Invited review

Cell Calcium (1996) 20(2), 105–121

Elementary events of $InsP_3$-induced Ca^{2+} liberation in *Xenopus* oocytes: hot spots, puffs and blips

Ian Parker, John Choi, Yong Yao*

Laboratory of Cellular and Molecular Neurobiology, Department of Psychobiology, University of California, Irvine, California, USA

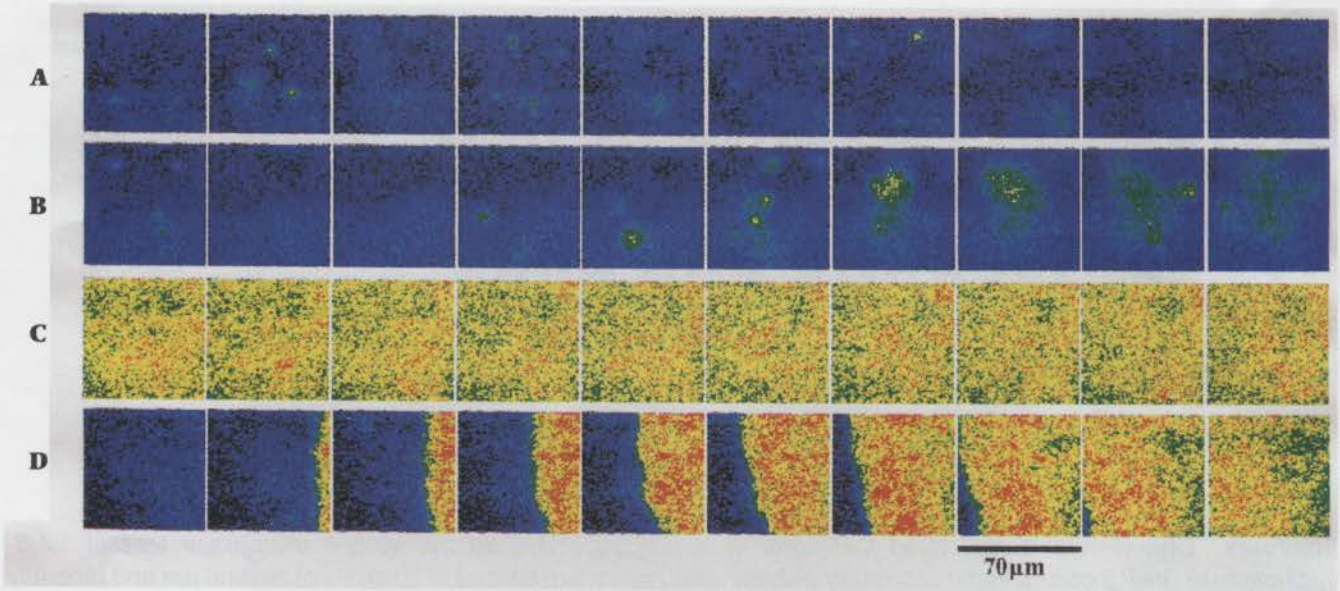
Summary Liberation of sequestered Ca^{2+} ions in *Xenopus* oocytes by the second messenger inositol 1,4,5-trisphosphate ($InsP_3$) occurs from functionally discrete sites, which are spaced at intervals of several μm and probably represent clusterings of $InsP_3$ receptor/channels ($InsP_3R$) in the endoplasmic reticulum. As well as requiring $InsP_3$, opening of release channels is regulated by dual positive and negative feedback by cytosolic Ca^{2+}, leading to regenerative Ca^{2+} transients. Because the sensitivity of this process is determined by [$InsP_3$], the ability of Ca^{2+} ions diffusing from one location to activate increasingly distant $InsP_3R$ is enhanced by increasing [$InsP_3$]. Together with the spatial distribution of receptors, this results in generation of a hierarchy of Ca^{2+} release events, which may involve individual $InsP_3R$ (Ca^{2+} 'blips'), concerted activation of several receptors within a single release site (Ca^{2+} 'puffs'), and recruitment of successive sites by Ca^{2+} diffusing over μm distances to produce propagating Ca^{2+} waves. Thus, Ca^{2+} signalling in the oocyte is organized as at least two sizes of elemental 'building blocks'; highly localized Ca^{2+} transients that arise autonomously and stochastically from discrete sites at low [$InsP_3$], but which become coordinated at higher [$InsP_3$] to produce global Ca^{2+} responses.

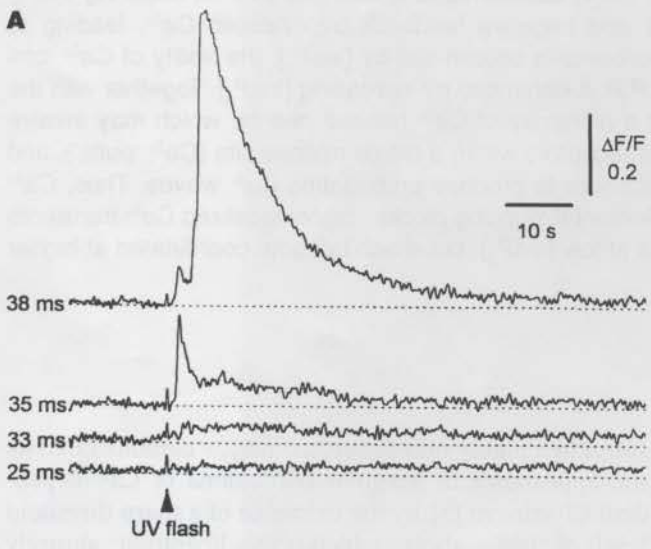
INTRODUCTION

The immature oocyte of *Xenopus laevis* has long been a favoured cell preparation in which to study inositol 1,4,5-trisphosphate ($InsP_3$)-mediated Ca^{2+} signalling [1], probably because its enormous size (> 1 mm diameter) facilitates many experimental procedures [2], the lack of Ca^{2+} release through ryanodine (Ry) receptors simplifies analysis of Ca^{2+} signals [3,4], and the presence of a Ca^{2+}-activated Cl^- current initially provided a simple endogenous monitor of cytosolic Ca^{2+}. A striking early finding was that $InsP_3$ causes liberation of Ca^{2+} from intracellular stores in a highly non-linear manner; as demonstrated by the appearance of irregular oscillations of Ca^{2+}-dependent Cl^- current [5], by the existence of a sharp threshold level of $InsP_3$ above which Ca^{2+} liberation abruptly increased [6] and, following the development of fluorescence Ca^{2+} imaging techniques, by the visualization of propagating waves of intracellular Ca^{2+} [7]. Explanations for these phenomena are provided by findings that gating of the $InsP_3$ receptor/channels which mediate Ca^{2+} liberation from the endoplasmic reticulum is regulated not only by $InsP_3$, but also by cytosolic Ca^{2+} ions acting in a biphasic manner. Moderate elevations of [Ca^{2+}] cause a rapid induction of Ca^{2+} release (CICR) whereas higher concentrations cause a more slowly developing inhibition [8–11]. Thus, in the presence of $InsP_3$, positive and negative feedback by Ca^{2+} ions released into the cytosol permits the oocyte cytoplasm to act as an excitable medium capable of repeatedly propagating regenerative Ca^{2+} waves (Fig. 1D). This process may be viewed as a chemical analogue of the electrical action potential, in which feedback by Ca^{2+} ions on the $InsP_3$ receptor is

Received and accepted 22 May 1996

Correspondence to: Dr I. Parker, Department of Psychobiology, University of California Irvine, CA 92717, USA
Tel. +1 714 824 7332: Fax. +1 714 824 2447
E-mail iparker@uci.edu
**Present address*: Dr Y. Yao, Howard Hughes Medical Institute, School of Medicine, University of California San Diego, La Jolla, CA 92093, USA.





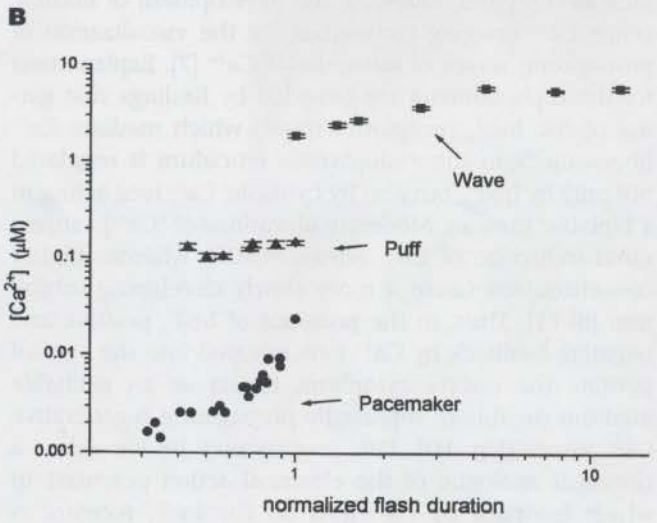


Fig. 1 (above) Pseudocoloured images showing different spatio-temporal patterns of Ca^{2+} liberation induced by increasing concentrations of 3-F-$InsP_3$. Frames within each row were captured at 1 s intervals by a video-rate confocal microscope, and depict a fixed 70 µm square region of an oocyte. The oocyte was loaded with Calcium Green-1 (30 µM) and increasingly 'warm' colours indicate increasing free [Ca^{2+}] above the resting level: the colour scale is uncalibrated, but consistent for all frames. (**A**) Images obtained about 9 min after loading 3-F-$InsP_3$ to a final intracellular concentration of about 30 nM, showing localized Ca^{2+} puffs. (**B**) Injection of additional 3-F-$InsP_3$ to raise the concentration to about 60 nM evoked abortive Ca^{2+} waves. (**C**) Injection of yet more 3-F-$InsP_3$ to give a final concentration of about 90 nM produced a sustained elevation of Ca^{2+} lasting several minutes, followed by a gradual decline and the appearance of Ca^{2+} waves (**D**). (Reproduced with permission of the *Journal of Physiology* from [13].)

equivalent to the voltage-dependent gating of Na^+ channels, and spatial transmission of the signal is achieved by diffusion of Ca^{2+}, rather than electrotonic spread of depolarization.

