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Picomolar sensitivity to inositol trisphosphate in *Xenopus* oocytes

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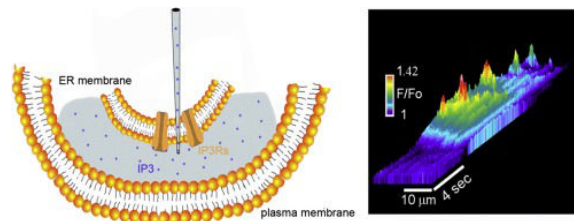
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Abstract

Ca²⁺ liberation from the endoplasmic reticulum mediated by inositol trisphosphate receptor/channels (IP₃Rs) in response to production of the second messenger IP₃ regulates numerous signaling pathways. However, estimates of resting and physiologically relevant cytosolic concentrations of IP₃ vary appreciably. Here we directly address this question, taking advantage of the large size of *Xenopus* oocytes to image Ca²⁺ liberation evoked by bolus intracellular injections of known concentrations of IP₃. Our principal finding is that IP₃ evokes both global and local Ca²⁺ signals in freshly isolated oocytes at concentrations as low as a few pM. A corollary is that basal, resting [IP₃] must be even lower, given the absence of detectable Ca²⁺ signals before injection. The dose/response curve for IP₃-activation of Ca²⁺ liberation suggests that freshly isolated oocytes express two distinct functional populations of IP₃ receptors with EC₅₀ values around 200 pM and tens of nM, whereas the high-affinity receptors are not apparent in oocytes examined later than about 3 days after isolation from the ovary.

Graphical Abstract



Keywords

inositol trisphosphate; calcium signaling; *Xenopus* oocyte; calcium puffs

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Conflict of interest

The authors declare that we have no conflicts of interest.

1. Introduction

Inositol 1,4,5-trisphosphate receptors (IP₃Rs) are intracellular Ca²⁺ channels in the membrane of the endoplasmic reticulum (ER) which mediate the release of sequestered ER Ca²⁺ into the cytosol in response to binding of IP₃ generated following activation of diverse cell surface receptors [1-3]. Here we address the questions of what minimal cytosolic concentration of IP₃ is required to evoke a detectable Ca²⁺ release and, by extension, what the maximal basal concentration of IP₃ may be in quiescent cells.

To do this, we employed a simple and direct method, taking advantage of the enormous size of the *Xenopus* oocyte (about 1 mm diameter, cytosolic volume about 1 μl) to inject a relatively large volume (10 nl) of solution containing known concentrations of IP₃ into oocytes previously loaded with a fluorescent Ca²⁺ indicator dye. The injection volume corresponds to a fluid bolus of about 100 μm diameter, and by imaging Ca²⁺ signals from a smaller cytosolic region within this we record the activity of IP₃Rs exposed to a concentration of IP₃ expected to approximate that in the original injection solution. Certainly, the concentration of IP₃ experienced by IP₃ receptors located within the imaging region would not exceed that in the injection solution, and control experiments injecting Ca²⁺-insensitive dye suggests that the large injected volume minimizes initial dilution by cytosolic fluid and slows the subsequent dilution resulting from diffusion into the bulk of the oocyte. This approach contrasts with previous experiments injecting much smaller volumes of high concentrations of IP₃ [4], where steep and time-varying concentration gradients around the pipette tip preclude any accurate estimation of local cytosolic IP₃ concentration.

Our principal finding is that oocytes examined within about two days after dissection from the ovary display a strikingly high sensitivity, generating robust Ca²⁺ signals following injections of as little as 10 pM IP₃, whereas oocytes examined at later times responded only to nanomolar concentrations of IP₃. Extrapolating the picomolar sensitivity of IP₃Rs in the oocyte to mammalian cells of more typical size (1,000 μm³ volume) leads to the surprising conclusion that Ca²⁺ release could be evoked by free concentrations of IP₃ corresponding to just tens of molecules per cell.

