Structural and functional relationships between Ca^{2+} puffs and mitochondria in *Xenopus* oocytes

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Marchant, Jonathan S., Viviana Ramos, and Ian Parker. Structural and functional relationships between Ca^{2+} puffs and mitochondria in *Xenopus* oocytes. *Am J Physiol Cell Physiol* 282: C1374–C1386, 2002. First published February 6, 2002; 10.1152/ajpcell.00446.2001.— Ca^{2+} uptake and release from endoplasmic reticulum (ER) and mitochondrial Ca^{2+} stores play important physiological and pathological roles, and these processes are shaped by interactions that depend on the structural intimacy between these organelles. Here we investigate the morphological and functional relationships between mitochondria, ER, and the sites of intracellular Ca^{2+} release in *Xenopus laevis* oocytes by combining confocal imaging of local Ca^{2+} release events (“ Ca^{2+} puffs”) with mitochondrial localization visualized using vital dyes and subcellularly targeted fluorescent proteins. Mitochondria and ER are localized in cortical bands ~ 6 – 8 μm wide, with the mitochondria arranged as densely packed “islands” interconnected by discrete strands. The ER is concentrated more superficially than mitochondria, and the mean separation between Ca^{2+} puff sites and mitochondria is ~ 2.3 μm . However, a subpopulation of Ca^{2+} puff sites is intimately associated with mitochondria ($\sim 28\%$ within <600 nm), a greater number than expected if Ca^{2+} puff sites were randomly distributed. Ca^{2+} release sites close to mitochondria exhibit lower Ca^{2+} puff activity than Ca^{2+} puff sites in regions with lower mitochondrial density. Furthermore, Ca^{2+} puff sites in close association with mitochondria rarely serve as the sites for Ca^{2+} wave initiation. We conclude that mitochondria play important roles in regulating local ER excitability, Ca^{2+} wave initiation, and, thereby, spatial patterning of global Ca^{2+} signals.

endoplasmic reticulum; calcium release

FUNCTIONAL INTERACTIONS between Ca^{2+} stores within the endoplasmic reticulum (ER) and mitochondria play important roles in determining the kinetics and, thereby, the physiological action of cellular Ca^{2+} signals (for reviews see Refs. 10, 16, 37, and 39). Ca^{2+} release from inositol 1,4,5-trisphosphate (IP_3) or ryanodine-sensitive ER Ca^{2+} stores results in an increased intramitochondrial Ca^{2+} concentration (8, 18, 36, 38, 40, 47), which upregulates mitochondrial metabolism and ATP production (18, 27, 36, 41). Reciprocally, mitochondrial Ca^{2+} sequestration influences the spatio-temporal profile of cytosolic Ca^{2+} signals generated by

Ca^{2+} release and Ca^{2+} entry pathways (12, 20, 22, 45, 49). In particular, mitochondrial Ca^{2+} uptake slows the rise and accelerates the decline of cytosolic Ca^{2+} transients, while delayed Ca^{2+} extrusion from mitochondria slows the return of cytoplasmic Ca^{2+} concentration to its resting level (12, 19). Because the gating of IP_3 receptors and ryanodine receptors is Ca^{2+} sensitive, perturbation of local Ca^{2+} levels by adjacent mitochondria is likely to influence the probability of their activation (17) as well as their interactions through Ca^{2+} -induced Ca^{2+} release to establish a propagating Ca^{2+} wave. Furthermore, in situations where mitochondria are exposed to high levels of cytosolic Ca^{2+} , mitochondria can amplify IP_3 -evoked Ca^{2+} release through concurrent cycles of mitochondrial depolarization and Ca^{2+} release triggered by Ca^{2+} -stimulated gating of the permeability transition pore (21). Finally, the level of mitochondrial ATP production may influence the activity of Ca^{2+} -ATPases that refill intracellular Ca^{2+} stores as well as the local sensitivity of intracellular Ca^{2+} release channels (23, 25).

As a result of this functional interrelationship between mitochondria and ER Ca^{2+} stores, attention has focused on the structural basis of these interactions. Given the presumed low affinity of mitochondrial Ca^{2+} uptake mechanisms (14, 37) and the demonstration of large increases in mitochondrial matrix Ca^{2+} concentration on cell stimulation (16, 37–40), ER Ca^{2+} release sites and mitochondria have been hypothesized to be closely apposed. Indeed, evidence from many cell types suggests that a portion of the cellular mitochondrial network is intimately associated with ER membranes. For example, electron micrographs in smooth muscle (31), cardiac muscle (43), neurons (42), oocytes (48), and hepatic tissue (44) show that ER and mitochondria membranes can be as close as 10–300 nm. Most elegantly, by employing ER- and mitochondria-targeted GFP fusion constructs with high-resolution fluorescence microscopy, Rizzuto et al. (40) imaged the three-dimensional morphological relationship between these organelles. Their results in HeLa cells show that ER and mitochondria form a finely intertwined network, with ~ 5 – 20% of the surface of the mitochondrial

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network in close apposition (<60 nm) to the ER (40, 41). These morphological estimates are consistent with functional measurements employing Ca^{2+} buffers to interfere with the transmission of Ca^{2+} signals between the ER and mitochondria, which points to an average separation in the 100-nm range (6, 47).

However, because only a small part of the ER network appears to be in close apposition to the mitochondria, it is important to determine whether active Ca^{2+} release channels are clustered within such regions. High-resolution confocal Ca^{2+} imaging (33, 46, 51) and electrophysiological analysis (24) have shown that IP_3 receptors are not homogeneously distributed throughout the ER but, rather, are clustered to form discrete Ca^{2+} release sites. These sites generate local, "elementary" Ca^{2+} signals during stimulation with submaximal IP_3 concentrations (33, 46, 51). Therefore, the following questions arise: 1) What proportion of active Ca^{2+} release sites are in intimate association with mitochondria? 2) What might the functional consequences be for the generation of local and global Ca^{2+} signals?

The *Xenopus laevis* oocyte provides an excellent system in which to investigate these questions. First, confocal imaging techniques have resolved a microscopic architecture of local IP_3 -dependent Ca^{2+} signals, " Ca^{2+} puffs" (33), which represent the activity of several IP_3 receptors clustered within a functional Ca^{2+} release unit (24, 33, 46, 51). These signals arise at fixed positions in the ER over long periods of time (46), facilitating their mapping relative to the morphological architecture of the cell. Second, functional interactions between mitochondrial activity and global Ca^{2+} signals have been demonstrated in the *Xenopus* oocyte, where intracellular injection of oxidizable substrates increased the amplitude, velocity, and period of repetitive IP_3 -evoked Ca^{2+} waves (22) and disrupted their spatial patterning (11).

