarization induces oscillations. Na\(^{+}\) replaced with Li\(^+\) in A, B, and E and N-methyl-glucamine (NMG) in C, D, and F.](image-url)
reverse mode operation. Because the removal of Na$^{+}$, by reversing the normal Na$^{+}$ gradient, would favor this mode of exchange, we looked closely to see if this mechanism of Ca$^{2+}$ influx was contributing to the changes in [Ca$^{2+}$], dynamics seen in Fig. 1.

Comparison of the dynamics of dendritic [Ca$^{2+}$] in pairs of records with and without Na$^{+}$ (Fig. 2A) showed that the rate of rise in [Ca$^{2+}$], was very similar, whether or not Na$^{+}$ was present. Were reverse mode exchange generating a Ca$^{2+}$ influx, the [Ca$^{2+}$], rise rate in 0 Na$^{+}$ solution might be expected to be greater. A second consistent feature of this comparison was that in the absence of Na$^{+}$, dendritic [Ca$^{2+}$] was seen to continue to increase, reaching a peak later than in the presence of Na$^{+}$. Following the peak, the decay of [Ca$^{2+}$], as already described, was slower when Na$^{+}$ was absent. The longer time to peak in the absence of Na$^{+}$ does not immediately suggest reverse mode exchange but would be consistent with reverse mode exchange continuing at a high rate after the closure of Ca$^{2+}$ channels. If so, then removal of Ca$^{2+}$ during this phase ought to accelerate the decline of [Ca$^{2+}$]. This possibility was ruled out experimentally. Depolarization in these experiments was achieved by a 2-s-long, high-K$^{+}$ puff applied to unclamped cells immediately followed by the removal of Ca$^{2+}$ by blowing 0 Ca$^{2+}$ solution over a cell. As shown in Fig. 2B, no change in the kinetics of [Ca$^{2+}$] decline was seen in the trials in which Ca$^{2+}$ was removed (time to half decay, 6.88 ± 3.51 s in 0 Na$^{+}$, 7.24 ± 2.73 in 0 Na$^{+}$, 0 Ca$^{2+}$, no significant difference, P > 0.4, paired t-test, n = 6 cells). Taken together, these results suggest that reverse mode exchange does not contribute significantly to the kinetics of Ca$^{2+}$ handling in dendrites.

Disabling forward-mode exchange generates [Ca$^{2+}$] gradients in dendrites

A striking feature of our experiments was that, in the absence of Na$^{+}$ [Ca$^{2+}$] within dendrites was frequently seen to be inhomogeneous over periods of tens of seconds, following a depolarization. In those cells in which a plateau was reached, long time averaging increased signal-to-noise ratio to the extent that it was possible to resolve stable hotspots and cool spots for Ca$^{2+}$ with separations of as little as 1 μm. As seen in Fig. 3A, adjacent hot- and cool spots differed in [Ca$^{2+}$] by as much as several hundred nanomolar. On return to normal external solution, spatial gradients collapsed and were absent prior to depolarization. In addition to steady-state gradients we frequently observed gradients that changed dynamically when the exchanger was disabled (Fig. 3B). Because extensive temporal averaging is precluded in these cases, spatial resolution is not so good; nevertheless it is possible to see that over a few micrometers [Ca$^{2+}$] appears to be independently regulated over a time course of seconds or tens of seconds. In some cases, [Ca$^{2+}$] oscillations were seen in one part of the dendrite, whereas in other parts of the dendrite, these oscillations were extremely damped, and in some cases, uncorrelated fluctuations were seen.

If the Ca$^{2+}$ entering a dendrite upon depolarization was able to diffuse freely, either in its free form or else bound to a mobile buffer, [Ca$^{2+}$] would quickly become homogeneous within the dendrite. Assuming that Fura-2 with a diffusion coefficient of 100 μm$^2$ s$^{-1}$ (Gabso et al. 1997; Murthy et al. 2000) is the main buffer in our cells, we calculate that homogeneity of a 10-μm stretch of dendrite would be achieved within 500 ms. The fact that [Ca$^{2+}$], gradients are maintained for considerably longer periods than is compatible with diffusive mixing implies that there must be Ca$^{2+}$ sources within a dendrite that continue to release Ca$^{2+}$ after the closure of Ca$^{2+}$ channels. Additionally it implies that there must also be Ca$^{2+}$ sinks operating within a dendrite. Together, sources and sinks are able to set up the stable or dynamic gradients seen in Fig. 3. The sources within dendrites are linked to the ER, as shown in the following text, but the nature of the sinks is unclear and might be intradendritic organelles or the PMCA or a combination of these. The one process that cannot be part of the sink mechanism though is the plasmalemmal Na$^{+}$-Ca$^{2+}$ exchanger.

