Acceleration of Intracellular Calcium Waves in Xenopus Oocytes by Calcium Influx

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Many cell membrane receptors stimulate the phosphoinositide (IP) cycle, which produces complex intracellular calcium signals that regulate diverse processes such as secretion and transcription. A major messenger of this cycle, inositol 1,4,5-trisphosphate (IP3), stimulates its receptor channel on the endoplasmic reticulum to release calcium into the cytosol. Activation of the IP3 cycle also induces calcium influx, which refills the intracellular calcium stores. Confocal microscopy was used to show that receptor-activated calcium influx, enhanced by hyperpolarization, modulates the frequency and velocity of IP3-dependent calcium waves in Xenopus laevis oocytes. These results demonstrate that transmembrane voltage and calcium influx pathways may regulate spatial and temporal patterns of IP3-dependent calcium release.

Propagating waves of elevated intracellular Ca2+ may be triggered in Xenopus oocytes by the activation of muscarinic receptors or by the microinjection of either guanosine-5’-O-(3-triphosphate) or IP3 isomers (1–4). Calcium release from IP3-sensitive intracellular stores (5) is necessary for both initiation and propagation of these waves (2–4). IP3-dependent Ca2+ waves persist in the oocyte for up to 30 min in the absence of extracellular Ca2+ (2, 3). We used confocal fluorescence microscopy with conventional two-electrode voltage clamp to study Ca2+ influx and its influence on IP3-dependent Ca2+ waves (6–9). Fluorescence from Calcium Green was recorded from superficial optical sections (628 by 419 μm) within ~30 μm of the plasma membrane (10).

To investigate the effects of Ca2+ influx on receptor-induced Ca2+ transients, we applied acetylcholine (ACH) to oocytes that expressed human m3 muscarinic acetylcholine receptors (m3AChRs) (Fig. 1). Saturating concentrations of ACH (50 μM) triggered Ca2+ release from intracellular stores, which resulted in increased concentration of Ca2+ that slowly decayed to base line over ~10 min (Fig. 1, A to D) (11). Hyperpolarization in the presence of 2.5 mM extracellular Ca2+ induced Ca2+ influx up to ~13 min after the application of atropine (200 μM) (12). In contrast to the generalized Ca2+ transient induced by high concentrations of ACh, submaximal stimulation (0.2 to 1 μM ACh) triggered regenerative Ca2+ waves (Fig. 1, E to G) that appear as streaks of increased fluorescence in a volume projection (Fig. 1F). The Ca2+ waves and Ca2+-activated Ca2+ current (ICa,CL) oscillations stopped within seconds after the application of atropine (200 μM at 550 s), whereas the hyperpolarization-induced influx of extracellular Ca2+ persisted for more than 7 min (Fig. 1F) (13). Unstimulated cells showed no Ca2+ influx with hyperpolarization (n = 5). Because Ca2+ influx persisted for minutes after the Ca2+ waves were abolished, we speculate that other second messenger mechanisms triggered Ca2+ entry or that the concentrations of IP3 were sufficient to bind to a receptor site that caused Ca2+ influx (14). These results also show that Ca2+ influx caused a generalized increase in Ca2+ but did not itself cause regenerative waves.

We examined the influence of Ca2+ influx on Ca2+ waves induced by injection of a nonmetabolizable IP3 analog, inositol 1,4,5-trisphosphorothioate (IP3S) (15). In the absence of extracellular Ca2+, the waves induced by IP3S (5 μM) had a period that increased because of the delayed interval between transients, which slowly increased over time (Fig. 2A and D). The Ca2+ wave velocity was independent of membrane potential in ten experiments conducted in the absence of extracellular Ca2+ (Fig. 2E), which indicates that the kinetics of the IP3 receptor (IP3R) channel are not directly altered by changes in membrane potential.