2. Methods

2.1. Oocyte preparation and IP₃ injection

Stage V-VI *Xenopus laevis* oocytes were purchased from Ecocyte Bioscience International (Austin, Texas). The supplier describes the following procedures for isolation and treatment of oocytes: Ovarian lobes from *Xenopus laevis* frogs (vendor: Xenopus Express) are removed surgically and transferred into Ca²⁺-free Barth's solution. Tissue is divided with scissors into smaller parts, placed into 50 ml Falcon tubes with collagenase (7.5 mg/ml collagenase type II (approx. 300 U/mg) in Ca²⁺-free Barth's solution and agitated gently for 90 minutes at room temperature to remove follicular and other surrounding cells. Defolliculated oocytes are then washed extensively with Ca²⁺-free Barth's solution, selected to exclude those with visible damage, and maintained in Ca²⁺-containing Barth's solution supplemented with Gentamicin (600 μg/ml). For shipment, oocytes are transferred into 10

ml glass tubes (50 per tube) completely filled with Ca²⁺-containing Barth's solution supplemented with gentamicin, and packed together with a cooling pack in a carton filled with expanded polystyrene beads. Ovaries are removed from the frog at about 8:30 am, and oocytes are shipped the same day for overnight delivery by UPS.

Upon receipt the following morning, oocytes were visually inspected and divided into batches of 15-20 per vial in 5 ml of regular Barth's solution containing 1.8 mM Ca²⁺. Some oocytes were used immediately for imaging, and others were maintained at 18 °C for use on following days. With the exception of experiments in Fig. 4, all recordings were made using oocytes within about 24-48 hrs of their isolation from the ovary.

2.2. Intracellular injections into oocytes

About 1 hr prior to Ca²⁺ imaging, oocytes in Ca²⁺-free Barth's solution were injected with fluo-4 dextran (low affinity; K_d for Ca²⁺ about 3 μM) to a final concentration of 40 μM, assuming even distribution throughout a cytosolic volume of 1 μl. Microinjection of IP₃ into oocytes during imaging was performed using a Drummond nanoinjector mounted vertically on a hydraulic micromanipulator affixed to the microscope frame. A glass pipette filled with IP₃ solution was inserted vertically down through the entire oocyte to a pre-established position, with the tip positioned a few μm inward from the plasma membrane and centered within the image field [5]. In some instances a given pipette was emptied and re-used to inject different concentrations of IP₃, but always in a sequence of increasing concentrations.

2.3. Imaging

Following injection of fluo-4, oocytes were mechanically stripped of the vitelline membrane [6], placed animal hemisphere-down in a chamber with a base formed by a fresh, ethanol-washed microscope cover glass, and were bathed in a Ca²⁺-free Ringer's solution (composition in mM: NaCl, 110; KCl, 2; EGTA, 5; HEPES, 5; at pH 7.2.). Oocytes were imaged at room temperature by total internal reflection fluorescence [TIRF] microscopy using an Olympus inverted microscope (IX 71) equipped with a 60X TIRF oil-immersion objective, a 488 nm solid-state laser for fluorescence excitation, and an electron-multiplied ccd camera (Cascade 128+: Roper Scientific) for imaging fluorescence emission (>510 nm) at frame rates of 10 to 100 s⁻¹. Fluorescence was imaged within a 40 × 40 μm region within the animal hemisphere of the oocyte. Measurements of Ca²⁺-dependent changes in fluo-4 fluorescence are expressed as a ratio (F/F₀) of the change in fluorescence at each pixel (F) relative to the mean resting fluorescence at that pixel before stimulation (F₀) obtained by averaging over several [50-100] frames before stimulation. Measurements of calcein fluorescence in Figs. 3A, B (used as a Ca²⁺-independent reporter) are expressed as arbitrary camera units. MetaMorph (Molecular Devices) was used for image processing, and measurements were exported to Microcal Origin version 6.0 (OriginLab, Northampton, MA, USA) for analysis and graphing.

Materials—Fluo-4 dextran, IP₃ and calcein were purchased from Molecular Probes/Invitrogen (Eugene, OR); all other reagents were from Sigma. A stock solution of 2 mM IP₃ was prepared by adding 771 μl of intracellular-like solution (composition in mM: KCl: 135; HEPES:10; Na-ATP:4 pH 7.4 with NaOH) to a 1 mg vial of lyophilized D-myo-Inositol

1,4,5-trisphosphate, hexapotassium salt. Serial dilutions in intracellular solution were then performed before each experiment to obtain concentrations of IP₃ as low as 10 pM, using fresh tubes and pipette tips for each dilution to preclude the possibility of carry-over of IP₃.

3. Results

3.1 Experimental approach

Oocytes loaded with fluo-4 dextran (low affinity), were positioned animal hemisphere-down on a coverglass forming the base of the imaging chamber and allowed to settle for few minutes. Injections of various concentrations of IP₃ dissolved in 10 nl of intracellular solution were made into the oocyte using a Drummond nanoinjector mounted on a hydraulic micromanipulator. A glass pipette pulled with a thin, tapering shank was inserted vertically down so the tip passed through the entire oocyte to a pre-established position, a few μm inward from the plasma membrane. Ca²⁺-dependent changes in fluo-4 fluorescence were imaged in TIRF mode using a 60× oil-immersion objective, with the imaging field centered on the pipette tip.