Ca$^{2+}$ is released from internal stores

A diagnostic criterion for Ca$^{2+}$ induced Ca$^{2+}$ release (CICR) from ER is that it should be abolished once ER stores are empty of Ca$^{2+}$. Thapsigargin (tg) is a membrane-permeant,
specific inhibitor of the SERCA pump that refills the ER with Ca\(^{2+}\) from the cytoplasm. Sufficiently long incubation in tg should deplete the ER of Ca\(^{2+}\) and reduce or eliminate the effect on the [Ca\(^{2+}\)]\(i\) transient of removing Na\(^{+}\) from the external solution.

To determine the duration of treatment with tg that would be sure to empty the ER stores completely, we tried different incubation times before challenging cells with 10\(\mu\)M thimerosal. Thimerosal is a non-specific sulfhydryl reagent that promotes Ca\(^{2+}\) efflux from the ER through both RyRs and InsP3 receptors (Elferink 1999). Because it also has effects on plasma membrane channels, we performed the challenge in the absence of Ca\(^{2+}\)o. Based on preliminary experiments with 12 cells exposed to tg for various times, we found that 1 h of tg was sufficient to deplete the dendritic ER totally, although interestingly cell bodies retained some releasable Ca\(^{2+}\) even after this treatment (Fig. 4).

Using linescan imaging of dendrites loaded with OGB-1, we replicated qualitatively the results of Fig. 1 using a 2-s K\(^+\) depolarization of unclamped cells. As expected, Na\(^{+}\) removal prolonged the Ca\(^{2+}\) transient, measured as time to half decay (\(P < 0.031, n = 5\), Wilcoxon signed-rank sum test). On average the increase in \(t_{1/2}\) was 80% (range 28–207%) in 0 Na\(^{+}\) versus normal (Fig. 5). After treatment with tg, results were different in two ways. First, Na\(^{+}\) removal no longer extended the duration of the transients (\(P > 0.3, n = 5\), Wilcoxon signed-rank sum test). On average change in \(t_{1/2}\) was 10%. Second, the [Ca\(^{2+}\)]\(i\) transient, even in normal external solution, was shorter and smaller than in untreated dendrites (\(P < 0.025\), Mann-Whitney test, \(n = 5\)). The difference in

**Fig. 3.** Gradients in dendritic [Ca\(^{2+}\)] can be induced by disabling Na\(^{+}\)-Ca\(^{2+}\) exchange. Two voltage clamped, Fura-2-loaded amacrine cells were stepped to 0 mV for 500 ms (†), while a dendrite was imaged. A, static and B, dynamic [Ca\(^{2+}\)] gradients. In the absence of forward-mode Na\(^{+}\)-Ca\(^{2+}\) exchange, dendritic [Ca\(^{2+}\)] often reaches a spatially inhomogeneous plateau following depolarization. A, top: time course of [Ca\(^{2+}\)] in 4 dendritic segments; bottom: segment locations. When Na\(^{+}\)-Ca\(^{2+}\) exchange was disabled by removing Na\(^{+}\)o, [Ca\(^{2+}\)] was the same in all 4 segments prior to depolarization. After depolarization, the segments reached approximately steady, but different, plateaus. When the cell was returned to normal Na\(^{+}\)o solution, [Ca\(^{2+}\)] returned to baseline, and differences between segments were abolished. Bottom: pseudocolor map of spatial [Ca\(^{2+}\)] profile time averaged over the periods indicated (\(\Delta t\)). Resting [Ca\(^{2+}\)] is seen to be initially uniform everywhere but when all frames were averaged over a 50-s period during the plateau, [Ca\(^{2+}\)] gradients on the order of 100 nM over <2 \(\mu\)m are revealed. In B, a similar treatment shows an initial uniformity in [Ca\(^{2+}\)] with dynamically changing gradients following depolarization.
fluorescence change between tg-pretreated cells and controls was not due to dye saturation or nonlinear behavior due to an increase in basal [Ca\(^{2+}\)], with tg. From Fura-2 imaging, we found that tg not only failed to increase resting [Ca\(^{2+}\)], when measured after 1 h of treatment, but caused a slight but significant decrease compared to control cells (35 \(\pm\) 7 vs. 71 \(\pm\) 30 nM in controls; \(P < 0.005; n = 6\) Mann-Whitney sum rank test).