In this study, we exploit these advantages of the *Xenopus* oocyte as a model system to map Ca^{2+} puff sites in relation to mitochondria. By correlating these morphological data with functional analyses of Ca^{2+} puff behavior, we show that Ca^{2+} release sites located close to mitochondria exhibit a lower frequency of Ca^{2+} puffs than sites in regions where mitochondria are scarce. Ca^{2+} puff sites close to mitochondria rarely trigger Ca^{2+} waves; instead, waves are initiated at sites with low surrounding mitochondrial density. These results suggest that mitochondrial Ca^{2+} handling plays an important role in determining the local excitability of the ER as well as the global cellular architecture of IP_3 -evoked Ca^{2+} release.

MATERIALS AND METHODS

Preparation and Use of *Xenopus* Oocytes

Experiments were performed using defolliculated *X. laevis* oocytes, isolated as described previously (26, 46). Female frogs were anesthetized by immersion in a 0.15% solution of MS-222 (3-aminobenzoic acid ethyl ester) for 20 min, and several ovarian lobes were removed by surgery. Epithelial

cell layers were removed from the oocytes manually using watchmakers' forceps and then briefly (30 min) exposed to collagenase (0.5 mg/ml; type I, Sigma) for removal of follicular cells. Defolliculated oocytes were cultured at 16°C in Barth's solution [88 mM NaCl, 1 mM KCl, 2.4 mM NaHCO_3 , 0.83 mM MgSO_4 , 0.33 mM $\text{Ca}(\text{NO}_3)_2$, 0.41 mM CaCl_2 , 10 mM HEPES, 550 mg/l sodium pyruvate, 0.05 mg/ml gentamicin]. For Ca^{2+} imaging studies, oocytes were microinjected 30 min before recording with Oregon green 488-1,2-bis(2-aminophenoxy)ethane-*N,N,N',N'*-tetraacetic acid-1 (OG-1, dissociation constant for Ca^{2+} of ~ 170 nM) or fura red (dissociation constant for Ca^{2+} of ~ 140 nM) to final intracellular concentrations of ~ 40 and ~ 200 μM , respectively, together with caged IP_3 (5 μM final concentration). Confocal Ca^{2+} imaging was performed using oocytes incubated in Barth's solution at room temperature from albino and pigmented donors.

For expression studies, a Drummond microinjector was used to inject plasmid cDNA (~ 2 μg in 5 nl) encoding an ER-targeted yellow fluorescent protein (ER-EYFP) and a mitochondrially targeted red fluorescent protein (mito-dsRED) into the nucleus of pigmented *X. laevis* oocytes. Injected oocytes were separated into 96-well plates and incubated in Barth's solution, with repeated changes of solution at least every 12 h. Control oocytes from the same donor frogs were injected with the same volume of intracellular solution alone (in mM: 140 KCl, 10 HEPES, 3 MgCl_2 , 1 EGTA, 0.5 CaCl_2). Typically, the distributions of ER-EYFP [wavelength (λ) $> 530 \pm 20$ nm] and mito-dsRED ($\lambda > 620$ nm) were imaged ≥ 48 h after injection, and the intensity of fluorescence expression in the control cells was $<1\%$ of that in positively expressing oocytes.

For labeling of mitochondria, oocytes were incubated in 100 nM MitoTracker Green (MTG) or 500 nM MitoTracker Red (MTR) for 30–60 min on a rotator at room temperature and then washed for 30 min with two changes in Barth's solution to remove extracellular dye. For 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolecarbocyanine iodide (JC-1) staining, oocytes were incubated in 10 $\mu\text{g}/\text{ml}$ JC-1 for up to 30 min. For $\text{C}_{26}\text{H}_{27}\text{ClN}_2\text{O}_7$ [tetramethyl rhodamine ethyl ester (TMRE)] staining, background staining of oocyte lipid was noticeably high and was best minimized by incubating for short periods of time (<10 min) with much higher concentrations of dye (up to 1 μM) than routinely used for staining mitochondria in mammalian cells. Emitted fluorescence was then monitored at $\lambda > 560$ nm for TMRE and by dual emission ($\lambda = 530 \pm 20$ nm and $\lambda > 560$ nm) for JC-1. For MTG, OG-1, and ER-EYFP, emitted fluorescence was monitored at $\lambda = 530 \pm 20$ nm. For MTR, fura red, and mito-dsRED fluorescence, fluorescence emission was collected with a 620-nm long-pass filter.

Confocal Imaging

Confocal imaging was performed using custom-built slow-scan (32) and video-rate confocal microscopes (3). The slow-scan system was used in line-scan (x - t), frame-scan (x - y), and axial-scan (x - z) modes. Fluorescent signals in line-scan mode were monitored along a fixed line formed by repeated scans of a focused laser spot from an argon ion laser ($\lambda = 488$ nm), with emitted fluorescence monitored through confocal pin holes using avalanche diode photon counting modules. For frame scanning (x - y), the scan line was driven in the lateral plane using a motor linked to the confocal scan head mount, and for axial scans (x - z), a motorized focus unit was used to drive the scan line in the vertical plane (32).

The video-rate system employed a resonant scanner coupled to an Olympus IX50 microscope to image a $65\text{-}\mu\text{m}^2$

region (450×450 pixels; 30 Hz) focused at various depths into the oocyte (3). Excitation was by a 488-nm laser line, and fluorescence emission was detected by a photomultiplier through a confocal aperture providing an optical section of $\sim 1 \mu\text{m}$. The lateral resolution of this system is $\sim 0.3 \mu\text{m}$ (3). This system was also used in video-rate axial (x - z) mode by turning off the y -scan mirror and, instead, focusing the objective lens at a rate of ~ 30 Hz using a piezoelectric transducer (3).

For Ca^{2+} imaging experiments on both microscopes, flashes of ultraviolet light (340–400 nm) from a mercury arc lamp were used to photorelease IP_3 from a caged precursor over a wide field area ($\sim 150 \mu\text{m}$ diameter) centered around the laser scan. Ca^{2+} images are displayed as ratios ($\Delta F/F_0$), depicting the change in fluorescence at a pixel during the response (ΔF) relative to the resting fluorescence at the same pixel before stimulation (F_0).