3.2 Ca²⁺ signals evoked by picomolar concentrations of IP₃

Injections of IP₃ evoked transient fluorescent signals, the amplitude and temporal evolution of which varied on IP₃ concentration. Surprisingly, a concentration of IP₃ as low as 10 pM consistently evoked signals in freshly prepared oocytes (n = 26 out of 32 oocytes examined within about 24-48 hr after isolation), whereas control injections of the same volume of solution without IP₃ uniformly failed to give responses (n = 6 oocytes). Fig. 1A shows a linescan image depicting a typical response to 10 pM IP₃, consisting of a slow, spatially extensive fluorescence rise persisting for many seconds, upon which transient, localized Ca²⁺ puffs [7] were superimposed. Puffs arose at multiple locations throughout the imaging field, often recurring at the same location. These characteristics are further illustrated in Fig. 1B, showing representative fluorescence traces measured simultaneously from small (1 × 1 μm) regions of interest positioned on puff sites.

Higher concentrations of injected IP₃ generated progressively larger fluorescence signals, characterized by faster rising and falling phases of the global signal, upon which local Ca²⁺ puffs became increasingly difficult to resolve. This is illustrated in the linescan images in Fig 1C showing representative responses to 100 pM (top) and 1 nM IP₃ (bottom); and in Fig. 1D showing traces of fluorescence measurements averaged across the entire imaging field in response to injections of vehicle alone and containing various concentrations of IP₃ between 10 pM and 100 μM.

3.3. Characteristics of puffs evoked by differing IP₃ concentrations

Fig. 2A shows superimposed traces of representative puffs evoked by 10, 30 and 100 pM IP₃, and Figs. 2B,C plot mean data showing puff amplitudes and durations at IP₃ concentrations between 10 pM and 10 nM. Mean puff amplitudes increased progressively with increasing IP₃ concentration over this range (Fig. 2B), but we found no appreciable concentration-dependence of puff durations, measured as duration at half-maximal amplitude (Fig. 2C).

3.4. Time course and spatial spread of injected IP₃

Our experiments involved the abrupt (a few hundred ms) intracellular injection of a relatively large volume (10 nl) of IP₃-containing solution, corresponding to a droplet with a radius of about 50 μm. Our expectation was that this bolus of solution would ensure that the restricted region of the oocyte we imaged (about 40 × 40 μm) initially experienced a spatially near-uniform concentration of IP₃ with minimal dilution, and that subsequent diffusion of IP₃ would result in only a slow decline in concentration within the imaged volume. To verify this, we made identical injections utilizing calcein as a surrogate fluorescent marker to estimate how injected IP₃ would initially spread and subsequently become diluted. The trace in Fig. 3A shows a representative record of fluorescence averaged over the imaging field following injection of 10 nl of solution containing 1 μM calcein, and the inset panels show individual image frames acquired at times marked on the trace. Following injection the fluorescence increased abruptly and Fig. 3Ab shows a broad and uniform spread throughout most of the imaging field after 1s. The fluorescence then decayed progressively over several tens of seconds (trace, Fig. 3A), while remaining diffusely distributed across the imaging field (Figs. 3Ac-e). We expect that injections of IP₃ would mirror this behavior, with the exception that the local [IP₃] might decay faster than by diffusion alone owing to enzymatic degradation of IP₃. Concordant with the time course and distribution of the calcein fluorescence, IP₃-evoked Ca²⁺ signals imaged from the central 20 μm of the imaging field were spatially uniform (eg. Fig. 1C), and responses to low concentrations of IP₃ decayed over about 20s (Fig. 1B). At progressively higher concentrations the decay became increasingly rapid, likely because Ca²⁺-dependent inhibition of IP₃Rs came to dominate the time course of Ca²⁺ liberation.