[Ca\(^{2+}\)], gradients exist when exchange is enabled

Because the [Ca\(^{2+}\)], transient in normal solution is much smaller following tg pretreatment, Ca\(^{2+}\) release from the ER is not a phenomenon solely associated with the disabling of the exchanger but occurs in normal conditions as well. It is reasonable then to ask to what extent the local [Ca\(^{2+}\)], gradients seen when exchange is disabled (Fig. 3), are also found in normal conditions. Fura-2 imaging indicates, as shown in Fig. 3, that at rest there are generally no detectable [Ca\(^{2+}\)], gradients within dendrites. Following depolarization, however, transient local Ca\(^{2+}\) gradients were often seen in both the Fura-2 imaging and the OGB-1 linescan experiments. In Fig. 5 (1st trial in normal solution, untreated cell) depolarization causes a substantial increase in fluorescence that is non-uniformly distributed along the dendrite. One region (red spot) shows an unusually prolonged [Ca\(^{2+}\)], increase lasting tens of seconds. This region of the dendrite is interesting because it has sharp spatial boundaries on either side, implying active sources of Ca\(^{2+}\) within the region and active sinks for Ca\(^{2+}\) at its margin. Toward the end of this first trial, another region of the dendrite (blue spot) shows a transient increase in [Ca\(^{2+}\)], not seen elsewhere in the dendrite. In a second trial, in the absence of Na\(^{+}\), [Ca\(^{2+}\)], recovery is, as expected, greatly slowed down. In the third trial, back in normal external solution, neither the region marked with the red spot nor the region marked with the blue spot show higher [Ca\(^{2+}\)], than the rest of the dendrite. The implication of these observations is that not only are [Ca\(^{2+}\)], gradients normally present after depolarization but also the sources and sinks that give rise to them are labile and behave in a history-dependent manner. This general conclusion was supported by experiments (not shown) in which [Ca\(^{2+}\)], hot-spots identified in linescans of dendrites during depolarization were compared with the [Ca\(^{2+}\)], profile seen when thimerosal was applied in Ca\(^{2+}\)-free medium so as to release Ca\(^{2+}\) from all ER stores. Many of the hotspots identified in depolarization were present in thimerosal-evoked release but some were absent, perhaps implying that stores at these sites were empty.

As shown in Fig. 1, brief spontaneous increases in [Ca\(^{2+}\)], could often be seen in normal external solution. The same observation was made using linescan of dendrites in which the spatial and temporal resolution was increased. Spontaneous events usually had a clear locus of origin from which Ca\(^{2+}\) spread in both directions (Fig. 6). Generally the total extent of an event was <10 \(\mu\)m, and the time course was \(\sim\)1 s, although we frequently saw events originating at the same site following so closely after each other as to be difficult to separate. The similarity of these [Ca\(^{2+}\)], events seen here in amacrine cells to elementary [Ca\(^{2+}\)], events seen in cultured hippocampal neurons (Koizumi et al. 1999) leads us to suppose that these may be the basic units from the CICR responses are built.

Interaction of Na\(^+\)-Ca\(^{2+}\) exchange and CICR

Exchanger molecules are thought to be inhomogeneously distributed on the plasma membrane of neurons (Reuter and Porzig 1995), and as we have described, Ca\(^{2+}\) can be very locally distributed in a dendrite; it seems possible therefore that the exchanger might have a close functional association with the Ca\(^{2+}\) sensor of CICR, as is the case for the smooth muscle (Nazer and van Breemen 1998). On this view, the exchanger might not actually contribute very much to the clearance of Ca\(^{2+}\) from the bulk of the dendritic cytoplasm. To investigate this possibility, we performed experiments to monitor the activity of the Na\(^+\)-Ca\(^{2+}\) exchanger while simultaneously imaging [Ca\(^{2+}\)],. Exchange was monitored by recording the small inward current generated by the exchanger as a consequence of the fact that three positive charges, carried by Na\(^+\), enter the cell for every two charges, carried by Ca\(^{2+}\), that leave the cell. We have shown previously that following a depolarizing step in ionic conditions similar to those used here, the slow “tail” current seen at the holding voltage is dominated by the exchange current (Geleason et al. 1995). A complication in this approach is that the exchange current measured in our patch-clamp configuration contains contributions from the cell body as well as the dendrites. Because direct observation (data not shown) indicated that the timecourse of [Ca\(^{2+}\)], in the cell body...
can be significantly different from that in the dendrites, the correlation of dendritic [Ca\(^{2+}\)] with whole cell exchange current is unlikely to be very meaningful. To avoid this problem, we used local perfusion of a dendrite by a small-tipped pipette to enable exchange along the dendrite under observation while it was disabled elsewhere in the cell.