However, there is evidence that the cytoplasm of the oocyte does not simply act as a uniform excitable medium, comprised of densely packed homogeneous

Fig. 2 (Left hand column) Pacemaker Ca^{2+}, puffs and waves evoked by increasing photorelease of $InsP_3$. (**A**) Traces show Ca^{2+}-dependent fluorescence signals averaged over a 3 µm square region of an oocyte in response to photolysis flashes of various durations (indicated in ms next to each trace). Flashes of 25 and 33 ms evoked increasing pacemaker Ca^{2+} signals, the 35 ms flash evoked a puff superimposed upon a pacemaker signal, and the 38 ms flash gave a Ca^{2+} wave following an initial puff. (**B**) Double-logarithmic plot showing peak free Ca^{2+} elevations during pacemaker Ca^{2+}, puffs and waves evoked by increasing photorelease of $InsP_3$. Stimulus strength is indicated as flash intensity normalized relative to the threshold required to evoke a wave in each experiment. Measurements of pacemaker and puffs were obtained in oocytes loaded with Calcium Green-1 and calculated assuming a dissociation constant of 250 nM; waves were measured in other oocytes loaded with Calcium Green-5N (dissociation constant 12 µM). (Reproduced with permission of the *Journal of Physiology* from [14].)

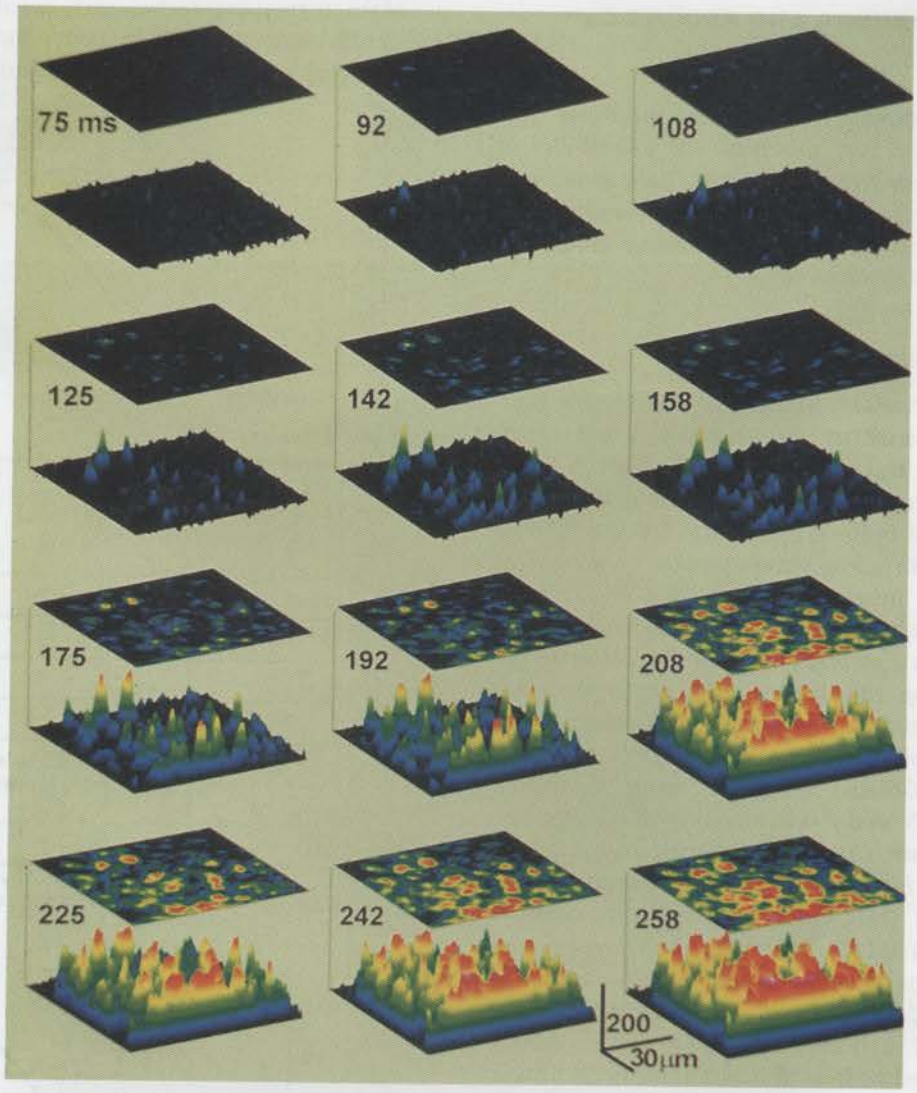


Fig. 3 Rapid confocal imaging of Ca^{2+} release induced by flash photorelease of $InsP_3$. Images show Calcium Green-1 fluorescence signals at various times (indicated in ms next to each frame) following a 10 ms photolysis flash. Frames are composites in which increasing Ca^{2+} levels are depicted by colour in 2-D images of a region of the oocyte (upper) and by elevations in 3-D surface projections of the same region (lower). Calibration bars represent 30 μm for the horizontal axis and 200 intensity units for the vertical axis of the surface projections.

Ca^{2+} stores. In particular, low concentrations of $InsP_3$ may evoke irregular abortive Ca^{2+} waves (Fig. 1B) or transient Ca^{2+} 'puffs' (Figs 1A & 5) which remain localized within a few μm of their origin [12,13], suggesting that $InsP_3$ acts on functionally discrete release sites separated by distances appreciable in comparison to the diffusional spread of Ca^{2+} ions in the cytosol. Intracellular Ca^{2+} signalling is, therefore, more analogous to the saltatory transmission of an action potential along a myelinated nerve than to continuous conduction along an unmyelinated axon. Further, the characteristics of Ca^{2+} wave propagation, and even whether a wave can propagate at all, will depend upon spatial factors, including the separation between release sites and the diffusivity of Ca^{2+} ions, as well as on the properties of the $InsP_3$ receptors themselves.

We review here studies from our laboratory [12–14] on spatio-temporal aspects of Ca^{2+} signalling in oocytes, concentrating particularly on the unitary release events including Ca^{2+} puffs, and yet smaller events ('blips') that may represent Ca^{2+} flux through individual $InsP_3$-gated channels. The overall picture that emerges is of a hierarchical organization of discontinuous Ca^{2+} release events involving single channels (blips), multiple channels clustered in functional units (puffs), and recruitment of successive units (waves). This organization may be applicable to other cell types utilizing the $InsP_3$ signalling pathway, and is similar also to calcium liberation mediated through CICR at ryanodine receptors [15–18].

TECHNIQUES TO EVOKE AND RECORD Ca^{2+} LIBERATION

The ability to detect and characterize subcellular Ca^{2+} signals has depended very much on the availability of appropriate Ca^{2+}-sensitive fluorescent indicators, and upon successive improvements in optical techniques (particularly confocal microscopy) allowing enhanced spatial and temporal resolution.

Fluorescent indicators

We have almost invariably used long-wavelength fluorescent indicators, such as Fluo–3 and Calcium Green [19] for monitoring and imaging cytosolic Ca^{2+}. A major reason for preferring these over UV-excited indicators, such as Fura–2 and Indo–1, is that the UV spectrum is thereby kept free to allow simultaneous photolysis of caged intracellular messengers, including caged $InsP_3$ [20]. Further, the long-wavelength indicators provide a brighter signal with a greater change in fluorescence following Ca^{2+} binding and, for use with confocal microscopy, can be efficiently excited by the 488 nm line of an argon ion laser, avoiding the need for a UV laser. In our hands, Calcium Green-1 provides more 'contrasty' images than Fluo–3, and the availability of other members of the Calcium Green family with varying Ca^{2+} affinities is helpful for recording signals covering a wide range of free $[Ca^{2+}]$. Thus, Calcium Green-1 (dissociation constant about 250 nM [20]) provides good resolution of small Ca^{2+} elevations during pacemaker release and puffs [13,14], whereas Calcium Green-5N (dissociation constant about 12 μM [21]) allows peak Ca^{2+} levels during waves to be followed without saturation of the indicator. A drawback of all currently available visible wavelength indicators is that they fail to show spectral shifts that would allow ratiometric calibration of fluorescence signals in terms of absolute free $[Ca^{2+}]$. However, this is not crucial when the main objects are to study the spatial and temporal distribution of Ca^{2+} rather than its absolute magnitude. In any case, although approximate calibrations can be made by taking a 'pseudo' ratio with the basal resting fluorescence [14], any calibrations of localized, transient Ca^{2+} signals derived from steady-state cuvette measurements are likely to be seriously in error (*see* later).

Confocal microscopy

Most studies of local Ca^{2+} transients have utilized confocal fluorescence microscopy, since the optical sectioning ability of this technique allows recordings to be obtained from a reasonably well defined depth into the cell, with good rejection of out-of-focus fluorescence [22]. However, use of this (expensive) technique is not absolutely mandatory: puffs were first seen by conventional fluorescence video-microscopy [12], and the localization of puff sites in