Cell Culture

3T3 fibroblasts were grown (37°C , 5% CO_2) in Dulbecco's modified Eagle's medium containing L-glutamine (4 mM), sodium bicarbonate (1.5 g/l), glucose (4.5 g/l), and 10% bovine calf serum. Cells were grown on poly-D-lysine-coated petri dishes, stained with 50 nM MTG for 20 min, and subsequently imaged by confocal microscopy at room temperature.

Materials

MTG ($\text{C}_{34}\text{H}_{28}\text{Cl}_5\text{N}_3\text{O}$), MTR ($\text{C}_{32}\text{H}_{33}\text{ClN}_2\text{O}$), JC-1, OG-1, fura red, caged IP_3 (caged *myo*- IP_3), and TMRE were obtained from Molecular Probes (Eugene, OR), nocodazole from Calbiochem (La Jolla, CA), ER-EYFP and mito-dsRED from Clontech, and 3T3 fibroblasts from American Type Culture Collection (CCL-92). All other reagents were obtained from Sigma Chemical (St. Louis, MO).

RESULTS

To investigate mitochondrial organization in the *Xenopus* oocyte, we first established the basic morphology and distribution of mitochondria using confocal microscopy and then proceeded to correlate mitochondrial localization with the spatial distribution and functional properties of IP_3 -evoked Ca^{2+} release sites in the ER.

Staining of Mitochondria in the *X. laevis* Oocyte

We examined the suitability of a variety of fluorescent dyes (Figs. 1 and 2), as well as subcellularly targeted fluorescent protein variants (Fig. 3), to selectively stain mitochondria in the *Xenopus* oocyte. With the vital dyes, the selectivity of mitochondrial staining was consistently better with MTG than with MTR; both of these dyes exhibited far less background staining than JC-1 or TMRE, which accumulated within lipid vesicles. The basis of this difference is unknown but may relate to the dependence on mitochondrial membrane potential for dye accumulation. TMRE and JC-1 are potentiometric stains, while MitoTracker staining, especially with MTG, appears to be less sensitive to membrane potential (35). We also used mito-dsRED to visualize mitochondrial organization (Fig. 3). Although the specificity of staining was presumably improved, two factors limited the utility of mito-dsRED

as a tool for studying mitochondrial morphology. First, the time for maturation of red fluorescence (1) was considerably longer (maximal fluorescence ~ 100 h after injection) than that for visualization of ER-EYFP fluorescence (< 30 h) and well within a time frame in which oocyte viability is decreasing after nuclear cDNA injection. Second, in accordance with the tendency of dsRED to aggregate (1), mitochondrial organization appeared less well defined than that observed with any of the vital stains.

Organization of Mitochondria Within the *X. laevis* Oocyte Cortex

Oocytes stained with MTG were imaged using a line-scan confocal microscope (32) operated in axial (x - z) or lateral (x - y) scanning modes. Low-magnification axial scans into oocytes from albino frogs revealed a cortical band of fluorescence that extended several micrometers into the animal and vegetal hemisphere of all oocytes examined (Fig. 1A). In higher-magnification axial scans from albino oocytes (50- μm laser scan line, Fig. 1, B and C), the peak fluorescence was consistently brighter in the vegetal than in the animal hemisphere (vegetal peak fluorescence was $148 \pm 11.0\%$ of that in the animal pole, $n = 30$ cells, 3 donors), although the width of the mitochondrial band was similar in the vegetal [$6.6 \pm 0.4 \mu\text{m}$, full width at half-maximal intensity (FWHM)] and animal hemispheres ($6.2 \pm 0.4 \mu\text{m}$). However, these estimates of organelle distribution are probably limited by decreases in optical resolution with increasing depth into the turbid cytoplasm of the oocyte. Similar results were obtained in albino oocytes stained with MTR (data not shown).

In pigmented oocytes, relatively little mitochondrial staining could be detected in the animal hemisphere. However, it is likely that the heavy pigmentation in the animal pole attenuates fluorescence emission from the mitochondria located there, and not that the asymmetric distribution of mitochondria is more pronounced in pigmented oocytes. Consistent with this, treatment of pigmented oocytes with the microtubule depolymerizing drug nocodazole (10 μM , 6–10 h), which disorganizes the pigment distribution across the animal pole, revealed mitochondrial structures that appeared brighter and more radially extended than those in control cells (data not shown).

Lateral (x - y) images obtained at various focal depths within the vegetal hemisphere of pigmented and albino oocytes revealed a similar morphology of mitochondrial organization (Fig. 1D). At the depth of peak mitochondrial fluorescence (0 in Fig. 1D, ~ 5 – $8 \mu\text{m}$ inward from the highly convoluted oocyte plasma membrane), MTG staining revealed large "island" masses comprising many densely packed intertwining strands (Fig. 2A). Individual masses appeared to be interconnected by one or more individual strands, thereby establishing an extensive mitochondrial network. In more superficial sections (labeled in positive micrometer increments), individual mitochondria extended upward from the densely packed islands, ramifying radially

Fig. 1. Confocal imaging of mitochondria in *Xenopus laevis* oocyte. **A**: low-magnification axial confocal scan ($\times 10$ dry objective) of MitoTracker Green (MTG) fluorescence in an albino oocyte. Oocyte was positioned with its equator resting on the microscope cover glass (flattened side at *right*), and the animal-vegetal axis was previously identified by transillumination of the germinal vesicle in the animal hemisphere. Regions enclosed in boxes correspond to location of scan areas in **B**. **B**: high-magnification axial scans in vegetal and animal hemispheres. These and other axial scan images represent a “sideways” view into the oocyte, depicted with the oocyte membrane near the *top* and the interior of the oocyte toward the *bottom* of each image. **C**: profiles of average fluorescence intensity from images such as those in **B** showing radial distribution of mitochondria in animal (red) and vegetal (blue) poles of the albino oocyte. Traces represent fluorescence intensity relative to depth, averaged across the 50-μm laser line. Decline in fluorescence with increasing depth into the cell results, in part, from increasing absorption and scatter of light. **D**: stack of lateral (x - y) confocal images showing mitochondrial morphology within a 50×50 -μm region, recorded at successive 1-μm depths into the vegetal pole (blue numbers), starting ~ 3 μm into the cell, where mitochondria are first resolved and numbered relative to the peak MTG fluorescence. Stack depth (blue lines) corresponds to region illustrated in **B**.

outward toward the plasma membrane. In deeper cross sections, the mitochondrial islands extended downward into the oocyte beyond the limit of our resolution (~ 15 μm into the oocyte, owing to the optical turbidity of the lipid-filled cytoplasm).