To then estimate the extent to which IP₃ might initially be diluted as the bolus of solution expanded through the imaged region during injection, we pre-loaded oocytes with calcein about 1 hr before imaging, allowing the dye to diffuse evenly throughout the cytosol (to a final intracellular concentration about 1 μM). We then monitored the decrease in fluorescence produced by injection of 10 nl of vehicle without added dye (Fig. 3B). The fractional decrease in fluorescence thus provides a measure of the extent to which calcein in the cytosol was diluted by the added bolus of unlabeled solution, and conversely provides an estimate of how much the IP₃ in our injection solutions may have been diluted. Within about 1 s following injection, the fluorescence fell to about one half throughout the imaging field (Fig. 3Bb), and subsequently recovered over several tens of seconds. Measurements of fluorescence made within 1s of injection showed a mean decrease to 57% of the initial level (n = 6 oocytes), suggesting that cytosolic IP₃ concentrations would initially be diluted to about one-half the initial concentration in the pipette.

3.5. Ca²⁺ signals arise from IP₃-mediated intracellular liberation

Fluorescence recordings during injections could be subject to artifacts including mechanical movement, and might reflect activation of Ca²⁺ liberation via mechanisms in addition to activation of IP₃Rs (eg. via Ca²⁺-permeable stretch-activated channels). To exclude possible signals arising from influx of Ca²⁺ across the plasma membrane, we performed all experiments in an extracellular solution containing no added Ca²⁺ and 5 mM EGTA. Moreover, IP₃ was dissolved in a solution with an ionic composition and osmolarity

approximating the cytosolic composition, and control injections of this vehicle alone into fluo-4 dextran-loaded oocytes typically evoked only a small, transient mechanical artifact, followed by a slower, small decrease in fluorescence that likely resulted from dilution of the indicator (upper trace, Fig. 3C; left bar, Fig. 3D). Further evidence pointing to Ca^{2+} liberation through IP_3 Rs as source of the fluorescence signals includes their suppression by bath application of 10 mM caffeine, a competitive inhibitor of IP_3 -mediated Ca^{2+} liberation (lower traces Fig. 3C; Fig. 3D), and the observation of transient, local signals characteristic of IP_3 -evoked Ca^{2+} puffs.

3.6. Dose-response relation for IP_3 -mediated Ca^{2+} liberation

Fig. 4A shows dose-response curves for IP_3 -evoked Ca^{2+} signals, obtained from records like those in Fig. 1D by measuring the peak overall Ca^{2+} fluorescence signals (F/F_0) averaged over the entire imaging field in response to intracellular injections covering a 7 decade range of IP_3 concentrations (10 pM to 100 μM). Our initial experiments (including all data in Figs. 1-3) were done on oocytes within 24-48 hr after isolation of oocytes from the ovary. Mean data from these oocytes are plotted as black squares in Fig. 4A, and reveal an apparently biphasic dose response relationship. Small but robust Ca^{2+} signals were evoked in 26/32 oocytes by injection of 10 pM IP_3 , and the mean response amplitudes increased with increasing concentration before reaching an intermediate plateau at concentrations around 1-10 nM (green curve, Fig. 4A). However, further increases in concentration over a range from 100nM to 100 μM then evoked yet further increases in Ca^{2+} signal (blue curve, Fig. 4A).

3.7. Oocytes lose picomolar sensitivity to IP_3 with increasing time after isolation

Subsequently, we noticed that oocytes examined at later times after removal from the ovary showed diminished or absent Ca^{2+} signals in response to injections of low pM concentrations of IP_3 . This is illustrated in Fig. 4B, showing a progressive reduction in Ca^{2+} responses to injections of 30 pM IP_3 into oocytes examined between 24 to 96 hr after isolation. We therefore repeated measurements of the dose-response relationship for oocytes examined 72-96 hr after isolation (red circles and curve in Fig. 4A). This showed a single-component relationship, with little or no signal detected for IP_3 concentrations between 10 - 300 pM, whereas increasing responses were evoked by progressively higher concentrations.

4. Discussion

Our principal finding is that *Xenopus* oocytes, when examined within one or two days after isolation from the ovary, display a remarkably high sensitivity to IP - typically showing Ca^{2+} liberation at concentrations as low as a few picomolar IP_3 . A corollary of this result is that the resting concentration of IP_3 must be even lower than this, given that we did not observe any ongoing, constitutive Ca^{2+} release signals before injecting IP_3 .