Experiments of this kind were automated so that the several solution changes could be carried out reliably and the entire sequence repeated in order that the currents and [Ca\(^{2+}\)] estimates could be averaged. One experiment is illustrated in Fig. 7. Figure 7A shows currents from the whole cell along with [Ca\(^{2+}\)] in a segment of dendrite. Leak-subtracted Ca\(^{2+}\) current is shown for a 500-ms depolarization from −70 to 0 mV. The tail current, which is seen on return to −70 mV and decays over 2 s, is largely generated by Na\(^{+}\)-Ca\(^{2+}\) exchange (Gleason et al. 1995). As previously described, [Ca\(^{2+}\)] in the dendrite under examination shows a rapid rise in concentration that has almost returned to baseline within 10 s. In the ubiquitous absence of Ca\(^{2+}\)o (B), depolarization was ineffective in raising dendritic [Ca\(^{2+}\)] transient is largely derived from Ca\(^{2+}\) released from the ER. Disabling the exchanger has no effect on the [Ca\(^{2+}\)] dynamics, supporting the idea that the exchanger is chiefly a mechanism for controlling Ca\(^{2+}\) release from the ER.

FIG. 5. Emptying endoplasmic reticulum (ER) Ca\(^{2+}\) stores abolishes the Na\(^{+}\)-Ca\(^{2+}\) exchanger’s control of dendritic [Ca\(^{2+}\)] dynamics. Color panels show the time course of [Ca\(^{2+}\)] along a dendrite examined in confocal linescan mode. Stimuli were high-K\(^+\) puffs applied over the cell body for 2 s (heavy black bars). Top: 4 repetitions of the stimulus on 1 cell, alternating between normal and 0 Na\(^{+}\)o. Plots (below top panels) show the [Ca\(^{2+}\)] time course of 3 different positions along the dendrite (position shown by arrows, color spots identify each trace). Disabling the exchanger causes a prolongation of the response, though even in the first response in normal solution some distinct regions (e.g., red spot) show prolonged responses. In subsequent trials, the red spot region is no longer a [Ca\(^{2+}\)] hotspot but is actually slightly lower than its immediate surroundings. In the 1st trial, the blue spot location shows a “spontaneous” local [Ca\(^{2+}\)] increase on the falling phase of the [Ca\(^{2+}\)] transient. The green spot location shows a relatively rapid return to resting [Ca\(^{2+}\)]. Bottom: identical experimental protocol in a typical tg-pretreated cell. Transients are much reduced in amplitude and duration, implying that in dendrites untreated with tg the [Ca\(^{2+}\)] transient is largely derived from Ca\(^{2+}\) released from the ER. Disabling the exchanger has no effect on the [Ca\(^{2+}\)] dynamics, supporting the idea that the exchanger is chiefly a mechanism for controlling Ca\(^{2+}\) release from the ER.
conformational coupling between L channels and RyRs as is well established in skeletal muscle. When Ca\(^{2+}\) was removed from all of the cell except for the perfused dendrite (C), Ca\(^{2+}\) current was smaller, about one-fifth the size of the current entering the whole cell. Despite this, the magnitude of the [Ca\(^{2+}\)] transient in the dendrite was almost as great as it was.

**FIG. 6.** A spontaneous Ca\(^{2+}\) event seen in the linescan of a resting dendrite in an unclamped amacrine cell. As in Fig. 5, relative fluorescence change of Oregon Green Bapta-1 (OGB-1) from baseline (ΔF/F₀) is color mapped in the top panel. Bottom: a profile of the same event derived from the average of a 1.5-μm-wide strip (→). External solution contained Na\(^+\).