Several lines of evidence suggest that MTG faithfully reports mitochondrial morphology in *Xenopus* oocytes. First, the fluorescence-staining pattern in the oocyte is similar to the morphology reported by other cell-permeant mitochondrial dyes. Figure 2 shows the similar staining pattern at the level of the mitochondrial islands in oocytes incubated with MTG (Fig. 2A), MTR (Fig. 2B), TMRE (Fig. 2C), and JC-1 (Fig. 2D). Second,

the staining pattern of individual mitochondria with MTG closely resembles that observed in mammalian cell types; e.g., 3T3 fibroblast cells (Fig. 2F, *inset*). The width of individual mitochondrial strands measured in the oocyte ($\text{FWHM} \sim 0.44 \pm 0.08$ μm; Fig. 2E) is consistent with measurements of mitochondrial size from mammalian cells ($\text{FWHM} \sim 0.3$ μm; Fig. 2F).

Morphological Relationship of ER and Mitochondria

To visualize the distribution of ER relative to mitochondria, we expressed ER-EYFP together with mtdsRED by injecting appropriate cDNA constructs into

Fig. 2. Mitochondrial morphology in the oocyte as revealed with different probes. Mitochondrial islands in the vegetal hemisphere of an albino *Xenopus* oocyte were stained with MTG (A), MitoTracker Red (MTR, B), 1 μM tetraethyl rhodamine ethyl ester (TMRE) for 10 min (C), and 10 $\mu\text{g/ml}$ 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolecarbocyanine (JC-1) for 30 min (D). All images were acquired using the line-scan confocal microscope in frame-scan mode. JC-1 image is a dual-color overlay corresponding to the dual emission of this vital stain [green fluorescence, wavelength (λ) = 530 ± 20 nm; red fluorescence, $\lambda > 560$ nm]. E: enlarged region of mitochondria from an MTG-stained oocyte, with associated fluorescence profile averaged along a 3-pixel ($\sim 0.1 \mu\text{m}$)-wide line (red line). F: enlarged region from a 3T3 fibroblast (yellow box) stained with MTG, with associated fluorescence profile (red line).

the nuclei of pigmented oocytes. Oocytes expressing both green and red fluorescence were selected ($n = 20$ cells), and the morphology of both organelle networks was visualized by confocal imaging with dual-wavelength emission. In axial (x - z) scans in the vegetal hemisphere, ER-EYFP fluorescence was distributed in a band $8.2 \pm 0.5 \mu\text{m}$ wide (Fig. 3A; FWHM). The red mitochondrial fluorescence was similarly localized to a band $7.9 \pm 0.3 \mu\text{m}$ wide, with the peak mitochondrial intensity located, on average, $3.3 \pm 1.4 \mu\text{m}$ deeper within the oocyte than the peak ER fluorescence (Fig. 3A). This axial separation of ER and mitochondria was further confirmed in oocytes ($n = 6$ cells) expressing ER-EYFP alone after staining with MTR (mitochondrial peak fluorescence was $2.5 \pm 0.4 \mu\text{m}$ deeper within the oocyte than the peak ER fluorescence intensity).

Figure 3, B and C, illustrates lateral (x - y) sections showing dual-fluorescence overlays taken at the level of peak ER-EYFP fluorescence (green arrow, Fig. 3A)

and at the level of peak mito-dsRED fluorescence (red arrow, Fig. 3A). At the depth into the cell where the anastomosing ER network was most dense (Fig. 3B), mitochondrial tubules were sparsely distributed and predominantly orientated as individual strands projecting outward from the deeper mitochondrial islands. In deeper sections (Fig. 3C), ER and mitochondrial fluorescence were more closely associated, with large areas devoid of either organelle that were presumably filled by lipid vesicles in the ooplasm.

Mitochondria in Xenopus Oocytes Are Relatively Immobile

To map the relative locations of mitochondria and Ca^{2+} release sites in the oocyte, we first needed to establish that both of these structures remain immobile throughout the time required (< 3 min) for imaging. Using line-scan (46) and video-rate (26) confocal

Fig. 3. Relative localization of mitochondria and endoplasmic reticulum (ER). A: dual-wavelength axial (x - z) section within the vegetal hemisphere of a pigmented oocyte previously (72 h) microinjected with cDNA encoding ER-targeted yellow fluorescent protein (EYFP, green) and mitochondrially targeted red fluorescent protein (mito-dsRED, red). Profiles represent respective averaged fluorescence intensities across the 50- μm laser-scan line with depth into the cell. Arrows, depth of peak mitochondrial and ER fluorescence. B and C: lateral (x - y) images showing fluorescence of ER-EYFP (green) and mito-dsRED (red) at the level of peak ER fluorescence (green arrow in A) and peak mitochondrial fluorescence (red arrow in A).

microscopy, we previously showed that Ca^{2+} puffs can be reproducibly mapped to discrete locations in the cytoplasm, with the same sites repeatedly responding from the same fixed positions over long periods (≤ 20 min).

To assess mitochondrial motility, we compared high-magnification ($10 \times 10\text{-}\mu\text{m}$ regions) time-lapse images of mitochondria stained with MTG in 3T3 fibroblasts with similar images from *Xenopus* oocytes (Fig. 4). Figure 4 shows two montage panels of successive images (3 s apart) of mitochondria in a 3T3 fibroblast (Fig. 4A) and a *Xenopus* oocyte (Fig. 4B). In the fibroblast, a large degree of mitochondrial movement was evident, with existing mitochondria moving around within the confocal plane and previously out-of-focus mitochondrial strands entering and traversing the section during the recording period. To better convey the extent of mobility, up to 180 individual frames from a 3-min time-lapse stack (1 s/frame) were projected as a maximal-intensity overlay on top of the original image of mitochondrial location. Colored regions represent the original mitochondrial position, whereas white areas reflect the extent of organellar travel during the recording period. The same algorithm was applied to records from 3T3 fibroblasts (Fig. 4C) and *Xenopus* oocytes (Fig. 4D). Figure 4, C and D, shows that whereas mitochondria are dynamic organelles in fibro-

blasts (even at room temperature), mitochondrial position in the oocyte is largely static. These data reporting a predominantly static location of mitochondria and puff sites over the time frame of our experiments facilitated our attempts to map their respective positions.