The method we employed, involving intracellular injection of a relatively large (10 nl) bolus of dilute solution yields a direct estimate of the resulting cytosolic concentration of IP_3 that, if anything, will overstate that value. Experiments injecting calcein as a fluorescent surrogate showed that the bolus of fluid expanded rapidly (a few hundred ms) throughout

the imaging field, and that fluorescence then declined over several seconds. Conversely, injection of vehicle (without dye) into oocytes pre-loaded with calcein produced an immediate drop in fluorescence (mean fall to 57%) followed by a recovery over several seconds. The latter result indicates that the injected bolus of fluid substantially displaces the cytosolic fluid as it expands. From the decrease in fluorescence it seems that the cytosolic concentration of IP₃ immediately following injection may be diluted to about one-half of that in the pipette, and that the concentration subsequently falls further as the injected solution mixes with cytosolic constituents. We thus conclude that detectable Ca²⁺ liberation may be evoked by IP₃ concentrations of <5 pM. However, throughout this paper we refer only to the initial concentration in the injection solution, as representing a conservative upper bound for the resulting cytosolic concentration of IP₃. A further possible consequence of the dilution of cytosolic contents is that some putative inhibitory factor becomes diluted, reducing constitutive inhibition of IP₃Rs and hence allowing responses to be generated at lower concentrations of IP₃ than under physiological conditions. We consider it unlikely that such mechanism would appreciably affect our results, given the modest (~50%) dilution factor, and that Ca²⁺ signals persisted for many seconds when cytosolic concentrations would re-equilibrate.

Previous approaches to determine the resting and stimulated concentrations of IP₃ in the *Xenopus* oocyte have been subject to much greater uncertainties and have yielded considerably higher values than we report here. Earlier experiments injecting IP₃ into the oocyte were done using much smaller volumes (pico liters) of much higher concentrations of IP₃ (hundreds of μM) [8, 9]. Although the *amount* of IP₃ injected could be calibrated, the resulting effective intracellular *concentration* is difficult to estimate. For example, Ivorra et al [9] observed Ca²⁺-activated Cl⁻ currents evoked by as little as 0.1 fmoles of IP₃. That corresponds to a concentration of about 100 pM if IP₃ had distributed uniformly throughout the ~1 μl cytosolic volume of the oocyte, but it is likely that the transient responses were evoked by higher concentrations close to the injection site before IP₃ had time to diffuse far. To avoid such spatial non-uniformities in [IP₃], Parker & Ivorra [10] estimated the threshold concentration of IP₃ needed to evoke a detectable Ca²⁺-activated Cl⁻ current to be about 60 nM, utilizing photolysis of a uniformly distributed caged precursor. Activation of native *Xenopus* oocyte IP₃Rs has also been extensively studied by patch-clamp recording from isolated nuclei [11]. The lowest concentration of IP₃ employed was 10 nM, which produced only a small open probability (P_o <0.1) even at optimal [Ca²⁺] [12]. Direct measurements of cytosolic [IP₃] in the oocyte were made using capillary electrophoresis in combination with a biological detector cell by Luzzi et al [13], yielding estimates of resting concentrations of tens of nM, increasing to a few μM during G-protein coupled stimulation of IP₃ signaling.

In addition to differences in methodologies, an important factor that may contribute to these discrepant findings is our observation that the picomolar sensitivity to IP₃ diminishes greatly within a few days after oocytes are removed from the ovary. At early times the dose-response curve for Ca²⁺ liberation approximates a two-component sigmoidal relationship with EC₅₀ values of roughly 200 pM and 100 nM IP₃. After a few days in culture only the latter component remains. This appears not previously to have been appreciated, so it is

difficult to compare published data from experiments where the 'age' of the oocytes is not stated.

Oocytes in the ovary are exposed to numerous environmental factors - including the presence of follicular cells which couple to the oocyte via gap junctions - that are lost following isolation and collagenase treatment, and the physiological state of the oocyte is known to change after isolation [14]. For example, freshly isolated oocytes show a large Cl^- current on hyperpolarization, which completely disappears after 4 days in culture [15]. We do not know the mechanism underlying the loss of IP_3 sensitivity, but the picomolar sensitivity to IP_3 is presumably important for the functioning, maintenance and possibly maturation of oocytes in the ovary. The time course of loss over a few days is similar to that of the hyperpolarization-activated Cl^- current [15], and would be compatible with turnover of IP_3R protein. The *Xenopus* oocyte expresses primarily the type 1 IP_3R isoform [16], so the loss in sensitivity might result from turnover of a type 1 IP_3R variant with unusually high sensitivity, or perhaps the disappearance of other sub-types with high sensitivity. Alternatively, it might also be explained by loss or gain of modulating factors. In the latter context, it is interesting that recombinant type 3 IP_3Rs show a much higher sensitivity to IP_3 when expressed in *Xenopus* oocytes as compared to DT40-KO cells, suggesting that some unknown factor(s) in the oocyte enhances their sensitivity [17].