In the presence of 2.5 mM extracellular Ca2+, hyperpolarization triggered Ca2+ influx in 35 of 46 oocytes injected with IP3S (5 μM) (16), and to Ca2+ influx was observed in noninjected oocytes under similar conditions (n = 10). The entry of extracellular Ca2+ reversibly affected the frequency and velocity of Ca2+ waves triggered by IP3S (5 μM) (Fig. 3), an effect that was blocked by extracellular La3+ (1 mM) (17). In one oocyte, the wave velocity varied with the holding potential after the concentration of extracellular Ca2+ was raised to 2.5 mM (Fig. 3D). The mean velocity increased from 25.1 ± 0.8 μm s−1 (n = 5) to 33 ± 2 μm s−1 (n = 3) during a second hyperpolarizing pulse from −15 to −70 mV with 2.5 mM extracellular Ca2+, and after depolarization to −15 mV the velocity returned to 25.4 ± 0.9 μm s−1 (n = 2). The wave velocity in this experiment ranged from 19 to 37 μm s−1 and was correlated with the local base line fluorescence before each wave (Fig. 3F). These data suggest that the influx of extracellular Ca2+ may alter the propagation of IP3-dependent Ca2+ waves by increasing the basal Ca2+ concentration. This modulation of the velocity of Ca2+ waves was best observed with large changes in the membrane potential (~70 mV), which correlate to larger Ca2+ influxes. Depolarization, which reduces the driving force for Ca2+ entry into the cell, decreased the resting concentration of Ca2+ and increased the period between successive wave fronts (Fig. 4, A to H). These alterations in wave frequency were reversible (Fig. 1, E and F, and Fig. 3, G and H). Calcium Green signals from 33-μm square regions (Fig. 4A) showed that the frequency of Ca2+ waves increased from 1.8 ± 0.2 min−1 at 0 mV to 5.2 ± 1.4 min−1 at −50 mV (mean ± SEM; n = 9). A comparison of the local Calcium Green signal from one region (Fig. 4G) and the signal averaged over the entire slice (Fig. 4H) showed that averaging over the entire section obscures the high frequency waves. Regions capable of initiating waves at −50 mV but not at more depolarized potentials appear as short segments of fluorescence in the volume projection (Fig. 4E), which suggests that different regions have unique threshold cytosolic Ca2+ concentrations that must be reached waves before are initiated.

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recruitment of initiation foci was, in part, responsible for the increase in frequency. Strong hyperpolarization in some oocytes arrested the IP$_3$S$_2$-induced waves (Fig. 4, I to L), which demonstrates that increases in cytosolic Ca$^{2+}$ concentration over a critical level reversibly inhibit waves (18).

Calcium waves depend on the regenerative production of a diffusible molecule that triggers Ca$^{2+}$ release from adjacent IP$_3$-sensitive stores. Because both IP$_3$ and cytosolic Ca$^{2+}$ influence Ca$^{2+}$ release at the oocyte IP$_3$R channel (19), either could function as this propagating signal. IP$_3$ diffuses more rapidly than Ca$^{2+}$ in cytosolic extracts from *Xenopus* oocytes (20), but our experiments with high concentrations of IP$_3$S$_2$ (100 μM) indicate that oscillations in the amounts of IP$_3$ are not required for Ca$^{2+}$ waves (21). Rather, these data suggest that activation of m3AChRs leads to a steady increase in IP$_3$ and that Ca$^{2+}$ released from one store propagates through the cytoplasm and triggers release from neighboring stores. Here, we have demonstrated that Ca$^{2+}$ entry from the extracellular space can modulate the frequency and propagation rates of this dynamic process. The modulatory role for Ca$^{2+}$ influx provides control and allows diversity of signaling end points for the otherwise convergent phosphoinositide cascade. The spatial and temporal regulation of intracellular Ca$^{2+}$ waves by Ca$^{2+}$ influx means that the combination of membrane voltage and receptor-activated Ca$^{2+}$ influx pathways greatly expands the range of signal transmission control for Ca$^{2+}$-dependent events ranging from transcription to secretion.

![Fig. 1. Demonstration of m3AChR-activated Ca$^{2+}$ influx. In (A) to (D), a Xenopus oocyte that expressed m3AChRs and was injected with Calcium Green (12 μM) (22) 20 min before study was stimulated with ACh (50 μM) 20 s into the experiment and then treated with atropine (200 μM) at 200 s. The concentration of extracellular Ca$^{2+}$ and the membrane potential were manipulated to study Ca$^{2+}$ influx after the application of antagonist. To follow the spatiotemporal transients of intracellular Ca$^{2+}$, we scanned a single 628 by 419 μm optical section (1 Hz) for 1000 s and the digital images (192 by 128 pixels) were stored with 256 gray scale values. The confocal section covered ~8% of the oocyte's surface area. (A) Extracellular Ca$^{2+}$ concentration ([Ca$^{2+}$]$_{o}$). (B) Membrane potential (V$_{m}$) was clamped at −70.0, −100.0, and −100.0 mV. (C) ANALYZE software (d) was used to stack the confocal images into a volume (192 by 128 by 1000 pixels) of digital data. This panel (192 by 1000 pixels) represents a two-dimensional projection (view) of this volume, shown with time increasing to the right. The gray scale values of this panel represent the fluorescence intensity along the reader's line of sight into the volume. A pseudocolor scale bar for fluorescence intensity is next to (C); gray scales of 0 are blue, and gray scales of 255 are yellow-green (22). (D) The average Calcium Green signal of the 24,576 pixels in each confocal image (192 by 128 pixels) was calculated, and this average was displayed with increasing time. In (E) to (G), an m3AChR-expressing oocyte was stimulated with ACh (1 μM) −1 min before imaging. The membrane potential was manipulated in the presence of 2.5 mM extracellular Ca$^{2+}$ to study the effect of influx before and after the application of atropine (200 μM) at 550 s [denoted by the arrow on (F)]. (E) Membrane potential (V$_{m}$) was pulsed from the holding potential of −20 mV to −90, −95, −95, −80, −100, and −115, and −115 mV. (F) Volume projection of the 1000 images displayed with time increasing to the right. Propagating Ca$^{2+}$ waves appear as arcs of increased signal above a baseline fluorescence. Preliminary experiments with indo-1 suggest that the amplitude of a typical Ca$^{2+}$ wave is greater than 300 nM. (G) Average Calcium Green signal in the confocal section.