Localization of Ca^{2+} Puff Sites With Mitochondria by Confocal Microscopy

To correlate the localization of mitochondria with the sites of Ca^{2+} release from the ER, we used two different methods, each of which afforded specific advantages. First, we exploited the high spatial resolution [$\sim 0.3\text{-}\mu\text{m}$ lateral resolution (32)] of the line-scan confocal microscope to map elementary Ca^{2+} release sites (Ca^{2+} puffs) relative to mitochondria. This afforded the highest-resolution measurements but has the drawback that information is restricted to a single spatial dimension (the laser-scan line), making it difficult to appreciate the three-dimensional relationship between sites of Ca^{2+} release and mitochondria. Therefore, as an additional method, we employed a video-rate confocal microscope used in lateral (x - y) and axial (x - z) scanning modes (3). Although the spatial and temporal resolution of the video-rate system was poorer than that of the line-scan system, the ability to image rapidly (30 Hz) in two spatial dimensions facilitated data

Fig. 4. Confocal imaging of mitochondrial movement in 3T3 fibroblasts and *Xenopus* oocytes. Mitochondria stained with MTG in a 3T3 fibroblast (A) and the vegetal hemisphere of a *Xenopus* oocyte (B) were imaged at 3-s intervals. Arrows, clear movements of mitochondria. C and D: maximum-intensity overlay projections, derived from 1 min (60 frames) of recording in a 3T3 fibroblast (C) and from 180 frames (3 min of recording) in an oocyte (D). Each image is superimposed with a color image of the original mitochondrial position at the start of each recording interval. White areas represent extent of mitochondrial movement within the confocal plane during recording.

measurement and reconstruction of the three-dimensional organization of Ca^{2+} puff sites in relation to mitochondrial morphology.

Line-scan experiments. To allow simultaneous monitoring of the localization of mitochondria and Ca^{2+} puff sites, we used combinations of fluorescent dyes with well-separated fluorescence emission spectra. For the line-scanning experiments, we monitored green fluorescence from mitochondria stained with MTG, together with Ca^{2+} -dependent changes in red fluorescence emission of fura red (note that fura red fluorescence decreases on binding Ca^{2+}). Figure 5A shows fluorescence line-scan images from oocytes microinjected with caged IP_3 together with fura red. Whereas strong photolysis flashes evoked a Ca^{2+} wave shortly after the photolysis flash (Fig. 5A, top), weaker photolysis flashes (~ 50 – 80% of the intensity that evoked a Ca^{2+} wave) evoked discrete, localized Ca^{2+} puffs at various positions along the laser-scan line (Fig. 5A, bottom).

To image Ca^{2+} puff sites and mitochondria, an x -y image of mitochondrial distribution (MTG, $\lambda = 530 \pm 20$ nm) was first obtained with the confocal section focused at a depth corresponding to the mitochondrial islands (~ 5 – 8 μm into the oocyte; Fig. 5B). The microscope was then switched to line-scan mode, and fura red fluorescence ($\lambda > 620$ nm) was monitored at the same depth along a scan line at a defined lateral (y) position on the mitochondrial x -y image. Analysis was restricted to bright, sharply resolved Ca^{2+} release events, which presumably arise from Ca^{2+} puff sites located on, or very close to, the laser-scan line. The x -coordinate of the center of the fluorescence signal

from individual Ca^{2+} puffs (presumably corresponding to the site of Ca^{2+} release) was then transposed onto the mitochondrial image (Fig. 5B), and the lateral distance from the edge of the nearest mitochondrial fluorescence was recorded (whether mitochondrial island or discrete mitochondrial strand). The resulting distribution from a total of 292 discrete Ca^{2+} puff sites is shown in Fig. 5C (21 oocytes, 5 donor animals). This was compared with the distribution expected if Ca^{2+} puff sites were randomly located throughout the imaging field, without any preferred association with mitochondria (Fig. 5C). The mean separation between Ca^{2+} puff sites and mitochondria was slightly smaller (2.33 μm) than expected from a random distribution (2.80 μm), owing to a larger proportion of Ca^{2+} puff sites closely juxtaposed to mitochondria (50.2% of Ca^{2+} puff sites within <2 μm of a mitochondrion compared with only 40.0% expected from the control distribution).

Video-rate lateral scan (x -y) imaging. For video-rate experiments, we monitored red fluorescence from mitochondria stained with MTR, together with Ca^{2+} -dependent increases in green fluorescence emission of OG-1. Repeated photolysis flashes were used to evoke Ca^{2+} puffs at numerous sites throughout the imaging field during a 3-min recording period. The locations of Ca^{2+} puffs were then mapped onto a composite mitochondrial image formed as a maximum-intensity projection of confocal sections at the depth used for Ca^{2+} imaging and at 0.5 μm above and below the site (Fig. 6A). Measurements of the lateral separation between individual Ca^{2+} puff sites and the edge of the nearest mitochondrial fluorescence are shown in Fig. 6B, together with a control distribution derived from ran-

Fig. 5. Mapping of Ca^{2+} puff sites relative to mitochondria using line-scan confocal microscopy. A: line-scan (x - t) images of Ca^{2+} release resolved using fura red showing a Ca^{2+} wave evoked by photorelease (arrowheads) of a saturating concentration of inositol 1,4,5-trisphosphate (IP_3 , top) and Ca^{2+} puffs evoked by photorelease of a lower IP_3 concentration (bottom). Distance along the scan line is depicted vertically, and time runs from left to right. Fluorescence profiles are expressed as a pseudoratio ($\Delta F/F_0$), relative to the average fluorescence at each pixel (F_0) preceding the photolysis flash. B: methodology for colocalization of puff sites with mitochondria. An x - y image of MTG staining (top) was first obtained using the microscope in frame-scan mode, using a green band-pass filter to image mitochondria. The microscope was then switched to line-scan mode, and Ca^{2+} -dependent changes in fura red fluorescence were recorded through a red long-pass filter, with the scan line (red bar) positioned at a defined y -position within the previously imaged area. Several line-scan Ca^{2+} images were recorded in response to weak photolysis flashes that evoked discrete Ca^{2+} puffs (bottom). Centers of Ca^{2+} puff sites were mapped to positions along the laser-scan line during successive recordings (white lines) and were then superimposed on the mitochondrial image (white asterisks). Finally, the distance from each puff site was measured to the edge of the nearest mitochondrial structure (mitochondrial "island" or individual strand). C: histogram showing separation of Ca^{2+} puff sites ($n = 292$ independent sites) from mitochondria (open bars), together with measurements between the same number of randomly selected coordinates and mitochondria (solid bars).