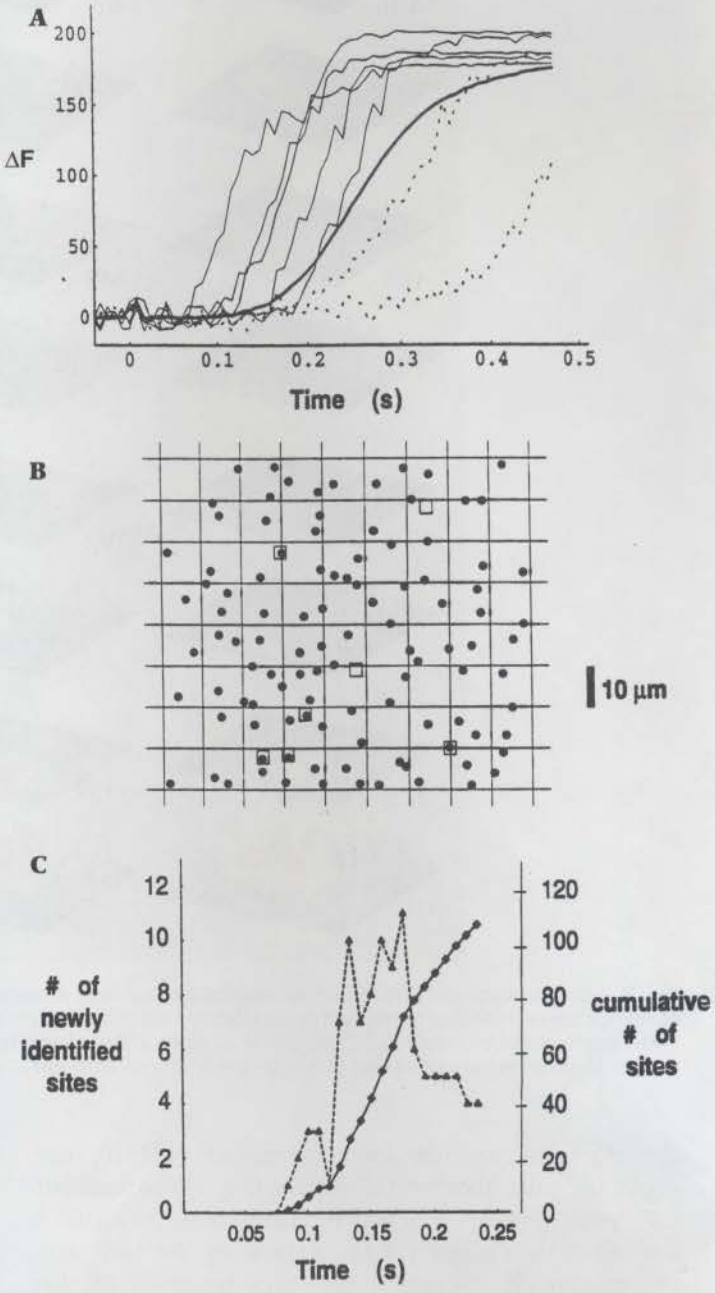


Fig. 4 Kinetics and localization of Ca^{2+} release at hot spots. (**A**) Superimposed traces show initial rise of Ca^{2+} signals (ΔF) at specific locations (2 μm square regions of interest) in the record of Figure 3 following a photolysis flash delivered at time 0. Thin solid lines represent signals at selected short latency hot spots, and dashed lines show regions between release sites (boxes in (**B**)). Thick trace is an average throughout the entire recording field (80 × 80 μm). (**B**) Mapping of unambiguously identified hot spots from the images in Figure 3. Grid spacing is 10 μm. (**C**) Appearance of Ca^{2+} release at new hot spots (dashed line) and cumulative number of release sites (solid line) as a function of time following photorelease of $InsP_3$.

the oocyte to a narrow circumferential band (*see* later) reduces the need to exclude out-of-focus signals. The time course of puffs in the oocyte (roughly 50 ms rise and a few hundred ms decay) is too fast for satisfactory 2-dimensional imaging by the majority of confocal microscopes, which scan at only a few frames per second but, unlike the more rapid sparks in cardiac muscle, is slow enough to allow good resolution by video-rate scanning confocal microscopes. We employ a Noran Odyssey system (Noran Instruments, Middleton, WI, USA) which uses an accousto-optical device for rapid x-scanning, and other slit-scanning systems should also be suitable. By de-interlacing video frames, images can be obtained at 16.66 ms intervals. If even better temporal resolution is desired a single line can be repeatedly scanned every 64 μs with a video-rate system [13], and galvanometer-based confocal microscopes allow line scanning at about a 2 ms rate. Finally, all spatial information can be sacrificed for an improved signal-to-noise ratio and temporal resolution by confocal monitoring of fluorescence from a stationary spot encompassing a volume of 1 fl or smaller within the cell [14].

Caged $InsP_3$

To regulate intracellular levels of $InsP_3$, we have found two techniques particularly advantageous. The first involves injection of oocytes with a poorly metabolized analogue of $InsP_3$, 3-deoxy-3-fluoro-D-*myo*-inositol 1,4,5-trisphosphate (3-F-$InsP_3$) [23]. After allowing about 10 min for diffusional equilibration throughout the oocyte, this produces sustained responses (e.g. puffs or waves, depending upon dose) persisting for an hour or more [24], and the final intracellular concentration can be estimated from the amount injected and the cytosolic volume (roughly 1 μl). In the second technique, oocytes are injected with caged $InsP_3$, and flashes of UV light are used to produce a rapid, transient local elevation of [$InsP_3$]. The relative amount of photoreleased $InsP_3$ can be readily controlled by varying flash duration or intensity [20], though absolute concentrations are more difficult to estimate [25], and the photolysis light can be focussed to irradiate a wide area of the cell or a small spot as desired.

SPATIO-TEMPORAL PATTERNS OF $InsP_3$-EVOKED Ca^{2+} LIBERATION IN OOCYTES

Differing modes of Ca^{2+} liberation at increasing [$InsP_3$]

Imaging of intracellular Ca^{2+} reveals that increasing concentrations of intracellular $InsP_3$ evoke Ca^{2+} liberation in several 'modes', which differ in their magnitudes, spatial patterns of Ca^{2+} localization and time courses. We studied these processes both by injecting progressively increasing amounts of 3-F-$InsP_3$ into oocytes (Fig. 1), and by using UV light flashes of varying durations or intensities to transiently photorelease differing amounts of $InsP_3$ (Fig. 2).

Low concentrations (a few tens of nM) of $InsP_3$ evoke small 'pacemaker' elevations of Ca^{2+}, which rise slowly over a few seconds (Fig. 2A) and, with the resolution attainable by video or confocal imaging, appear spatially homogeneous. The magnitude of this pacemaker signal increases in a graded and non-linear manner with increasing [$InsP_3$] but, even with strong stimuli, overall free [Ca^{2+}] increases are only about 10 nM (Fig. 2B). Over roughly the same concentrations of $InsP_3$ that evoke the pacemaker signal, Ca^{2+} puffs are also apparent superimposed upon the diffuse rise of Ca^{2+}. These appear as transient (a few hundred ms) Ca^{2+} elevations, which arise at particular fixed locations and spread only a few μm (Figs 1A, 2A & 5). Their frequency increases as about the third power of [$InsP_3$] [13] but, unlike the steeply graded pacemaker signal, their amplitudes increase less than 2-fold (Fig. 2B). With increasing [$InsP_3$] some puffs act as foci to trigger Ca^{2+} waves, but these are initially abortive and die away after traveling a short distance (Fig. 1B). Finally, as the concentration of [$InsP_3$] is raised above a sharply defined threshold level, a 'tide' of Ca^{2+} sweeps across the cell (Fig. 1C) and after this declines over a few minutes repetitive propagating Ca^{2+} waves are seen (Fig. 1D). With injections of 3-F-$InsP_3$ this wave threshold is reached at a concentration of about 60 nM [13], and calibration of the amount of $InsP_3$ photoreleased from a caged precursor by threshold photolysis flashes yields a roughly similar value [25]. Near-threshold photolysis flashes uniformly irradiating a region of the oocyte evoke waves beginning at one or a few initiation sites, whereas strong flashes evoke Ca^{2+} release at numerous 'hot spots'. Sustained elevation of $InsP_3$ leads to repetitive Ca^{2+} waves, whose frequency increases steeply with increasing [$InsP_3$] [26], although their peak amplitudes grow over only a 3–4-fold range (Fig. 2B) [27].

'Hot-spots' of Ca^{2+} release mapped by fast confocal imaging

The appearance of Ca^{2+} puffs at localized foci and the irregular 'budding' propagation of abortive Ca^{2+} waves both suggest that Ca^{2+} release occurs at functionally discrete sites. This is further supported by observations that spatially uniform photolysis of caged $InsP_3$ evokes Ca^{2+} signals beginning at discrete 'hot-spots' [12]. Fast (60 frames s^{-1}) confocal imaging thus provides a way to explore the distribution of release sites, by visualizing Ca^{2+} at early times following its release, before diffusion has appreciably blurred the distribution of Ca^{2+}.

Figure 3 shows a sequence of confocal images captured at various times (indicated in ms) following a 10 ms flash which photolysed caged $InsP_3$ uniformly throughout the imaging field. No changes are apparent until after a latency of about 70 ms, which may arise because of a slow sensitization of $InsP_3$ receptors to Ca^{2+} following binding of $InsP_3$ [27]. Fluorescence then begins to rise at several discrete hot spots, and over a few hundred ms the signals at these spots grow in amplitude and spread over increasing distances, while additional hot spots appear elsewhere. Finally, after about 300 ms, Ca^{2+} signals from the numerous release sites begin to blend into one another, to produce a more spatially uniform elevation.

Local Ca^{2+} measurements from hot spots show sharply rising signals, beginning after varying latencies at different locations (thin traces in Fig. 4A) whereas other, appar-