**FIG. 7.** Na\(^+\)-Ca\(^{2+}\) exchange shapes dendritic [Ca\(^{2+}\)] dynamics but generates no measurable current. In this experiment an amacrine cell was depolarized to 0 mV in a sequence of 5 conditions, shown schematically (far left). A: the initial condition in which the entire cell was bathed in normal external solution containing Na\(^+\) and Ca\(^{2+}\). Second column: the Ca\(^{2+}\) current elicited by the 500-ms step, followed by the slow inward tail current generated by Na\(^+\)-Ca\(^{2+}\) exchange. The exchange current is shown at greater magnification in the 3rd column. Far right: the [Ca\(^{2+}\)] averaged over a small (≈15 μm long) segment of dendrite for a period including the depolarization. B: the absence of any dendritic [Ca\(^{2+}\)] increase when Ca\(^{2+}\)o was removed from the entire cell. A small outward leak current (not shown) has been subtracted from all other current traces. C: Ca\(^{2+}\) has been removed from the bath but is provided to the dendrite under examination by means of a puff pipette. The inward Ca\(^{2+}\) current is ≈20% of its magnitude in A, but the [Ca\(^{2+}\)] transient is very similar to that in A, implying that significant flows of Ca\(^{2+}\) from the dendrite to the rest of the cell do not occur. The tail current shows little evidence of Na\(^+\)-Ca\(^{2+}\) exchange activity, however, consistent with the results shown in D and E. D: the exchanger is disabled over the entire cell leading to a larger and slower [Ca\(^{2+}\)] transient and a much reduced tail current. E: the same conditions apply except that exchange has been enabled in the dendrite under examination. Clearly exchange within the dendrite is important since the [Ca\(^{2+}\)] transient now has its normal kinetics. Unexpectedly though, the tail current in E is no different from that in D. All traces are the averages of 4 trials. All external solutions contained 3 μM bicuculline. For 10 cells examined, the mean times to half decay of the [Ca\(^{2+}\)] transients were, by row, A, 2.17 ± 0.92 s; C, 2.1 ± 1.07; D, 4.51 ± 1.34; E, 3.39 ± 1.60. Paired t-tests show significant differences at P = 0.01 between A and D and D and E, but not between A and C or A and E.
when Ca\(^{2+}\) bathed the entire cell, implying that lateral diffusion along the dendrite is not a significant factor in determining [Ca\(^{2+}\)] following its influx. When Na\(^{+}\) was removed from the entire cell (D), the [Ca\(^{2+}\)] transient in the dendrite was slightly larger than in normal solution and, as described earlier, the peak occurred later and the fall of [Ca\(^{2+}\)] was slower. Without Na\(^{+}\), the inward exchange current was suppressed leaving only a very small slow component to the tail that we have previously shown is due to Ca\(^{2+}\)-activated Cl\(^{-}\) and K\(^{+}\) channels (Gleason et al. 1995). In E, Ca\(^{2+}\) was present over the whole cell but Na\(^{+}\) was applied only to the dendrite under observation. The [Ca\(^{2+}\)] transient seen in the dendrite resembles that seen in normal external solution, implying that Na\(^{+}\)-Ca\(^{2+}\) exchange locally within the dendrite is able to remove Ca\(^{2+}\) from that dendrite. Surprisingly though, the tail currents seen in this condition were not measurably different (shown enlarged in Fig. 8) from those seen when Na\(^{+}\) was removed from the entire cell (D) and in none of 10 cells examined was a difference in tail currents resolvable.

This result is unexpected because on the one hand, comparison of the Ca\(^{2+}\) transients in D and E shows that Na\(^{+}\)-Ca\(^{2+}\) exchange is effective in shaping Ca\(^{2+}\) dynamics in the dendrites but on the other hand, evidence of this exchange cannot be had by looking for its signature tail current. Because the Ca\(^{2+}\) current seen in C is about one-fifth the size of the Ca\(^{2+}\) current seen for the whole cell in A, the tail current produced by the perfused dendrite would be expected to be about one-fifth the size of that of the whole cell if Ca\(^{2+}\) current and exchange current scaled proportionally. This is not the case and, within the limits set by noise in our data, the exchange current cannot be more than 1/20 of the magnitude for the whole cell. From these experiments, we conclude that Ca\(^{2+}\) current and exchange current do not scale proportionately in the cell body and the dendrites. In dendrites, a small amount of Na\(^{+}\)-Ca\(^{2+}\) exchange, so small as to be electrically undetectable, is apparently able to exert a strong influence on dendritic [Ca\(^{2+}\)] by controlling Ca\(^{2+}\) release from internal stores.

**DISCUSSION**

The experiments described here allow a rejection of the simple picture that Ca\(^{2+}\) entering through open VGCCs elevates dendritic [Ca\(^{2+}\)] that is then reduced by the action of Na\(^{+}\)-Ca\(^{2+}\) exchange and other processes. Brief depolarizing steps in voltage clamp, or K\(^{+}\) depolarizations of unclamped cells, produce dendritic [Ca\(^{2+}\)] transients in which a substantial fraction, the majority as indicated in Fig. 5, is contributed by Ca\(^{2+}\) released from the ER by CICR, rather than Ca\(^{2+}\) entering through Ca\(^{2+}\) channels. This arrangement can be usefully thought of as a Ca\(^{2+}\) amplifier, taking the small amount of Ca\(^{2+}\) entering through Ca\(^{2+}\) channels and boosting it with internally stored Ca\(^{2+}\). Positive feedback, provided by the released Ca\(^{2+}\), tends to increase the gain of the amplifier while Na\(^{+}\)-Ca\(^{2+}\) exchange works to reduce the gain.