domly selected points on the same composite image. The mean distance between Ca^{2+} puff sites and mitochondria was smaller than expected if Ca^{2+} puff sites were randomly distributed (2.27 vs. 2.96 μm , $n = 355$ puff sites, 16 oocytes, 4 donor animals), and a greater proportion of Ca^{2+} puff sites (50.3%) were located within $<2 \mu\text{m}$ of the nearest mitochondrial mass than expected from a random distribution (37.6% of sites within $<2 \mu\text{m}$).

Data derived from the line-scan (Fig. 5) and frame-scan imaging techniques (Fig. 6) indicate that Ca^{2+} puff sites are located, on average, at a lateral distance of $\sim 2.3 \mu\text{m}$ from the closest neighboring mitochondrion but that a subpopulation of Ca^{2+} release sites is more closely localized with mitochondria than would be expected from a random distribution (Figs. 5C and 6B).

Video-Rate Axial Imaging

The experiments described above provided information on the spacing between Ca^{2+} puff sites and mitochondria in a lateral plane (i.e., parallel to the "plane" of the cell membrane) but give no information regarding their relative radial spacing (i.e., whether Ca^{2+} puff sites lie "above" or "below" mitochondria). To address this question, we performed experiments in which Ca^{2+} puffs were captured by axial video-rate scans in oocytes stained with MTR (3). Experiments were done as described above, except, instead of scanning the laser line laterally in the y -dimension, a piezoelectric translator was used to focus the microscope objective at 30 Hz in the axial (z) dimension. Each video frame thus represents a "side-on" (x - z) view into

Fig. 6. Mapping of Ca^{2+} puff sites in relation to mitochondria using video-scan confocal microscopy. **A**: Ca^{2+} images (OG-1 fluorescence imaged through a green band-pass filter) were recorded at video rate (30 frames/s) from a $65 \times 65\text{-}\mu\text{m}$ confocal section during stimulation with repeated photolysis flashes to evoke a low frequency of Ca^{2+} puffs. Individual video frames displaying sharply defined puffs (e.g., stack images at *left*) were combined to form a composite image providing the x - y coordinates of several puff sites. These were then superimposed on a composite mitochondrial image (MTR fluorescence imaged through a red long-pass filter) formed as a maximum-intensity projection of mitochondrial fluorescence at the same scan plane, as well as lateral regions $0.5\text{ }\mu\text{m}$ superficial and distal to the recording area (numbered at *right*). **B**: histogram showing separation of Ca^{2+} puff sites ($n = 355$ independent sites) from mitochondrial fluorescence in the composite image (open bars), together with comparable measurements from the same number of randomly selected coordinates (solid bars) and mitochondrial fluorescence. Distances were measured to the edge of the nearest mitochondrial fluorescence, identified as mitochondrial islands or individual mitochondrial strands.

the oocyte. Figure 7A shows a composite image of two Ca^{2+} puffs captured at times when their fluorescence intensities were maximal, superimposed on a image of mitochondrial distribution and resting OG-1 fluorescence in the same image field. The location of Ca^{2+} puffs was identified from the sites of initial fluorescence increase during the local Ca^{2+} transients measured with OG-1, before this became appreciably blurred by diffusion of Ca^{2+} within the cytoplasm. Figure 7B shows measurements of radial (z) separation between the position of Ca^{2+} puff sites (144 sites, 5 oocytes) and the peak axial mitochondrial fluorescence. The majority of Ca^{2+} puff sites ($\sim 70\%$) were localized in a band $\sim 2.5\text{ }\mu\text{m}$ wide, centered $\sim 2\text{ }\mu\text{m}$ superficial to the peak mitochondrial fluorescence.

Modulation of Ca^{2+} Puff Characteristics by Closely Apposed Mitochondria

The results presented above indicate that although the majority of Ca^{2+} puff sites are located a few micrometers from mitochondria, a subpopulation of deeper Ca^{2+} release sites may be more intimately apposed to mitochondria. We were therefore interested to determine whether the properties of the closely juxtaposed sites differed from the more-distant Ca^{2+} release sites.

From video-rate imaging experiments such as those shown in Fig. 6, the properties of Ca^{2+} puffs at particular Ca^{2+} release sites were measured and correlated with measurements of the surrounding density of mi-

Fig. 7. Radial positioning of Ca^{2+} puffs in the oocyte relative to mitochondria. *A*: 3-color overlay illustrating methodology used to map the radial separation between Ca^{2+} puff sites and mitochondria. Axial images were obtained at video rate using a piezoelectric translator to focus into the oocyte at 30 Hz. Different colors represent resting OG-1 fluorescence serving as a cytosolic marker ($\lambda = 530 \pm 20$ nm, depicted in gray scale as an average of 8 video frames), MTR fluorescence ($\lambda > 620$ nm, depicted in red), and 2 individual video frame images of Ca^{2+} puffs ($\lambda = 530 \pm 20$ nm, green "temperature" scale), captured at their times of peak fluorescence. The center of each Ca^{2+} puff site was identified as the center of fluorescence in the first frame in which a Ca^{2+} puff occurred. *B*: histogram of radial separations between individual Ca^{2+} puff sites (where positive values are more superficial) and the peak axial mitochondrial fluorescence (plotted as 0, dashed red line) measured from images such as those in *A*.