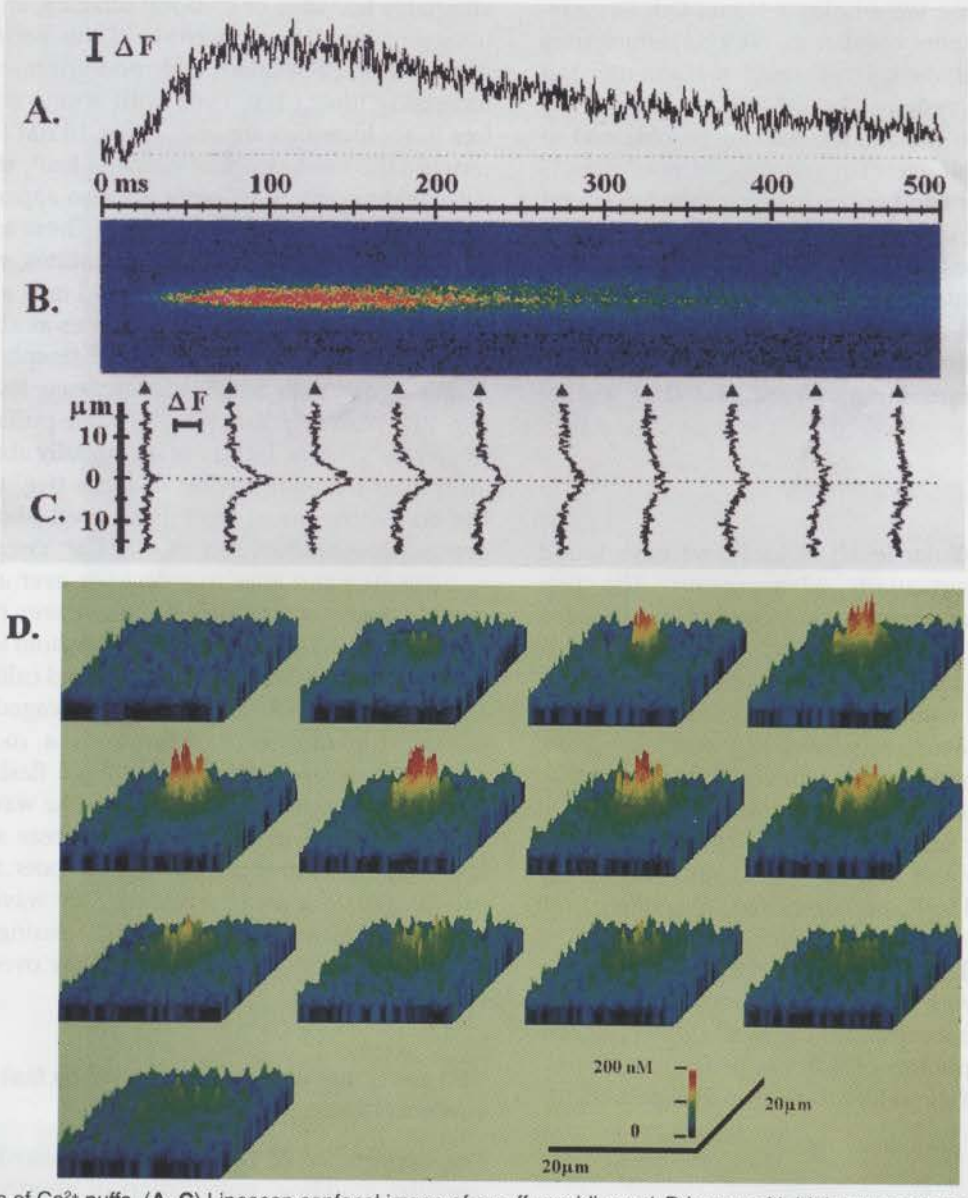


Fig. 5 Two views of Ca^{2+} puffs. (**A–C**) Linescan confocal image of a puff providing a 1-D image with high temporal resolution. The laser spot was repeatedly scanned across a line passing through a puff site at 64 ms intervals and successive lines were stacked from left to right to form a pseudocoloured image (**B**) in which the vertical axis denotes distance along the scan line, time runs horizontally, and increasing Ca^{2+} levels are denoted by increasingly warm colours. Upper trace shows time course of fluorescence monitored directly at the puff site. Lower traces (**C**) show profiles of fluorescence along the scan line at the times indicated by the arrowheads. Time and distance calibrations apply also to the image in (**B**). (**D**) Confocal 2-D images showing a puff evoked by photoreleased $InsP_3$. Successive frames (left to right, top to bottom) were captured at 16.66 ms intervals. The plane of the confocal section was focussed about 5 μm into an oocyte, and increasing Ca^{2+} concentrations above the resting level are depicted both by increasingly 'warm' colours and by increasing height of each pixel. The colour bar indicates Ca^{2+} concentrations on a linear scale, derived from steady-state calibrations. (Reproduced with permission of the *Journal of Physiology* from [13].)

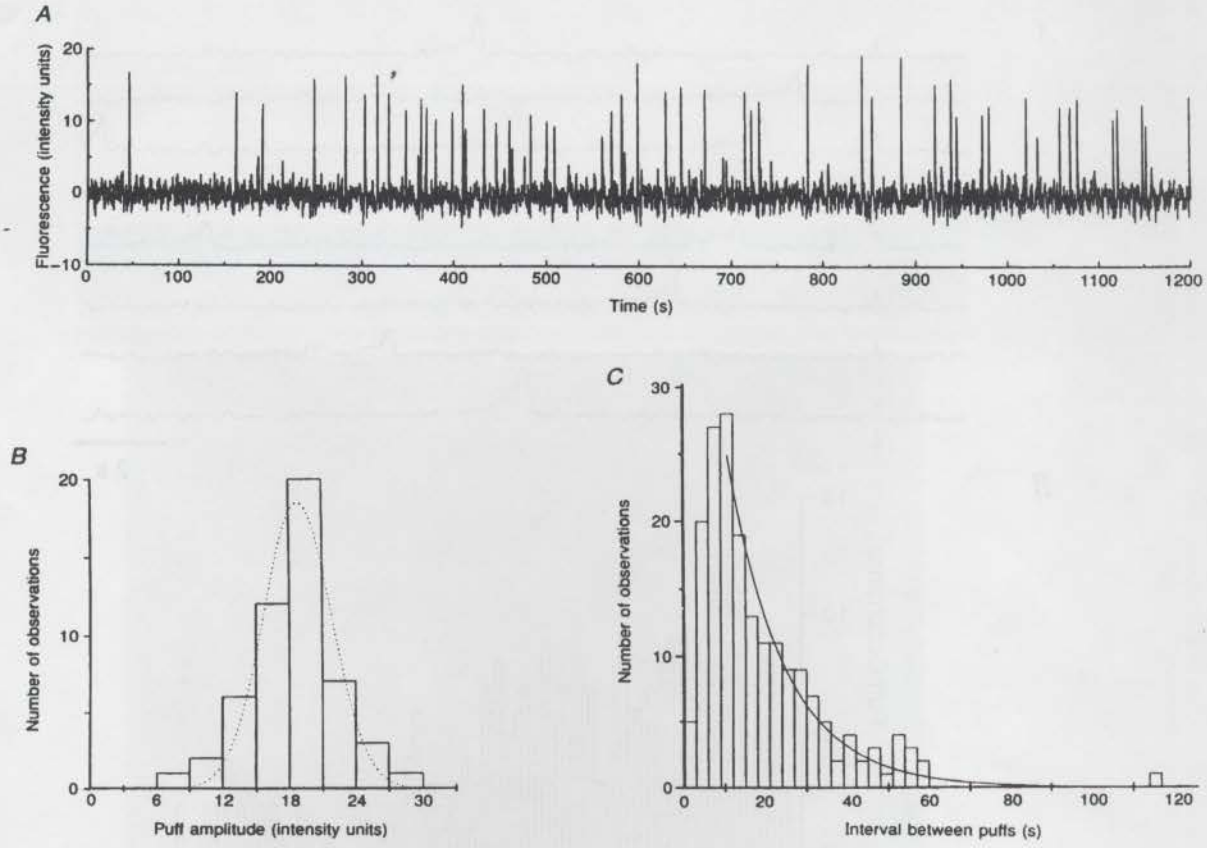


Fig. 6 Continuous record of puffs originating at a single site (**A**) and distributions of puff amplitudes (**B**) and intervals between successive puffs (**C**). (**A**) Trace shows fluorescence recorded from a 3 μm region centered on a puff site in an oocyte loaded with 25 fmol 3-F-$InsP_3$. (**B**) Distribution of puff amplitudes derived from the trace in (**A**). Amplitudes are expressed as arbitrary gray-scale units, and events smaller than 6 intensity units were not counted so as to exclude noise spikes and weak signals from Ca^{2+} diffusing from adjacent sites. (**C**) Pooled data showing distribution of intervals between successive puffs recorded at three sites showing similar frequencies of activity. Curve is an exponential with $\tau = 14$ s, fitted to measurements at intervals > 10 s. (Reproduced with permission of the *Journal of Physiology* from [13].)

ently passive, regions of the cell show more slowly rising signals (dashed traces) as Ca^{2+} diffuses in from surrounding release sites. Because of the variation in latencies between different sites, new hot spots continue to appear for over 100 ms (Fig. 4C), and the overall fluorescence signal integrated over the imaging field (thick trace in Fig. 4A) thus rises more gradually than the local signals at individual release sites. Further, individual sites show latencies that vary considerably between different trials, giving different patterns of Ca^{2+} release following successive flashes and indicating that particular sites do not consistently respond to $InsP_3$ with shorter latency.

Figure 4B shows a map of those Ca^{2+} releasing sites identified during the first 250 ms of the response in Figure 3. Since some sites were probably missed (particularly later in the response when the overall fluorescence level was high) this gives a minimal estimate of the density of Ca^{2+} releasing sites of about 1 per 65 mm^2.

Ca^{2+} PUFFS

Ca^{2+} liberation during puffs

Figure 5 shows the appearance of Ca^{2+} puffs evoked spontaneously in the presence of low concentrations of $InsP_3$ and viewed by one-dimensional line-scan imaging (Fig. 5A–C) and video-rate two-dimensional confocal imaging (Fig. 5D). Important characteristics are that Ca^{2+} release begins at an apparent point source smaller than our limit of resolution (< 1 μm), and the signal then continues to grow for about 50 ms (Fig. 5A), which presumably corresponds to the time for which Ca^{2+} flux continues. Calibration of the fluorescence signals yields an estimate of about 200 nM for the peak free [Ca^{2+}] during a puff [13] but, although this value is useful for comparison with measurements in other cells under similar conditions, it is probably a gross underestimate of the true local free [Ca^{2+}] at the release site (*see* later). At its