**Na\(^{+}\)-Ca\(^{2+}\) exchange and the Ca\(^{2+}\) amplifier**

Na\(^{+}\)-Ca\(^{2+}\) exchange clearly has a major influence on dendritic [Ca\(^{2+}\)] as evidenced by the, often dramatic, effect that removal of Na\(^{+}\) has on dendritic [Ca\(^{2+}\)] dynamics. The results shown here are similar in several ways to those described by Reuter and Porzig (1995) for presynaptic terminals of hippocampal neurons, although in that study neurons were not voltage clamped. In particular, hippocampal neurons showed prolonged but variable [Ca\(^{2+}\)] dynamics in the absence of [Na\(^{+}\)], and like amacrine cells could show stable plateau [Ca\(^{2+}\)] following depolarization. A major point of difference though is in the effect of thapsigargin which is reported by Reuter and Porzig (1995) to have no significant effect, whereas in our study, it clearly produces a large reduction in both the amplitude and duration of [Ca\(^{2+}\)] following depolarization, providing sufficient time is allowed for the ER stores to empty completely.

The experiments in which forward-mode Na\(^{+}\)-Ca\(^{2+}\) exchange was disabled employed the removal of Na\(^{+}\) and the substitution of a non-transportable ion. This does not, of course, prevent reverse mode exchange in which the Na\(^{+}\)-Ca\(^{2+}\) exchanger, being thermodynamically reversible, operates so as to import Ca\(^{2+}\) while expelling cytoplasmic Na\(^{+}\). However, because removal of [Ca\(^{2+}\)]\(_{o}\) in the experiments illustrated in Fig. 2B has no effect on the falling phase of [Ca\(^{2+}\)] transients, we conclude that reverse mode exchange contributes little to dendritic [Ca\(^{2+}\)] dynamics. Because forward-mode exchange has a consistent, and in many cells dramatic, influence on [Ca\(^{2+}\)] dynamics, this conclusion might seem surprising. Two considerations reconcile these observations. First, although thermodynamics requires that exchange is reversible, the rate of exchange in reverse mode might well be low. Second, as discussed in the following text, Na\(^{+}\)-Ca\(^{2+}\) exchange influences [Ca\(^{2+}\)] in an indirect and nonlinear way.

A striking feature of our results is the variability between cells seen in experiments in which [Na\(^{+}\)]\(_{o}\) was removed (see Fig. 1). In some cells, [Ca\(^{2+}\)] dynamics were drastically affected, whereas in a minority, the effect was relatively subtle. We cannot rule out the possibility that these differences characterize different cell types because our cultures may include more than one type of amacrine cell; but we have noticed no correlation with cell morphology. In contrast to this variability, the [Ca\(^{2+}\)] dynamics seen in normal [Na\(^{+}\)]\(_{o}\) were similar in cells drastically affected by [Na\(^{+}\)]\(_{o}\) removal and those much less affected. Very likely the implications of this is that in a minority of cells, some mechanism other than Na\(^{+}\)-Ca\(^{2+}\) exchange has a leading role in moderating the gain of the Ca\(^{2+}\) amplifier.

The precise way in which Na\(^{+}\)-Ca\(^{2+}\) exchange moderates

**FIG. 8.** A higher magnification view of the tail currents from D and E in Fig. 7. These tail currents, with and without local activation of Na\(^{+}\)-Ca\(^{2+}\) exchange are indistinguishable. The small tail current seen in both is carried by Ca\(^{2+}\)-activated K\(^{-}\) and Cl\(^{-}\) currents (Gleason et al. 1995).
Ca\(^{2+}\) release from ER stores remains to be elucidated. Several pieces of evidence suggest, however, that the interaction is more intimate than simply that the exchanger reduces [Ca\(^{2+}\)], in a well-stirred cytoplasmic compartment in which the ER is subject to CICR. As shown in Fig. 7, Na\(^+-Ca^{2+}\) exchange is able to reduce dendritic [Ca\(^{2+}\)], transients, even though the summed activity of the exchanger in a dendrite, shown by its signature current, is undetectably small. One interpretation of this, illustrated diagrammatically in Fig. 9, is that the exchanger is not the chief mechanism for clearance of Ca\(^{2+}\) from a dendrite but instead operates close to the Ca\(^{2+}\) sensor of CICR where it can have a disproportionate effect on [Ca\(^{2+}\)]. Implicit in this interpretation is that the exchanger sees a higher concentration range over which the exchanger is effective. On consideration would resolve a paradox concerning the apparent magnitude calculation. It might be thought