![Fig. 2. Periodic Ca$^{2+}$ waves triggered by IP$_3$S$_2$ propagate at a steady velocity in the absence of extracellular Ca$^{2+}$. This oocyte was injected with IP$_3$S$_2$ (5 μM) −6 min before imaging under voltage clamp in the absence of extracellular Ca$^{2+}$. (A) Membrane potential (V$_{m}$) was pulsed to −100 mV from a holding potential of 0 mV. (B) Volume projection of the 1000 images displayed with time increasing to the right. (C) Velocities of Ca$^{2+}$ waves ± SD. Planar Ca$^{2+}$ waves were studied if they sustained propagation for more than 4 s in a given direction and if they did not interact with other waves. A vector in the direction of propagation was constructed by eye with ANALYZE software (8), and the position of the wave front along this line was recorded for successive images. The slope of a linear best fit of the wave front position expressed as a function of time gave the wave velocity and an error of this estimate. (D) Average Calcium Green signal in a 33-μm square region centered in the confocal section. (E) Summary of 97 wave velocities (mean of data at each voltage ± SD) measured in ten oocytes at various holding potentials in the absence of extracellular Ca$^{2+}$. The number of waves observed at each voltage is given in parentheses.

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Fig. 3. Increased frequency and velocity of IP$_3$-induced Ca$^{2+}$ waves after Ca$^{2+}$ influx. The oocyte was injected with IP$_3$S$_2$ (5 μM) -10 min before imaging. (A) Extracellular Ca$^{2+}$ concentration ([Ca$^{2+}]_o$). (B) Membrane potential ($V_m$) was clamped at -10, -80, -5, -70, -15, -70, and -15 mV. (C) Volume projection of the 1000 images displayed with time increasing to the right. (D) Velocities of Ca$^{2+}$ waves ± SEM. Where error bars are not shown, the least squares estimate had a standard error less than the width of the symbol. (E) Average Calcium Green signal in a 33-μm square region centered in the confocal section. (F) Wave velocity as a function of the local resting signal averaged for the 4 s immediately before the initiation of each wave ($r = 0.8$).

Fig. 4. Recruitment of new initiation focus and increased frequency of IP$_3$-induced Ca$^{2+}$ waves after hyperpolarization. In (A) to (H), an oocyte was injected with IP$_3$S$_2$ (5 μM) -15 min before imaging, in the presence of 2.5 mM extracellular Ca$^{2+}$. (A to D) Confocal images of active Ca$^{2+}$ waves at 1, 179, 570, and 828 s, respectively. Nine local (33-μm square) regions are shown in (A). (E) Volume projection of the 1000 images displayed with time increasing to the right. (F) Membrane potential ($V_m$) was clamped at -50, 0, -10, -20, -30, and -40 mV. (G) Average Calcium Green signal in a 33-μm square region of the middle row in (A). (H) Average Calcium Green signal in the confocal section. In (I) to (L), an oocyte was injected with IP$_3$S$_2$ (5 μM) -8 min before experimentation. (I) Extracellular Ca$^{2+}$ concentration ([Ca$^{2+}]_o$). (J) Membrane potential ($V_m$) was clamped at -40, -7, -20, -30, -50, -60, -80, and -10 mV. (K) Volume projection of the 1000 images displayed with time increasing to the right. (L) Average Calcium Green signal in a 33-μm square region centered in the confocal section.