Extrapolation of our finding of picomolar IP_3 sensitivity in oocytes to cells of more typical dimensions leads to some interesting calculations. Taking a cytosolic volume of $1000 \mu\text{m}^3$ (1 pL) for the volume of a mammalian cell (BioNumbers; www.bionumbers.org), a concentration of 10 pM IP_3 corresponds to only about 6 molecules per cell! In contrast, neuroblastoma cells are estimated to contain about 30,000 IP_3 receptor monomers [18]; corresponding to a concentration of IP_3 binding sites of about 50 nM. Given that opening of a tetrameric IP_3R channel is believed to involve simultaneous binding of IP_3 to at least 3 subunits [19], the probability that 3 out of the 6 IP_3 molecules would bind to the same receptor/channel to evoke Ca^{2+} liberation is so low as to be effectively zero. How is it then that 10 pM IP_3 is able to evoke robust Ca^{2+} liberation in the oocyte? A probable explanation is that IP_3Rs in the oocyte are concentrated primarily in a thin band adjacent to the plasma membrane [20, 21], occupying only a small fraction of the volume of the injected droplet. Although the concentration of IP_3 in the droplet is very low, the total amount of IP_3 is relatively large - about 10^{-19} moles, or 60,000 molecules. The total number of IP_3 molecules may thus be more comparable to the number of IP_3 receptors within the imaged region of the oocyte, whereas the equivalent calculation for a mammalian cell with 10 pM IP_3 indicates that IP_3 receptors outnumber IP_3 molecules by a factor of about 5,000. A consequence of this would be that in cells expressing IP_3 receptors with high affinity (pM or low nM) affinity, the free concentration of IP_3 would be strongly buffered. The relationship between IP_3 production and Ca^{2+} liberation would thus more closely resemble a titration with the amount of IP_3 produced, rather than an equilibrium based on free IP_3 concentration. Moreover, binding of one or two IP_3 molecules to tetrameric receptor/channels would fail to open these channels but would hinder the diffusion of IP_3 , potentially slowing and restricting the range of action of this intracellular second messenger. Although the diffusion of IP_3 in the cytosol had previously been estimated to be little different from its diffusion in

free solution [22], we believe that result may not represent the true physiological situation. Those measurements were made of ^3H IP₃ diffusion in slabs of *Xenopus* oocyte homogenate where the density of IP₃ receptors would have been diluted by mixing with cytoplasm from the interior of the cell; a large excess of IP₃ was present bathing the side of the slab; and it is unknown whether oocytes were studied at a time after isolation when high-affinity IP₃ receptors would be present.

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Highlight

- Freshly-isolated *Xenopus* oocytes display picomolar sensitivity to IP3.
- The resting concentration of IP3 in *Xenopus* oocytes is < 10 pM
- IP3 sensitivity declines over a few days after oocytes removal from the ovary.

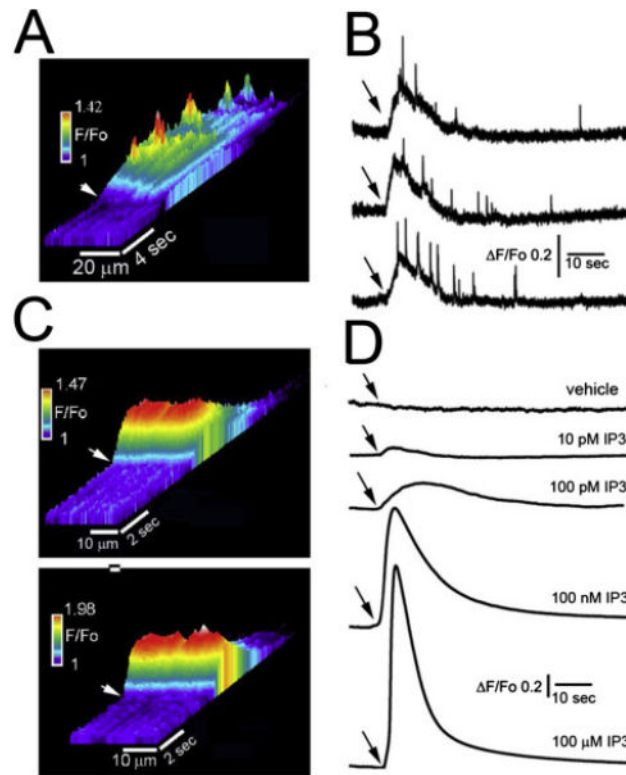


Fig. 1.