tochondria (Fig. 8). All measurements of puff activity were made in oocytes at rest, without specifically energizing mitochondria with oxidizable substrates (22). Under these conditions, no differences were apparent between the magnitude of Ca^{2+} puffs generated at sites most closely juxtaposed to mitochondria ($<1.25\text{-}\mu\text{m}$ separation) and the overall population of Ca^{2+} puff sites: $(F/F_0) = 1.62 \pm 0.3$ for peak fluorescence of Ca^{2+} puffs situated $<1.25\text{ }\mu\text{m}$ from mitochondria and 1.56 ± 0.1 for a random selection of Ca^{2+} puff sites ($n = 15$ and 42 puff sites, respectively). However, a marked difference was apparent in Ca^{2+} puff frequency. Figure 8A shows the frequencies of Ca^{2+} puffs generated at multiple sites during sustained photore-

lease of IP_3 as a function of their localization with mitochondria. Proximity was quantified in terms of a "mitomass" parameter, which provides a measure of the density of mitochondria throughout a volume of $\sim 27\text{ }\mu\text{m}^3$ surrounding each Ca^{2+} puff site. Ca^{2+} puff sites surrounded by a higher density of mitochondria displayed progressively lower frequencies of Ca^{2+} puffs (Fig. 8A). Furthermore, in each imaging frame, the Ca^{2+} puff site with the highest frequency of activity (average frequency = 3.5 puffs/min, $n = 11$ oocytes, 3 donor animals) was surrounded by a low mitochondrial density, whereas the Ca^{2+} puff site most intimately associated with mitochondria displayed a much lower frequency of activity (Fig. 8B; average frequency = 0.6 puffs/min).

Sites of Ca^{2+} Wave Initiation

We recently showed that a subset of Ca^{2+} puff sites repeatedly serves as the site of Ca^{2+} wave initiation during Ca^{2+} oscillations in the oocyte (26). Those Ca^{2+} puff sites where Ca^{2+} waves initiate, called "focal" Ca^{2+} puff sites, display a higher IP_3 sensitivity and a higher frequency of Ca^{2+} puff activity than other sites (26). We were therefore interested to compare mitochondrial density associated with focal puff sites with a random sample of "nonfocal" Ca^{2+} puff sites in the same record. Focal Ca^{2+} puff sites were identified in MTR-stained oocytes as the sites of Ca^{2+} wave initiation during repetitive Ca^{2+} oscillations evoked by sustained photorelease of low concentrations of IP_3 . Measurements of the mitomass associated with these focal sites showed that sites of Ca^{2+} wave initiation were associated with a lower mitochondrial density than Ca^{2+} puff sites that did not initiate Ca^{2+} waves (Fig. 8C).

DISCUSSION

Mitochondrial Morphology in the *Xenopus* Oocyte

There have been few previous studies documenting mitochondrial morphology in intact *Xenopus* oocytes commonly used for Ca^{2+} imaging (stages V and VI). Therefore, using confocal imaging, we began by examining the morphological relationship between mitochondria, ER, and sites of intracellular Ca^{2+} release within live *X. laevis* oocytes.

MTG and MTR staining revealed large islands of densely intertwined mitochondria, interconnected by discrete vermiform mitochondrial strands, establishing an extensive cortical network spreading a $\sim 6\text{-}$ to $8\text{-}\mu\text{m}$ -wide band around the entire oocyte cortex. The appearance of mitochondrial islands is reminiscent of the mitochondrial clustering shown to occur within peripheral corridors of yolk-free cytoplasm in oocyte electron micrographs (2). Denser mitochondrial staining was evident within the vegetal hemisphere than within the animal hemisphere of the oocyte (Fig. 1C), possibly reflecting the asymmetric dispersal of the mitochondrial cloud of previtellogenic oocytes to the cortex of the vegetal pole (50). Although the mitochondrial

Fig. 8. Mitochondrial proximity dampens Ca^{2+} puff activity. **A:** Ca^{2+} puff frequency as a function of the surrounding mitochondrial density at each site. Density of mitochondria was estimated in terms of a "mitomass" parameter, obtained by averaging the MTR signal intensity over a $3 \times 3\text{-}\mu\text{m}$ imaging plane parfocal, $0.5\text{-}\mu\text{m}$ superficial and $0.5\text{-}\mu\text{m}$ distal to the recording plane monitored for Ca^{2+} signals ($\sim 27\text{-}\mu\text{m}^3$ volume). This value, estimated from a composite overlay of these 3 focal planes, was expressed as a percentage relative to the maximal and minimal values of MTR fluorescence within an equivalent $\sim 27\text{-}\mu\text{m}^3$ volume anywhere within the same imaging field ($65 \times 65\text{-}\mu\text{m}$). Puff frequency was measured at discrete sites over a 3-min period. Data are from 122 puff sites in 11 oocytes. **B:** scatterplot showing the correlation between puff activity and mitomass for selected single Ca^{2+} release sites displaying the highest (■) and lowest (□) frequency of puffs within each imaging region. **C:** correlation of mitomass of Ca^{2+} sites where Ca^{2+} waves were initiated most frequently (focus) with the average mitomass associated with 20 other puff sites in the same record (average). Measurements were made during repetitive Ca^{2+} oscillations evoked by sustained photorelease of IP_3 in the vegetal hemisphere of pigmented oocytes.

network is highly dynamic, undergoing continuous rearrangement in many cell types (40), the mitochondrial network of unenergized oocytes was predominantly static (Fig. 4). Given the influence of cell cycle and metabolic state on mitochondrial morphology, the static nature of oocyte mitochondria is not unexpected; the oocyte is in G_2 arrest, and mitochondrial potentials are low at rest ($\sim 93\text{ mV}$) (22). Whatever the explanation, the lack of mitochondrial dynamics facilitated long-term mapping of mitochondria in relation to Ca^{2+} release sites.

Relationship of Ca^{2+} Puff Sites to Mitochondria

Three main conclusions can be drawn from the morphological and functional data presented in Figs. 5–7. First, the mean separations between Ca^{2+} release sites and their neighboring mitochondrial islands ($\sim 2.3\text{-}\mu\text{m}$) are appreciable compared with the spatial spread of Ca^{2+} during a Ca^{2+} puff (effective space constant for Ca^{2+} diffusion of $\sim 2.8\text{-}\mu\text{m}$). Most mitochondria are, therefore, located well away from the microdomain of high cytosolic Ca^{2+} concentration surrounding puff sites and, as a consequence of the low affinity for mitochondrial Ca^{2+} uptake, are unlikely to buffer the Ca^{2+} released during Ca^{2+} puffs. Second, a major factor determining the separation between Ca^{2+} puff sites and mitochondria is that most Ca^{2+} puff sites are concentrated in a band located $\sim 2\text{-}\mu\text{m}$ more peripheral in the oocyte cortex than the location of the mitochon-

drial islands (Fig. 7). Finally, a proportion of Ca^{2+} puff sites ($\sim 28\%$) are located in close juxtaposition with mitochondria (within the lateral resolution of the confocal microscope, $<400\text{ nm}$) (3, 32). These Ca^{2+} puff sites most likely represent the deeper sites in the cortical ER, which extends downward into the layer where mitochondrial density is highest.