REFERENCES AND NOTES

6. Albino oocytes (Dumont stage V) were prepared as reported (3) except that the incubation medium was supplemented with horse serum (5%) M. W. Quick, J. Naive, N. Davidson, H. A. Lester, BioTechniques 13, 360 (1987). Serum-free solutions were used to wash the oocytes at least 6 hours before study. For experiments with exogenous receptors, 5 ng of rMACH-rCRNA (in vitro transcript from a cDNA clone) was injected as a 47-nil bolus 48 hours before experimentation, and the oocytes were incubated at 19°C. From 30 to 240 min before imaging, 47 nil of Calcium Green (0.25 mM) was injected, which resulted in a final concentration of ~12 μM, assuming a 1-μl volume of distribution.
7. A Leitz Sharp Bio-Rad MRC-600 box adapted to a Zeiss IM55 inverted microscope was used with an Olympus DP20 participant objective (0.4 numerical aperture) for confocal imaging (C. Biiton, J. Lechleiter, D. E. Clapham, J. Microsc. 169, 15 (1993). Excitation provided by the 488-nm line of a 25-W argon laser was filtered to less than 5% transmission by neutral density filters (Omega Optical). Returning fluorescence was long pass-filtered (515 nm), and detected with the confocal aperture set to its maximal opening (7 mm) in the low signal mode.
8. The digitized images were stored on optical disk and studied with ANALYZE software (Mayo Foundation, Rochester, MN) on a Silicon Graphics Personal Iris Computer.
9. A 10-mm square patch of woven mesh (Spectrum, 750-μm spacing) was attached with silicon rubber to a cover slip that functioned as the bottom of a 2-ml chamber. The mesh improved the kinetics of the ACh response fourfold by allowing ACh better access to the space between the oocyte and the cover slip. Solution changes by addition of 750 μl to the bath (initial volume = 750 μl) were considered complete within 10 s. The extracellular solution with a low concentration of Ca$^{2+}$ contained 96 mM NaCl, 5 mM KCl, 2 mM HEPES, 5 mM Hepes (pH 7.5), and 0.1 mM EGTA (free Ca$^{2+}$ ~10 mM). EGTA was replaced with CaCl$_2$ to obtain the desired final concentration of extracellular Ca$^{2+}$. A conventional two-electrode voltage clamp technique was used in parallel with imaging. Oscillations in the Ca$^{2+}$-activated I$_{Ca,ov}$ reflected the full-field average concentration of Ca$^{2+}$ and are not shown. Experiments were conducted at 22° to 25°C (S. Girard and D. E. Clapham, Methods Cell Biol., in press).
10. Calcium Green (Molecular Probes, Eugene, OR) has a dissociation constant (K_d) for Ca$^{2+}$ of 189 mM in 100 mM KCl at 22°C.
12. I. Parker and R. Millie, Proc. R. Soc. London Ser. 8 191, 27 (1987). The activation of native muscarinic receptors has been shown to trigger the influx of extracellular Ca$^{2+}$ that is insensitive to rilpivirine (50 μM) (M. Lupu-Meli, H. Shapiro, V. Oron, FEBS Lett. 262, 165 (1990). In these experiments, Ca$^{2+}$-activated I$_{Ca,ov}$ was used as the assay for cytosolic Ca$^{2+}$, depletion to 0 mM blocks the influx of extracellular Ca$^{2+}$, which suggests that voltage-activated Ca$^{2+}$ currents are minor or that they inactivate during prolonged depolarization (R. Millie, Proc. R. Soc. London Ser. 8 191, 27 (1982); M. E. Barish, J. Physiol. (London) 342, 303 (1983).
13. The time constant of inhibition of the Ca$^{2+}$-activated I$_{Ca,ov}$ by atropine (200 μM) was less than 8 s in four rMACH-expressing oocytes that exhibited oscillations induced by ACh (1 μM).

15. S. DeLisle, K. H. Krause, G. Denning, B. V. L. Potter, M. J. Welsh, J. Biol. Chem. 265, 11726 (1990). IP3_S2 has high affinity for the IP3_R of the cerebellum (Kd = 31 ± 3 mM) and a median effective concentration (EC50) for Ca2+ release of ~5 μM in permeabilized Swiss 3T3 cells. In Xenopus oocytes, IP3 was five to ten times more potent than IP3_S2 in generating oscillations in intracellular Ca2+. IP3_S2 induced waves and the binding of IP3 to oocyte microsomes [the amount required for 50% inhibition (IC50) = 2 μM] (6-8, 10) [B. V. L. Potter, R. A. J. Chaliis, S. R. Nahorski, Du Pont Biotech. Update 5, 1 (1990); C. W. Taylor, M. J. Berndig, K. D. Soll, B. V. L. Potter, Biochim. Biophys. Res. Commun. 150, 626 (1988); J. E. Ferguson, B. Potter, R. Nuccitelli, ibid. 172, 229 (1986)].