Ca^{2+} responses evoked by bolus injections of IP_3 into *Xenopus* oocytes. (A) Linescan (kymograph) image illustrating spatio-temporal patterns of fluorescence Ca^{2+} signals evoked by intracellular injection of a 10 nl bolus of 10 pM IP_3 . The panel depicts fluorescence measured as a function of time along a line (40 μm long and 2 μm wide) on the video record. Increasing fluo-4 fluorescence (increasing free $[\text{Ca}^{2+}]$) is represented by warmer colors as depicted by the color bar (scaled as F/F_0) and by increasing height of each pixel. The arrow indicates the time of IP_3 injection. (B) Representative traces showing fluorescence signals monitored from small ($1 \times 1 \mu\text{m}$) regions of interest positioned sites of local activity (puff sites). (C) Corresponding linescan images recorded in response to injections of 10 nl of 100 pM (top) and 1 nM IP_3 (bottom). (D) Traces show averaged fluorescence signals measured throughout the entire image field in response to injections of 10 nl of solutions of vehicle alone and containing different concentrations of IP_3 , as indicated.

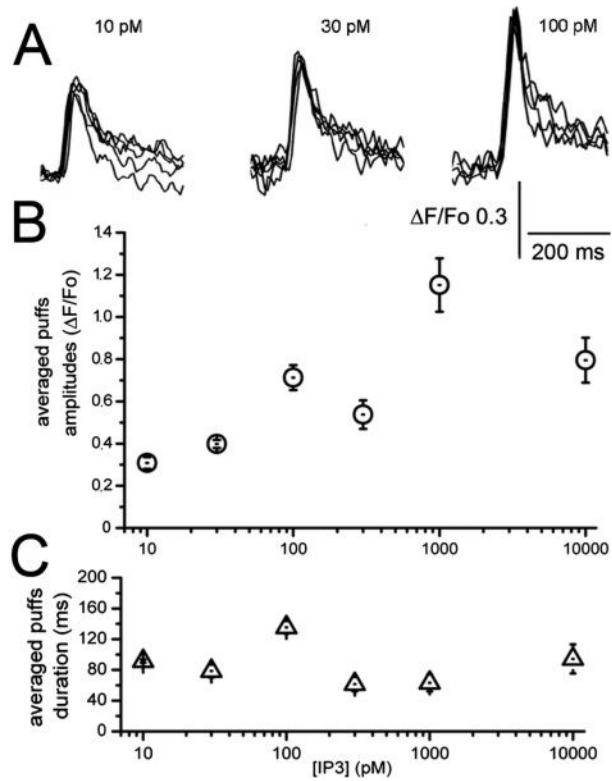


Fig. 2. Local Ca²⁺ puffs evoked by low concentrations of IP₃. (A) Selected traces recorded in response to intracellular injections of 10 nl of solution containing 10, 30 and 100 pM IP₃. Each panel shows 4-5 superimposed traces of fluorescence ratio signals from regions of interest (1 × 1 μm) centered on puff sites, with peaks aligned in time. (B) Scatterplot of mean peak puff amplitudes as a function of IP₃ concentration. Error bars indicate ± 1 SEM (n = 7). (C) Corresponding plot of mean puff durations (duration at half-maximal amplitude) as a function of [IP₃].

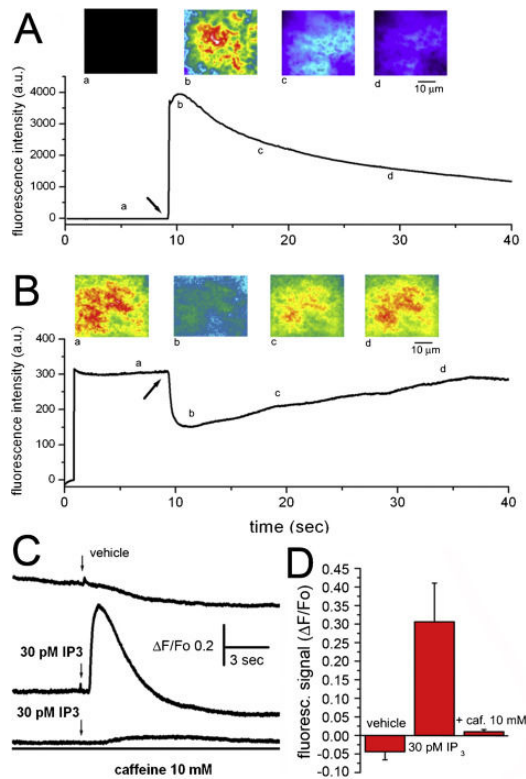


Fig. 3.