Our results thus demonstrate a heterogeneous spacing between Ca^{2+} puff sites and mitochondria (Figs. 5 and 6), which is expected to establish a broad range of functional cross talk between IP_3 receptors and mitochondria. If a comparable range of separation also occurs in other cell types, then such morphological heterogeneity could contribute to the diversity in agonist-evoked Ca^{2+} signals (5, 29, 36) and membrane potential responses observed from discrete mitochondrial regions (9).

Role of Mitochondria in the Spatial Patterning of Local Ca^{2+} Signals

Does mitochondrial proximity influence the behavior of local IP_3 receptor clusters in the *Xenopus* oocyte? The frequency of Ca^{2+} puffs at sites with low mitochondrial density was relatively high (Fig. 8, A and B), and these sites also preferentially served as initiation foci for Ca^{2+} waves (Fig. 8C). We recently reported that particular focal Ca^{2+} release sites tend to repeatedly initiate Ca^{2+} waves and that these sites are functionally distinguished by higher sensitivity to IP_3 and

greater Ca^{2+} puff frequency than are nonfocal sites (26). The present results indicate that mitochondrial proximity is likely to be an important factor underlying these differences, although the broad range of activity exhibited by Ca^{2+} puff sites in areas of sparse mitochondrial density suggests that other factors are also likely to be involved. The basis for these effects of mitochondria on Ca^{2+} puff activity is unclear but, speculatively, might include effects of local Ca^{2+} (34) or ATP gradients (25) on the Ca^{2+} -induced Ca^{2+} release sensitivity of the *Xenopus* IP_3 receptor. These results in the *Xenopus* oocyte parallel those of Gordienko et al. (13) in smooth muscle myocytes, where mitochondria are sparse around Ca^{2+} spark sites with a high frequency of activity that serve as the focus of Ca^{2+} wave initiation. A low mitochondrial density surrounding sites of Ca^{2+} wave initiation has also been reported in endothelia (30) and pancreatic acinar cells (49), whereas in hepatocytes, heightened IP_3 receptor sensitivity is evident in subcellular regions lacking mitochondria (17). However, in the processes of oligodendrocyte progenitors, local Ca^{2+} signals develop into Ca^{2+} waves not in regions defined by low mitochondrial density, but only at sites where mitochondria are present (15, 45).

A second question concerns whether local IP_3 -dependent Ca^{2+} puffs stimulate mitochondrial Ca^{2+} uptake. In the oocyte, the separation between Ca^{2+} puff sites and mitochondria suggests that this is unlikely to be the case for most mitochondria, but a direct answer to this question necessitates simultaneous measurement of cytoplasmic and mitochondrial Ca^{2+} or mitochondrial potential. This is difficult given the nonspecificity of mitochondrial staining in the oocyte with TMRE or acetoxymethyl ester (AM)-loading protocols using Ca^{2+} -indicator dyes such as rhod 2-AM (5, 7, 8, 29) and dihydro-rhod 2-AM (18). The presumed ineffectiveness of discrete Ca^{2+} puffs in signaling to mitochondria certainly does not preclude the existence of preferential, and even graded, communication between the organelles. However, such cross talk probably occurs under stronger conditions of stimulation, as demonstrated during global Ca^{2+} oscillations in the oocyte (11, 22).

In other cell types where the geometry is different and mitochondria are more closely apposed to Ca^{2+} release sites, discrete elementary Ca^{2+} release signals can be sensed by mitochondria. For example, in rat cardiac myocytes, local Ca^{2+} release events resulting from activation of ryanodine receptors in sarcoplasmic reticulum Ca^{2+} stores (" Ca^{2+} sparks") are sufficient to trigger transient depolarizations of mitochondrial membrane potential (9). In cardiac myocytes, ryanodine receptors are closer to mitochondria (~40–270 nm) (43), and the majority of mitochondria share such intimate contact with the ER. Similarly, Ca^{2+} sparks in smooth muscle appear competent in evoking transient mitochondrial depolarizations (7), where again both organelles are in close proximity (~10–20 nm) (31). For IP_3 -evoked Ca^{2+} puffs, the situation is less clear; for example, in mast cells, it has been suggested

that elementary Ca^{2+} signals may stimulate mitochondrial Ca^{2+} uptake on an individual basis (6), whereas in HeLa cells, Ca^{2+} puffs appear not to increase intramitochondrial Ca^{2+} concentration (5).

Role of Mitochondria in the Spatial Patterning of Global Ca^{2+} Signals

The emerging picture is that the relationship between ER and mitochondrial architecture is cell-type specific, with the respective morphology of both organelles molding the spatial patterning of Ca^{2+} release appropriate to cellular function. In the immature oocyte, the Ca^{2+} buffering capacity of the cortical mitochondrial band may serve to constrain Ca^{2+} waves to the oocyte periphery, as observed during axial imaging (4). More speculatively, in mature oocytes, the same morphological arrangement would aid establishment of the fertilization envelope, rather than allow the fertilization Ca^{2+} wave to dissipate into the internal volume of the egg. This situation resembles, albeit on a much different scale, that seen in frog sympathetic neurons (28), where circumferential propagation of a Ca^{2+} wave on top of a mitochondrial band occurs in preference to radial Ca^{2+} elevations. Another example of a cell-specific use of a mitochondrial barrier to mold the spatial patterning of Ca^{2+} signals occurs in the pancreatic acinar cell, where a band of mitochondria envelop the apical pole of the cell, restricting localized Ca^{2+} signals to the region where the secretory granules are localized (49). When mitochondrial function is inhibited, local Ca^{2+} oscillations are abolished and the agonist-evoked Ca^{2+} signal pervades the entire cell (49). In all these cases, the role played by mitochondria in cellular Ca^{2+} dynamics appears tailored to cellular function, with mitochondria influencing the spatial patterning of Ca^{2+} release through their global capacity for Ca^{2+} buffering and through more localized effects on ER excitability.

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