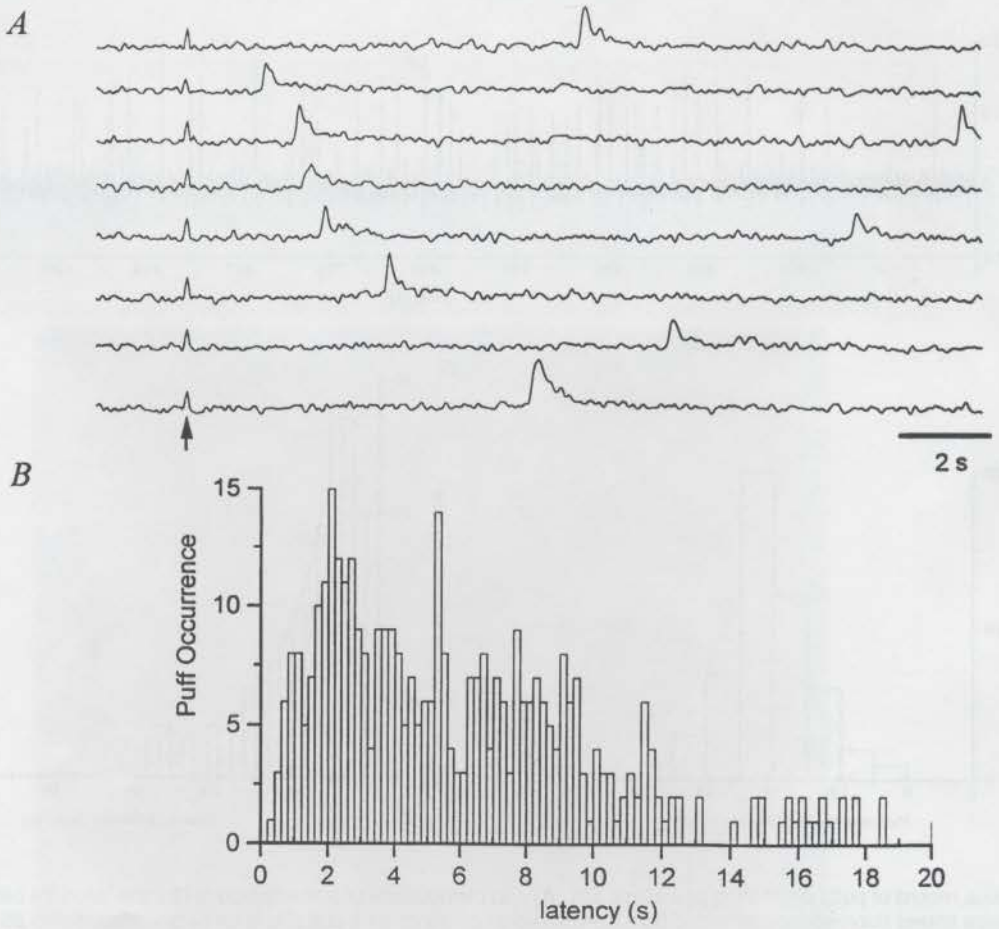


Fig. 7 Stochastic occurrence of puffs following photorelease of $InsP_3$. (**A**) Fluorescence records at a single puff site following repeated delivery of identical photolysis flashes (time marked by arrow). (**B**) Distribution of latencies to occurrence of first puff at a given site following photorelease of $InsP_3$. Data were derived from multiple sites within confocal images, in response to repeated, identical photolysis flashes

peak, the fluorescence is elevated throughout a region roughly 6 μm diameter, and the signal then becomes progressively fainter and more diffuse, becoming undetectable after several hundred ms. The major factor determining the decline of puffs appears simply to be diffusion of Ca^{2+} away from its localized source of release and into the enormous volume of the oocyte, rather than resequestration into the endoplasmic reticulum. Thus, measurements of Ca^{2+} signal integrated throughout a large volume of cytosol around a puff site show a much slower decay than highly localized measurements at the release site [13]. Radial profiles of Ca^{2+}-dependent fluorescence at different times throughout a puff show Gaussian distributions, whose widths increase as a square-root-function with time, as expected for diffusional spread from a point source. The rate of spread corresponds to an apparent diffusion coefficient of about 25 $\mu m^2.s^{-1}$ [13]. However, because this reflects the spread of Ca^{2+}-bound indicator rather than Ca^{2+} itself, the apparent diffusion coefficient for Ca^{2+} ions is likely to be slower as a result of binding to immobile buffers.

Puffs represent unitary, stochastic release events

Continuous recordings of activity at individual sites over tens of minutes show that successive puffs evoked in the presence of a steady concentration of 3-F-$InsP_3$ tend to be of similar sizes, and occur at apparently random intervals (Fig. 6A). Their amplitudes follow a Gaussian distribution (Fig. 6B), suggesting that puffs are 'quantal' events of roughly fixed sizes. An approximate calculation of the amount of Ca^{2+} involved can be made from the magnitude of the fluorescence change and the volume throughout which this is distributed [13]. Assuming endogenous buffering capacities between 10–100 and the presence of 50 μM indicator, this gives estimates of 3–6 attomoles (10^{-18} mol). Because this amount of Ca^{2+} is liberated during the 50 ms rising phase of a puff, the efflux rates would

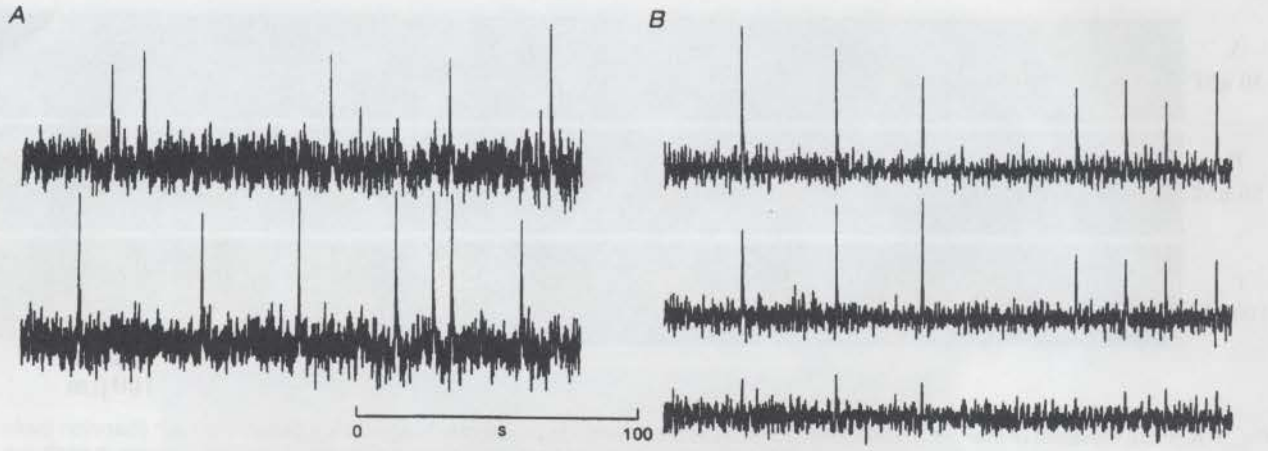


Fig. 8 Asynchronous and synchronous activity from puff sites at different spacings. (**A**) Traces show autonomous puff activity at two sites 6.4 μm apart in an oocyte loaded with 25 fmol 3-F-$InsP_3$. (**B**) Upper two traces show similar recordings a two sites only 2.2 μm apart. Lower trace shows passive Ca^{2+} signals at a point 2.2 μm distant from both puff sites. (Reproduced with permission of the *Journal of Physiology* from [13].)

be $0.6–1.2 \times 10^{-16}$ mol.s^{-1}, corresponding to Ca^{2+} currents of 11–23 pA. These currents are appreciably larger than single channel currents through oocyte $InsP_3$ receptor/channels (< 1 pA; [28,29]), suggesting that puffs do not originate as a result of single channel openings, but involve the concerted opening of a group of several clustered channels.

Intervals between successive puffs at individual sites (Fig. 6C) show a biphasic distribution, with the probability of observing a puff increasing steeply with intervals up to about 10 s following a preceding event, and then declining in a roughly exponential manner at progressively longer intervals. The relative low frequency of puffs at short intervals probably arises from a refractory process induced during the initial puff by Ca^{2+}-dependent inhibition of $InsP_3$ receptors [8,10]. Recovery from this inhibition leads to the probability of observing a second puff increasing to a maximum after about 10 s, after which the probability falls exponentially, suggesting that triggering of puffs is a random process, possibly triggered by stochastic binding of $InsP_3$ to single receptors.

Stochastic triggering of puffs is seen also following a step increase in [$InsP_3$] evoked by flash photorelease of $InsP_3$, since successive flashes evoke puffs with widely differing latencies (Fig. 7A) whose mean value shortens progressively with increasing stimulus strength. With the flash intensity used in Figure 7, no puffs were observed for 200 ms, and the probability of first observing a puff at any given site then increased to a maximum after about 2 s, before declining again over several s (Fig. 7B). The decline at longer intervals arises because of the increasing cumulative probability that a puff will already have occurred at each site, and because [$InsP_3$] falls after the flash due to metabolism and diffusion away from its site of photorelease. However, if the triggering of puffs depended simply upon a 1:1 binding of $InsP_3$ to a receptor, the probability of observing a puff is expected to be greatest immediately following the flash and to decline monotonically thereafter. Different to this, the observed increase in puff occurrence at times up to 2 s suggests that multiple reaction steps may be involved. The underlying mechanisms involved are not yet clear, but may include binding of $InsP_3$ to multiple receptor sites on a channel [30], a requirement for several channels in a cluster to simultaneously bind $InsP_3$, slow transition of channels to a Ca^{2+}-sensitive state [27] and facilitation of channel opening by a rising level of pacemaker Ca^{2+}.

Autonomous and synchronous puffs from sites at different spacings

Puffs arising from sites spaced several μm apart show virtually no correlation in their occurrence (Fig. 8A), indicating that different sites can function autonomously. This again is consistent with the idea that puffs are triggered stochastically, and indicates that there are no factors (e.g. fluctuations of $InsP_3$ level or voltage across the membrane of the endoplasmic reticulum) that tend to synchronize activity over wider areas of the cell. On the other hand, the occurrence of puffs at more closely adjacent sites can be highly correlated. For example, the upper two traces in Figure 8B show recordings at two sites 2.2 μm apart, where the appearance of a puff at one site was invariably accompanied by a puff at the other site. This did not arise simply through passive diffusion of Ca^{2+} from one site to the other, because control recordings