16. Inositol 1,4,5-triphosphate, inositol 2,4,5-triphosphate, and inositol 1,3,4-triphosphate have been shown to induce Ca2+ influx in Xenopus oocytes. In our experiments, the IP3-activated block of IP3 selected for assays in cystolic Ca2+ [S. DeLisle, D. Pittet, B. V. L. Potter, P. D. Lew, M. J. Welsh, Am. J. Physiol. 282, C1456 (1992); P. M. Snyder, K. H. Krause, M. J. Welsh, J. Biol. Chem. 283, 11048 (1988)].

17. Manganese has been used as an indicator of Ca2+ influx because it quenches indo-1 and fura-2 (Molecular Probes, Eugene, OR) fluorescence [G. Grynkiewicz, M. Poenie, R. Tsien, J. Biol. Chem. 260, 3440 (1985)]. We found in some cases that extracellular Mn2+ (1 to 10 mM) irreversibly increased the Calcium Green signal in IP3_S2-stimulated oocytes, which suggests that Mn2+ reacts with free manganese fluorophores and that the influx pathway is at least partially permeable to Mn2+. This makes it unsuitable as an influx channel marker in simultaneous imaging and electrophysiological experiments. Conversely, 1 mM La3+ reliably blocked influx in eight of eight oocytes without affecting the ability of the indicator to fluoresce. In the absence of extracellular Ca2+, the application of La3+ did not influence the Ca2+ waves induced by IP3_S2 (5 μM).


20. N. L. Albrighton, T. Meyer, L. Stryer, Science 285, 1271 (1999) and 2.8 (isobutyryl 1,4-benzoquinone (IBHQ), 0.8 mM) (this work). We observed regenerative Ca2+ waves for up to 7 min in the absence of extracellular Ca2+ after injection of IP3_S2 (100 μM) in 12 of 16 oocytes. We found that the peroxide does not yield either excitation or excitation spectrum upon binding Ca2+.

Biological forces in motile systems, such as those involving ciliary dynein and actomyosin, are usually studied as the sum of contributions from many force-generating units (1). However, the mechanochemical enzyme kinesin can drive an individual molecule. Kinesin that is adsorbed to glass moves microtubules so that they pivot around a single attachment point (2). The concentration dependence of the motility, calculated as an effective Hill coefficient of 1 for kinesin adsorbed to glass cover slips (2) or as a Poisson distribution for kinesin adsorbed to glass beads (3), indicates that one molecule alone can generate force and move microtubules. We have directly measured the force generated by an individual kinesin molecule using the single-beam optical gradient trap, also known as optical tweezers (4).

To characterize the optical trap, we used viscous drag to displace trapped microtubules that were 0.55 μm in diameter. All calibration experiments were performed at least 2 μm from the cover slip surface to minimize viscous coupling to the glass surface, so deviations from Stokes drag were <7% (5). For all biological force measurements, we calibrated the escape force (Fes) from the optical tweezers by using a laminar flow cell (6). However, optical forces during escape from the optical tweezers are spatially complex, requiring nanometer-level characterization of the optical trap to interpret subsequent experiments. Such characterization would be biased by the shear gradient of the flow cell, so we used viscous forces that were generated by moving the microscope specimen with a piezoceramic-driven stage (7). The stage had a maximal usable velocity of ~150 μm s−1 (~0.8 pN of viscous drag), making it less appropriate for direct calibration of biological force measurements. Trapped particles were alternately displaced by the piezoceramic stage moving at constant velocity, and their positions were monitored while laser irradiation was reduced (Fig. 1A). Normalized to the laser irradiation at the specimen, a force and displacement profile could be constructed (Fig. 1B). Force is proportional to the displacement for the first ~100 nm. This force-displacement profile qualitatively agrees with theoretical models of the optical trap (8). Because of video limitations, the region of maximum force has not been precisely determined, but it appears to be located at a radius of 150 ± 26 nm (SD) (Fig. 1C).

Optical forces were applied to streptavidin-coated latex microspheres that were attached as handles to biotinylated microtubules. The biotinylation procedure, both with and without the attachment of microspheres, did not alter kinesin-driven gliding velocities of the microtubules. After evaluating different procedures for constructing biotin-specific beads (9), we covalently attached bovine serum albumin to carbodim-