![Diagramatic summary of the interactions between voltage-gated Ca\(^{2+}\) channels (VGCCs), the ER Ca\(^{2+}\) stores and the Na\(^+-Ca^{2+}\) exchanger (NCX) within a dendrite. The opening of VGCCs permits Ca\(^{2+}\) entry, which in turn triggers release of Ca\(^{2+}\) from the ER. The role of the exchanger is to reduce the gain of this Ca\(^{2+}\) amplifier by removing Ca\(^{2+}\) thereby moderating the positive feedback that is an inherent property of this arrangement. As argued in the text, the exchanger probably experiences a higher [Ca\(^{2+}\)] than seen in the bulk of the dendritic cytoplasm and exercises control of Ca\(^{2+}\) release by relatively small fluxes of Ca\(^{2+}\) at a location near the ER and critical to Ca\(^{2+}\)-induced Ca\(^{2+}\) release. Synaptic vesicles are shown to indicate that transmitter release will be affected by this [Ca\(^{2+}\)] in a dendrite.

A surprising feature of our results though, is that long-lasting [Ca\(^{2+}\)], gradients of several hundred nanomolar can exist in smooth dendrites without spines or boutons to act as physical barriers to diffusion. Some evidence that this is a normal feature of amacrine cell physiology comes from a recent study using 2 photon microscopy to view cells in the intact retina (Denk and Detwiler 1999) in which amacrine cell dendrites are reported to show very local differences in [Ca\(^{2+}\)], when receiving normal synaptic input. Another finding of that study is that, as we report, local spontaneous increases in [Ca\(^{2+}\)], are sometimes seen. At least some of these probably represent elementary events from which more long-lasting [Ca\(^{2+}\)], transients are built up. Inspection of long-lasting [Ca\(^{2+}\)], transients, like those in Fig. 5, usually fails to reveal a temporal structure consistent with this idea but closer investigation is required to resolve this question.

Hotspots for [Ca\(^{2+}\)] in amacrine dendrites very likely correspond to sites of Ca\(^{2+}\) release through RyRs and possibly InsP3Rs. The identity of cool spots is less certain but might include the SERCA pumps of the ER itself. SERCA pumps are thought not to be co-located with RyRs, and there is growing evidence showing that the ER is quite inhomogeneous with regard to its Ca\(^{2+}\) handling molecules (Golovina and Blaustein 1997; reviewed in Meldolesi and Pozzan 1998; Pozzo-Miller et al. 2000). A recent study of Ca\(^{2+}\) handling in DRG neurons divides Ca\(^{2+}\) handling by the ER at low and moderate loads into two modes (Hongpaisan et al. 2001). At the lowest Ca\(^{2+}\) loads, the ER is a net importer of cytoplasmic Ca\(^{2+}\), whereas at higher loads the ER is a net exporter of Ca\(^{2+}\). We suggest that in those stable patterns such as Fig. 3A, the ER is balanced between these two modes, possibly allowing Ca\(^{2+}\) tunneling between the sinks and sources for Ca\(^{2+}\) in the ER (Petersen et al. 1999).

Ca\(^{2+}\) flux implied by local gradients

When Na\(^+-Ca^{2+}\) exchange is disabled, Ca\(^{2+}\) influx can generate stable spatial patterns of [Ca\(^{2+}\)]. It might be thought that the existence of standing gradients like that illustrated in Fig. 3A because they imply continuously active sources and sinks would place an unreasonable energetic burden on a cell and quickly empty internal stores. Neither of these suppositions is likely to be true because the fluxes required to sustain the observed gradients are small as shown in this order-of-magnitude calculation.

In the steady state, immobile buffers have no influence on flux, thus the total flux, \(J_T\), between a source and a sink is the
sum of the flux of free Ca\(^{2+}\) plus the flux of Ca\(^{2+}\) bound to mobile buffer
\[
J_F = J_{Ca} + J_{CaB}
\]

For simplicity, we neglect intrinsic mobile buffers and assume that Fura-2 is the only mobile buffer and is present at a total concentration, \([B]_T\), of 500 \(\mu M\) with a \(K_D\) of 200 \(nM\) and a diffusion coefficient \(D_B = 100 \mu m^2/s\) that we assume is identical for the bound and free forms of Fura-2.