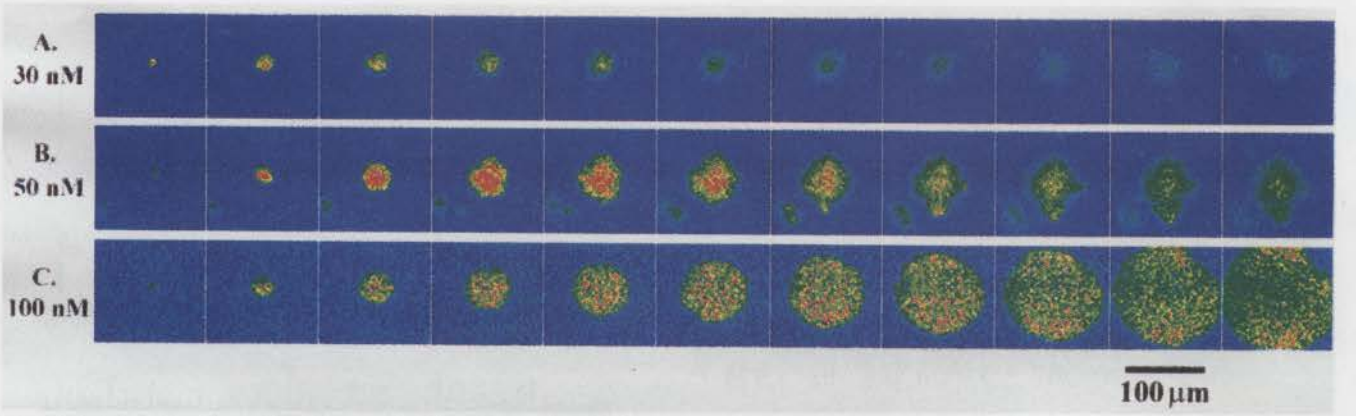


Fig. 9 A global elevation of $InsP_3$ is required in order to allow Ca^{2+} waves to propagate. Images show patterns of Ca^{2+} liberation evoked in an oocyte loaded with caged $InsP_3$ by flashes of photolysis light focussed to a small spot in the center of the field. Frames in each row were obtained at 1/3 s intervals, with the flashes applied 1 frame before the start of each sequence. Sequences in **A,B,C** were recorded from the same region of the cell, and in response to identical flashes after progressively injecting the oocyte with 3-F-$InsP_3$ to final intracellular concentrations of 30, 50 and 100 nM, respectively. About 10 min was allowed after each injection to allow for diffusion of 3-F-$InsP_3$ throughout the oocyte. (Reproduced with permission of the *Journal of Physiology* from [13].)

(lower trace) at a point 2.2 µm from each site showed only small signals. Instead, it seems that Ca^{2+} ions diffusing from a puff are able to trigger Ca^{2+} release from a closely adjacent site, but that the distances between sites are sufficiently great to preclude this happening in most instances.

Why do puffs fail to propagate?

As described above, propagating Ca^{2+} waves are observed only when the concentration of $InsP_3$ is above a certain threshold, and at lower levels Ca^{2+} liberation remains highly localized as transient puffs. We could envisage two explanations for this failure of puffs to initiate propagating waves at low [$InsP_3$]. Firstly, the amount of Ca^{2+} liberated may be inadequate to induce CICR from surrounding sites, and thus 'ignite' a Ca^{2+} wave until [$InsP_3$] is raised sufficiently to produce a high frequency of larger puffs. Secondly, the level of $InsP_3$ may determine the global sensitivity of release sites to Ca^{2+}, so that the cytoplasm is unable to support wave propagation at low [$InsP_3$], irrespective of the magnitude of an initial trigger.

Figure 9 illustrates a simple experiment demonstrating that the second of these hypotheses is correct. An oocyte was loaded with caged $InsP_3$, so that flashes of photolysis light focussed to a small spot could be used to evoke localized Ca^{2+} liberation, and injected with increasing amounts of 3-F-$InsP_3$ to progressively raise the global level of $InsP_3$. The magnitude of the Ca^{2+} signal increased with increasing flash intensity but, at intracellular concentrations of 3-F-$InsP_3$ less than about 30 nM, it remained localized and failed to initiate a wave no matter how large the localized response (Fig. 9A). However, as the oocyte was then loaded to progressively higher global concentrations of 3-F-$InsP_3$, even weak flashes began to evoke first abortive Ca^{2+} waves (Fig. 9B), and then regenerative waves (Fig. 9C)

Mapping of puff sites

Puffs are most clearly resolved when the plane of the confocal section is focussed a few µm below the surface of the oocyte, whereas at more superficial and deeper location the Ca^{2+} signals are fainter and more diffuse [13]. It thus seems likely that the sites of Ca^{2+} release lie about 5–7 µm into the cell, so that rapid Ca^{2+} liberation from localized sources gives sharp Ca^{2+} signals at this depth, but that signals recorded at other levels are attenuated and blurred by diffusion of Ca^{2+} ions. This conclusion is also in agreement with immunohistochemical localization of $InsP_3$ receptors in *Xenopus* oocytes, which show intense staining in a narrow band just under the plasma membrane [28,31,32]. Thus, Ca^{2+} release phenomena in the oocyte, including puffs and waves, may be largely two-dimensional events, restricted to a narrow band close under the surface membrane. However, additional, weaker Ca^{2+} liberation may also arise more deeply into the cell. A faint, diffuse staining of $InsP_3$ receptors is observed throughout the animal hemisphere of the oocyte [28], and confocal recordings at depths of 10–25 µm into the oocyte show Ca^{2+} transients in response to photoreleased $InsP_3$ that rise more rapidly than expected if Ca^{2+} ions had to diffuse from a source 5 µm below the surface.

Figure 10A shows a mapping of the lateral distribution of puff sites across a 100 µm region of the animal hemisphere of an oocyte, derived by marking the positions and numbers of puffs observed while imaging a confocal

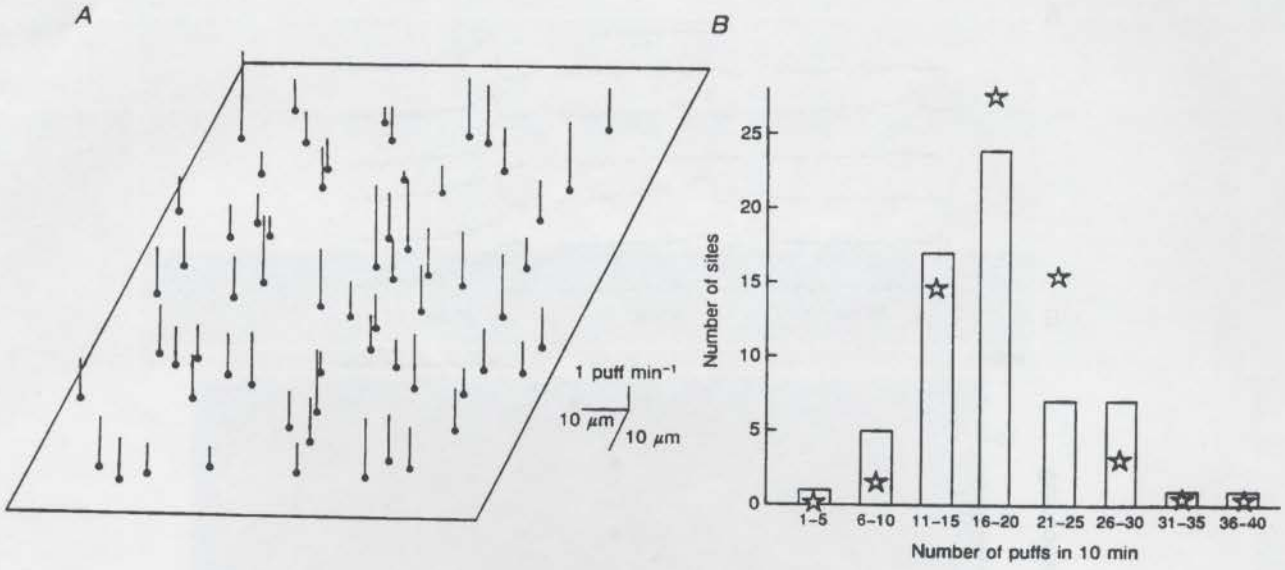


Fig. 10 Lateral distribution of puff sites and distribution of puff frequencies at different sites. (**A**) Diagram represent a 100 × 100 μm region of the animal hemisphere of an oocyte loaded with 35 fmol 3-F-$InsP_3$. Dots indicate sites where one or more puff were observed during a 10 min recording, and vertical bars represent the frequencies of puffs at each site. (**B**) Distribution of puff frequencies at the sites in (**A**). Stars indicate the Poisson distribution expected if the probability of occurrence of puffs was equal at all sites. (Reproduced with kind permission of the *Journal of Physiology* from [13].)

section at a depth of 7 μm for a period (10 min) long enough to ensure that most sites give at least one puff. Puffs are observed only at particular fixed locations, present at a density of about 1 per 30 μm^2, corresponding to a mean spacing between sites of roughly 6 μm, similar to values obtained by mapping hot spots (Fig. 4B).

The localization of puffs at discrete sites might arise if $InsP_3$-sensitive stores are physically separate, or through localized regions of high densities of $InsP_3R$ within an extensive, continuous organellar store. The latter appears to be the case, as dual injection of oocytes with Ca^{2+} indicator and a lipophilic dye (DiQ) which labels the endoplasmic reticulum shows a contiguous, though irregular reticulum, on which puff sites are superimposed (N. Callamaras & Y. Yao, unpublished data).

SENSITIVITY OF DIFFERENT RELEASE SITES TO $InsP_3$

Studies on permeabilized cell preparations have shown that sub-optimal concentrations of $InsP_3$ lead to the rapid release of only a fraction of the total releasable Ca^{2+}: a phenomenon christened 'quantal' release [33,34]. One explanation proposed to account for this characteristic is that $InsP_3$-sensitive stores are arranged as autonomous quantal units, which release their contents in an all-or-nothing manner at varying thresholds concentrations of $InsP_3$ (*see* [35] for review). Given our finding of all-or-none Ca^{2+} release as puffs originating at functionally autonomous sites, we were thus interested to determine whether different puff sites display differing sensitivities to $InsP_3$.

One approach was to measure the observed frequencies of puffs originating at different sites in the presence of a stable and spatially uniform concentration of 3-F-$InsP_3$ [13]. As illustrated in Figure 10A, the numbers of puffs seen during a 10 min recording period varies appreciably between different sites. However, much, if not all of this variability can be attributed to statistical variation, rather than to intrinsic differences in sensitivities between sites. Figure 10B plots the numbers of sites which showed various numbers of puffs during the recording period. These data are fitted reasonably well by a Poisson distribution (stars), calculated assuming that all sites show the same probability (0.55 min^{-1}) of generating puffs.