(A) Estimating the spatio-temporal profile of injected [IP₃] utilizing calcein as a fluorescent surrogate. The trace shows fluorescence averaged throughout a 20 × 20 μm region of interest centered on the pipette tip in response to injection of 10 nl of 1 μM calcein when marked by the arrow. The image panels show single frames captured at times as indicated on the trace. The fluorescence calibration bar and pseudocoloring of the images are in terms of arbitrary camera units. (B) Estimating the extent to which injected IP₃ would be diluted by mixing with cytosolic fluid by injection of vehicle into an oocyte previously loaded with calcein (final cytosolic concentration about 1 μM). The trace shows fluorescence monitored from a 40 × 40 μm region around the pipette tip. Baseline fluorescence was recorded for about 9s after opening the laser shutter, and an injection of 10 nl of vehicle (without dye) was then made when marked by the arrow. The image panels show single frames captured at times as indicated on the trace. (C) Fluorescence signals evoked by IP₃ injections arise from liberation of sequestered Ca²⁺ through IP₃Rs. Traces in C illustrate representative responses to 10 nl injections of vehicle alone (top), 30 pM IP₃ (middle), and 30 pM IP₃ after pretreating the oocyte with 10 mM caffeine in the bathing solution (bottom). Bars in C show mean fluorescence changes (n = 11 oocytes each) evoked by 10 nl injections of vehicle alone (control); 30 pM IP₃; and 30 pM IP₃ in the presence of 10 mM caffeine in the bathing solution.

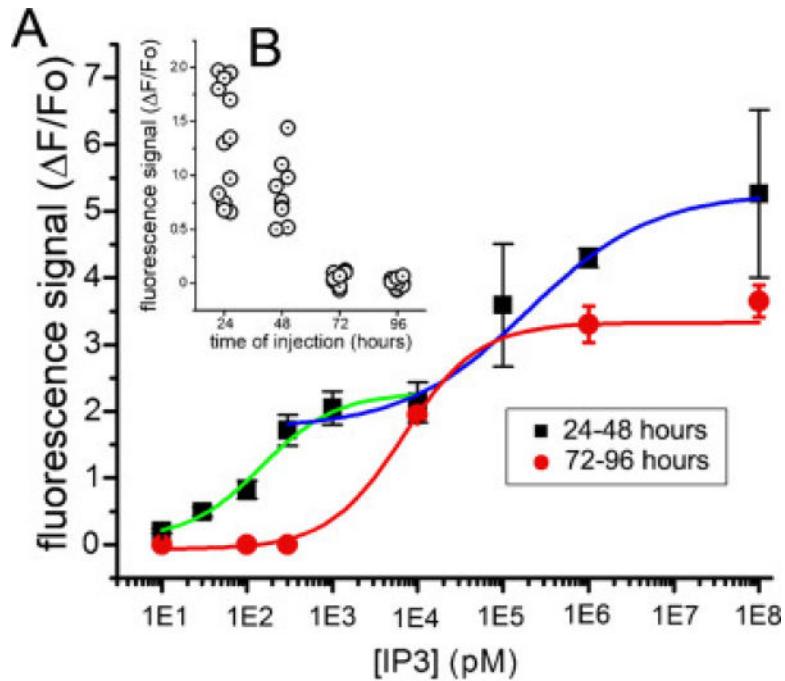


Fig. 4.

Dose/response relationship for IP₃-evoked Ca²⁺ liberation and loss of sensitivity with time after oocyte isolation. (A) Dose-response relationships show mean peak fluorescence signals (averaged across the imaging field) evoked by injections of 10 nl of solutions containing concentrations of IP₃ ranging from 10 pM to 100 μM. Data points represent mean \pm 1 SEM from 4-8 oocytes. Data were grouped by the time between isolation of the oocytes and recording: black squares = 24-48 hr after isolation; red circles = 72-96 hr after isolation. Colored curves are sigmoidal relationships fitted to the data. Fits to the 24-48 hr data (black squares) were done independently for concentrations between 10 and 1000 pM (green curve) and between 1 nM and 100 μM (blue curve). (B) The inset scatter plot shows measurements of Ca²⁺ fluorescence signals evoked by injections of 10 nl of 30 pM IP₃ into individual oocytes at different times after isolation from the ovary.