For a constant flux, \(J_F\), in a one-dimensional dendrite, Fick's equation can be integrated and rearranged to yield
\[
J_F = -D_{Ca} \frac{\Delta [Ca^{2+}]}{\Delta x} - D_{CaB} \frac{\Delta [CaB]}{\Delta x}
\]

Provided the binding kinetics of Fura-2 are rapid compared to diffusion (Smith et al. 1996; Wagner and Keizer 1994), Ca\(^{2+}\) will be in equilibrium with Fura-2 everywhere, and the concentration of Ca\(^{2+}\) bound to Fura-2, \([CaB]\), can be obtained from
\[
[CaB] = \frac{[Ca^{2+}]}{[Ca^{2+}] + K_B [B]_T}
\]

Considering a typical \([Ca^{2+}]\) gradient of 200 nM over a distance, \(\Delta x\), of 5 \(\mu m\), then \(\Delta [CaB] = 83 \mu M\), and the associated fluxes, assuming \(D_{Ca} = 300 \mu m^2/s\), are
\[
J_{Ca} = 12 \times 10^{-21} \text{mol} / \mu m^2/s
\]
\[
J_{CaB} = 1.66 \times 10^{-21} \text{mol} / \mu m^2/s
\]

Note that the flux due to free Ca\(^{2+}\), \(J_{Ca}\), is <1% of the total flux. Most of the Ca\(^{2+}\) is carried by Fura-2. We can express the total Ca\(^{2+}\) flux in a more intuitive form, as the Ca\(^{2+}\) current at the source
\[
I_F = 2F \times A \times J_F
\]

where \(F\) is Faraday's constant.

Therefore, in a dendrite with a cross sectional area, \(A = 1 \mu m^2\), the total current at the source is ~0.33 pA. This current is close to the estimate of 0.35 pA for the current through a single RyR (Mejia-Alvarez et al. 1999) and 44 times less than the peak current in a Ca\(^{2+}\) spark of skeletal muscle (Rios et al. 1999).

Synaptic transmission

What function might be served by the local release and uptake of Ca\(^{2+}\) in amacrine dendrites? In the dendrites of hippocampal pyramidal cells and cerebellar Purkinje cells, local [Ca\(^{2+}\)] regulation has been linked to long-term changes in synaptic efficacy (Nishiyama et al. 2000) and gene expression (Deisseroth et al. 1996). These mechanisms may also be relevant to amacrine cell dendrites but a peculiarity of amacrine cells is that, lacking an axon, dendrites act as pre- as well as postsynaptic structures, suggesting that local [Ca\(^{2+}\)] release controls local release of transmitter. Strong support for this supposition is provided by experiments showing that disabling Na\(^{+}\)-Ca\(^{2+}\) exchange extends the duration of asynchronous transmitter release (Gleason et al. 1994). Recent studies of both hippocampal CA3 pyramidal cell presynaptic boutons (Emptage et al. 2001), and Purkinje neurons from the cerebellum (Llano et al. 2000) suggest that in those neurons as well, Ca\(^{2+}\) release is coupled to transmitter release.

An implication of the work described here is that presynaptic sites, even when close together, might have different transmission characteristics. If, as we suggest here, the exchanger enjoys a privileged location relative to the ER, we speculate that local regulation of Na\(^{+}\)-Ca\(^{2+}\) exchange could be used to locally influence transmitter release. Clearly the location of synapses relative to the ER and the exchanger will be an important factor in this and remains to be determined. Small alterations in the exchanger, by phosphorylation, or allosteric binding of ATP (DiPolo and Beaugé 1999), would be expected to change the gain of Ca\(^{2+}\) amplification and thereby affect the time course of synaptic transmission. A clear expectation is that, because the rate of Na\(^{+}\)-Ca\(^{2+}\) exchange is voltage dependent (DiPolo et al. 1985; Hayashida et al. 1998; Kimura et al. 1987), CICR ought therefore to be voltage dependent in a way separate from the voltage dependence of Ca\(^{2+}\) influx. This possibility could lead to a multiplicative interaction between depolarization and Ca\(^{2+}\) influx on transmitter release.

Synaptic transmission is usually thought of as a process in which widespread voltage changes in a neuron are coupled rather rigidly to the influx of Ca\(^{2+}\) and the subsequent release of transmitter. Our results here, because they identify a local Na\(^{+}\)-Ca\(^{2+}\) amplification step, imply a looser coupling between membrane voltage and the Ca\(^{2+}\) available for transmitter release. Na\(^{+}\)-Ca\(^{2+}\) exchange is one mechanism that can influence this step but very likely there are others, including local [Ca\(^{2+}\)] increases brought about by the local action of transmitter acting on both ionotropic and metabotropic receptors. From this perspective, the mixing of pre and post synaptic sites along an amacrine dendrite would effectively allow a single amacrine cell to behave as multiple processing units loosely coupled to each other by membrane voltage but independent by virtue of their local [Ca\(^{2+}\)] regulation.

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