Another approach was to more directly estimate the relative $InsP_3$ concentration required to just evoke Ca^{2+} release at different sites. For this purpose, oocytes were loaded with caged $InsP_3$, and a pin-hole aperture was placed in the UV light source so as to focus the light to a spot of roughly 2 μm diameter and thus cause photorelease of $InsP_3$ within a highly localized region. Simultaneous confocal imaging with Calcium Green-1 showed that photolysis flashes with durations slightly above threshold evoke Ca^{2+} signals at individual sites that are usually not coincident with the center of the photolysis

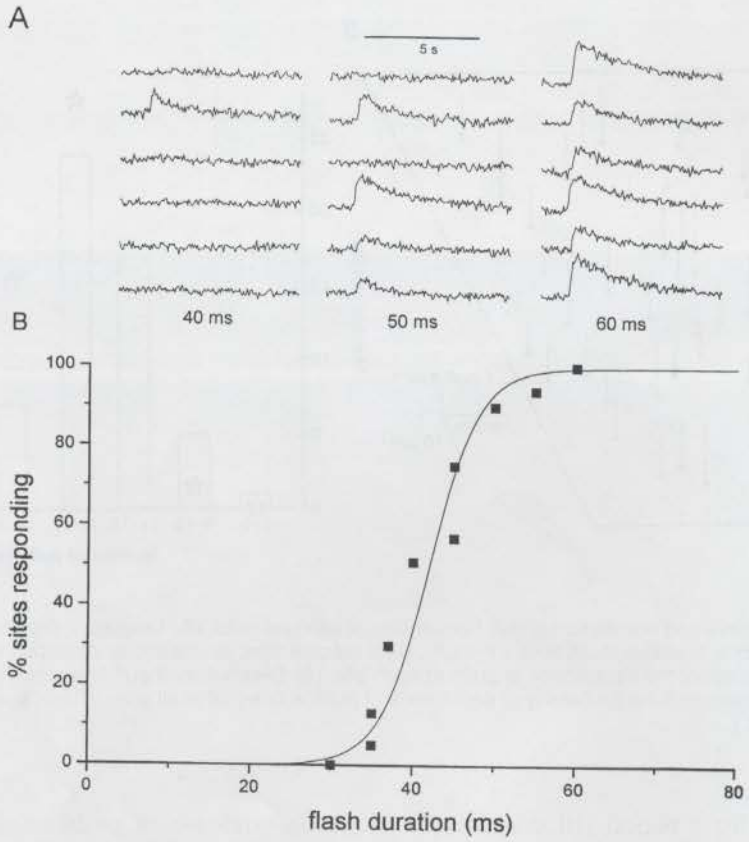


Fig 11 Local 'pin-hole' photorelease of $InsP_3$ reveals little variation in sensitivity to $InsP_3$ between different release sites. (**A**) Traces show Ca^{2+} fluorescence signals monitored by a stationary confocal spot in response to photorelease of $InsP_3$ evoked by flashes of UV light focussed to a spot of about 2 μm diameter concentric with the confocal spot. Each trace was obtained with the oocyte moved to a new, random position, and the columns of records show responses to three different flash durations as indicated. Note the all-or-none characteristic of responses. (**B**) Percentage of sites responding to photolysis flashes of varying durations. Data were obtained from traces like those in (**A**). Points indicates the percentage of trials ($n > 20$) with each flash duration in which responses were observed. All measurements are from a single oocyte, and each trial was made at a different, randomly chosen location within a roughly 200 × 200 μm area of the animal hemisphere.

spot, but are displaced by varying distances up to 2–3 μm. This further confirms the idea that Ca^{2+} release occurs at discrete sites separated by distances of several μm, such that $InsP_3$ formed in the tightly focussed photolysis spot (randomly positioned within a matrix of sites) diffuses on average 1 or 2 μm before activating the closest (or most sensitive) site. Responses show all-or-none activation, allowing threshold stimuli to be unambiguously determined for different sites. For example, in Figure 11A, flashes of 40 ms duration delivered at several random locations in an oocyte generally failed to evoke Ca^{2+} release, whereas 60 ms flashes almost invariably gave responses. We could thereby estimate the proportion of Ca^{2+} release sites responding to a given photorelease of $InsP_3$ by recording at many randomly chosen locations and counting the numbers of trials which show responses to any given light flash. Surprisingly little variability is observed. In Figure 11B only about 10% of sites respond to flashes of 35 ms duration, yet > 95% respond to flashes of 50 ms duration. Given the additional variation that may be expected from differences in displacements of release sites from the stimulus spot, as well as variations in light absorption and exclusion of caged $InsP_3$ by organelles, it seems that different release sites have very similar threshold sensitivities to $InsP_3$.

Ca^{2+} BLIPS: ELEMENTARY UNITS OF Ca^{2+} RELEASE?

Pacemaker Ca^{2+} involves discontinuous release events smaller than puffs

Two observations mentioned above suggest that the Ca^{2+} puff may not be **the** elemental unit of Ca^{2+} liberation, involving the opening of only a single $InsP_3$ receptor/channel. Firstly, the amount of Ca^{2+} involved in a puff

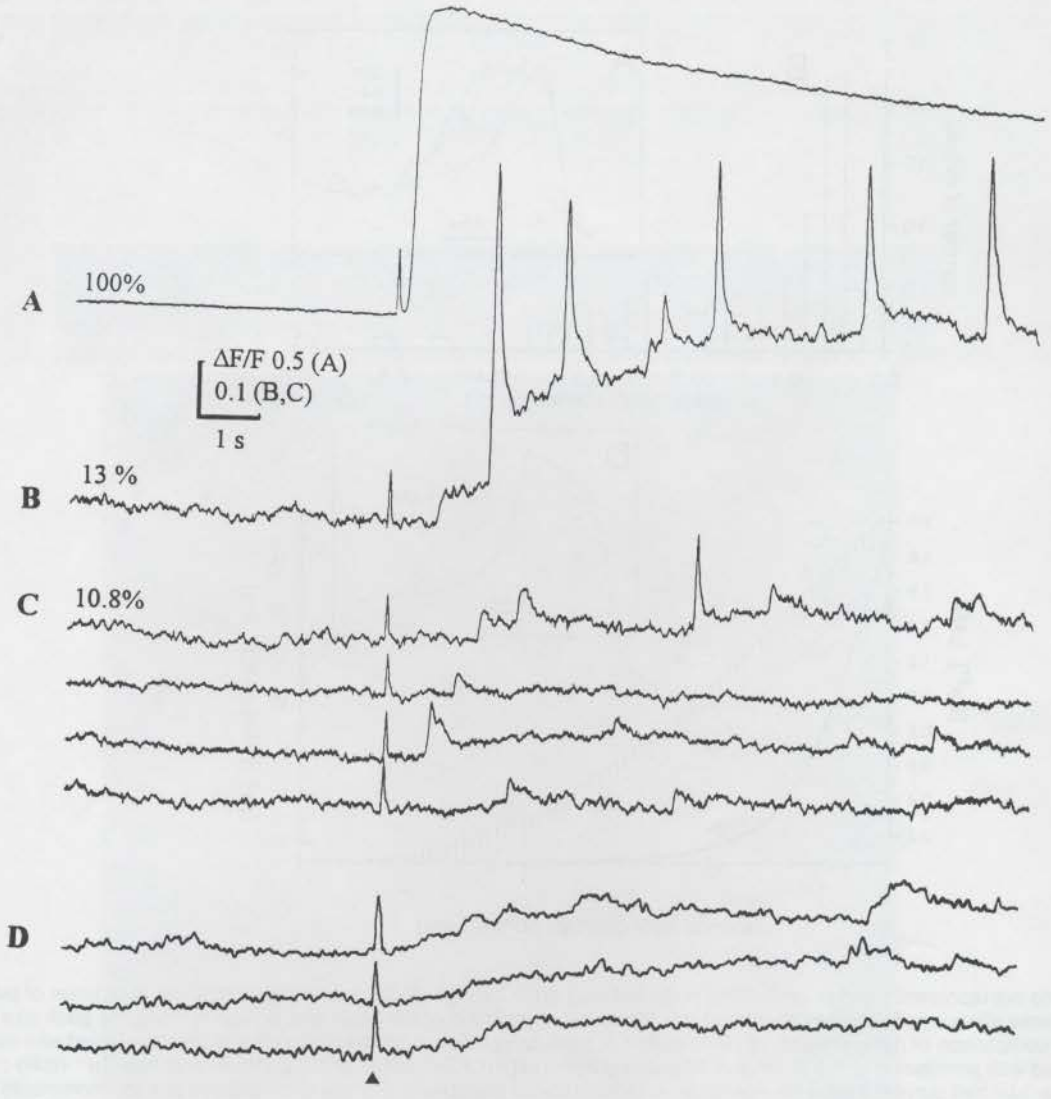


Fig. 12 Ca^{2+} waves, puffs and blip recorded by stationary-spot confocal microfluorimetry. Traces show Rhod–2 fluorescence from a confocal spot in response to photorelease of $InsP_3$ by light flashes of varying intensity which irradiated a region of about 80 μm diameter around the confocal spot. (**A**) Ca^{2+} wave evoked by a strong flash applied at the arrowhead. Note that the recording gain is 5× smaller than for other traces. (**B**) A flash 1% the strength of that in (**A**) evoked a pacemaker Ca^{2+} signal with superimposed puffs and smaller blips. (**C**) Four repeated flashes with intensities 10.8% that in (**A**) evoked small pacemaker signals, together with discrete Ca^{2+} blips. (**D**) Traces from a different, non focal site, illustrating pacemaker signals with poorly resolved fluctuations. Flash intensity was the same as (**C**). (Reproduced with kind permission of the *Journal of Physiology* from [14].)

appears too great to reconcile with likely values for the single channel current and, instead, may require the concerted openings of several channels. Secondly, the pacemaker signal points to the existence of Ca^{2+} flux smaller than that during the puff. Despite the smoothly graded nature of the pacemaker signal and its apparent homogeneous spatial distribution in confocal images, we wondered if it may arise through the summation of underlying elementary Ca^{2+} release events that are individually too small to resolve by that technique. To look for such discrete events, we used confocal microfluorimetry [26] to monitor Ca^{2+} signals with much improved signal-to-noise ratio from fixed points (femtolitre volumes) within the cell in response to photorelease of $InsP_3$ [14].

As illustrated in Figure 12, this approach allows resolution of discontinuous Ca^{2+} release signals at low levels of $InsP_3$. Photolysis flashes of appropriate strength invariably evoke pacemaker signals, but at most recording locations these show irregular, rounded fluctuations, suggesting an underlying stepwise Ca^{2+} release at sites some distance from the recording spot (Fig. 12D). Furthermore some recordings where the confocal spot presumably lies close

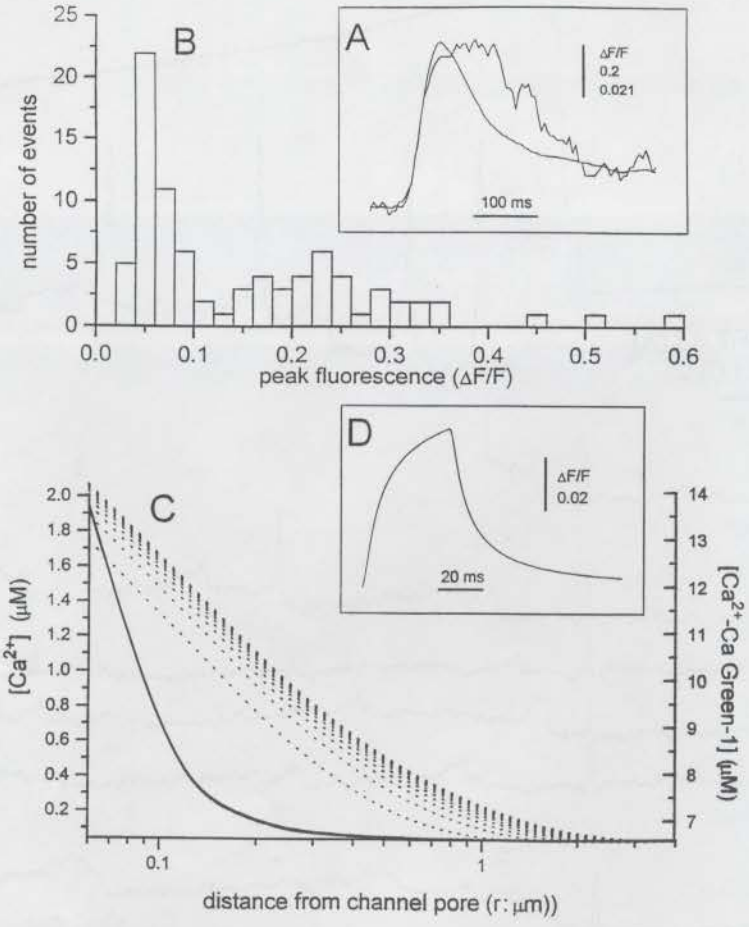


Fig. 13 Ca^{2+} blips do not represent small or attenuated puffs, but may arise through single channel openings. (**A**) Rise times of puffs and blips are similar. Traces show a puff (smooth trace) and blip (noisy trace) recorded at the same site, scaled to the same peak size and aligned to facilitate comparison of rising phases. (**B**) Distribution of peak amplitudes of 85 events (puffs and blips) selected with rise times < 70 ms). (Reproduced with permission of the *Journal of Physiology* from [14].) (**C**) Simulation of radial diffusion of free Ca^{2+} (solid curves) and Ca^{2+}-bound indicator (dashed curves) from a channel in an extensive planar membrane. Curves show profiles at 4 ms increments from 4–40 ms after onset of a Ca^{2+} flux corresponding to a Ca^{2+} current of 0.1 pA. Profiles were numerically simulated by diffusion-reaction equations [36] assuming 40 nM resting free Ca^{2+}, diffusion coefficient of free Ca^{2+} = 250 $\mu m^2.s^{-1}$, and parameters for various buffers as follows (buffer species; concentration (μM); on rate ($\mu M^{-1}.s^{-1}$); off rate (s^{-1}); diffusion coefficient ($\mu m^2.s^{-1}$)); immobile buffer, 300, 500, 5000, 0; fast mobile buffer, 100, 500, 5000, 250; slow mobile buffer, 100, 500, 5000, 15; Calcium Green-1, 50, 680, 180, 40. D, Confocal fluorescence signal corresponding to the simulation in (**C**). Fluorescence was calculated by averaging the Ca^{2+}-bound indicator concentration throughout a hemispherical volume of radius 1 μm around the channel, and is expressed as fractional fluorescence change relative to resting fluorescence at 40 nM free Ca^{2+}.

to a release site show more sharply defined events (Fig. 12C). These Ca^{2+} 'blips' resemble miniature puffs and occur at apparently random times following the photolysis flash. Weak stimuli evoke only infrequent blips, whereas slightly stronger stimuli evoke also larger puff signals, superimposed on a pedestal of pacemaker Ca^{2+} (Fig. 12B). Finally, yet stronger stimuli evoke Ca^{2+} spikes with a peak amplitude about 50-fold greater than the blips (Fig. 12A). Although we have not yet been able to map the distribution of sites showing sharply defined blips, they tend to occur at locations where stronger stimuli evoke puffs, suggesting that both signals arise from common sites.

An obvious concern is whether the blips could be artifactual, arising because the confocal spot records the attenuated 'tails' of Ca^{2+} signals diffusing from puffs that originate some distance from the recording site. Two observations indicate this is not the case. Firstly, confocal imaging shows that relatively weak flashes that evoke few or no puffs may still evoke frequent blips. Secondly, blips and puffs at a given site show similar rates of rise (Fig. 13A), whereas blips would be expected to be greatly

slowed (time to peak ~200 ms) if they arose from puffs at sites sufficiently distant (about 5 μm; Fig. 7A of [13]) to result in a 5-fold attenuation.

Figure 13B shows the distribution of amplitudes of Ca^{2+} release events (puffs and blips), selected with rapid (< 70 ms) rise times to minimize inclusion of distant events. The blips form a separate population, rather than representing a tail of small puffs. Their mean size is roughly one-fifth that of the puffs, though this value may be a overestimate since small events could have gone undetected in the noise of the recordings.

Amount of Ca^{2+} liberated during blips

The question thus arises of whether the blip may arise as a result of Ca^{2+} flux though a single $InsP_3$-gated channel. Based on our earlier calculation that a puff may involve concerted opening of ~20 channels [13], it seems that a few (~4) channels would still be needed to account for the blip. However, the measurements on which this estimate are based are subject to considerable uncertainties; in particular, the buffering capacity of the oocyte is not well characterized, errors in measuring radial spread of the puff will be raised to the third power, and the ratio of puff/blip sizes may be underestimated if small blips lie undetected below our noise floor. As an alternative approach, we computed the approximate fluorescence signal that would be detected within the confocal spot by simulating the radial diffusion of Ca^{2+} and Ca^{2+}-bound indicator from a channel opening for 40 ms (Fig. 13C,D). This indicated that a current of only 0.1 pA would be sufficient give a Calcium Green-1 fluorescence signal ($\Delta F/F = 0.05$) greater than that observed during blips (mean $\Delta F/F = 0.022$; 8 events). Although this comparison is again subject to uncertainties, it seems plausible that blips may represent single channel activity.

Another question concerns whether blips arise through stochastic gating of channels which remain open for random, exponentially-distributed durations, or whether the duration of Ca^{2+} flux during each event is roughly constant and determined by feedback processes like those regulating the termination of release during puffs and Ca^{2+} waves. The approximately Gaussian distribution of blip amplitudes (Fig. 13B) is consistent with each event arising from release of a roughly constant amount of Ca^{+}, but we cannot exclude the possibility that many small events escaped detection so that the observed blips represents only the tail of a skewed distribution.

Relation of fluorescence signals to local Ca^{2+} gradients

It is evident from Figure 13C that the predicted magnitude and spatial distribution of fluorescence signal (concentration of Ca^{2+}-bound indicator) bear no simple relationship to the profile of free $[Ca^{2+}]$. Although this issue is not new (for example, [36,37]), it is worth reemphasizing in light of increasing interest in local Ca^{2+} signals. Because of factors including the greater mobility of the dye as compared to the restricted diffusion of Ca^{2+} in the presence of immobile buffers, the fluorescence signal is more diffuse than the tightly localized free Ca^{2+} profile and underestimates free $[Ca^{2+}]$ near the channel. For example, at a distance of 100 nm, free $[Ca^{2+}]$ was estimated to be about 1 μM, yet the proportion of bound dye at equilibrium (about 25%) corresponds to a steady-state free $[Ca^{2+}]$ of about 125 nM; and even greater discrepancies are apparent closer to the channel. For these reasons, and because the dimensions of even a diffraction-limited confocal spot are large in comparison to diffusional spread around a channel, simple calibrations of indicator signals during localized Ca^{2+} transients give little (or even misleading) information about local free $[Ca^{2+}]$ levels.

CONCLUSIONS

Figure 14 summarizes our working model for the mechanisms underlying the different modes of $InsP_3$-evoked Ca^{2+} liberation seen in *Xenopus* oocytes. This hinges on two key assumptions – that $InsP_3$ receptor/channels are clustered into groups in the endoplasmic reticulum membrane to form discrete functional release sites and that, in the presence of $InsP_3$, Ca^{2+} released through one channel will tend to promote the opening of further channels. At low concentrations of $InsP_3$ only one or a few receptors in a cluster may be binding $InsP_3$, so that the majority are unable to respond to a local rise in cytosolic $[Ca^{2+}]$. Thus, stochastic binding of $InsP_3$ may cause the opening of a single channel, but the Ca^{2+} that is released would be unlikely to activate neighbouring channels within the cluster, giving rise only to a small, localized Ca^{2+} signal apparent as a blip. As the $InsP_3$ concentration is then progressively raised, more receptors will bind $InsP_3$, so that Ca^{2+} flux through a single channel is able to activate regenerative release from other channels at nm spacings within a cluster, producing a larger puff signal. Each puff, however, may remain localized if neighbouring receptor clusters are insufficiently sensitive to respond to Ca^{2+} diffusing across the much greater (~5 μm) distances between functional release sites. Finally, at yet higher concentrations of $InsP_3$, the excitability of $InsP_3$ receptors is enhanced sufficiently that a puff originating at one site is able to trigger regenerative release from adjacent sites, giving rise to a propagating Ca^{2+} wave.

In order that discrete sites function independently, the spacing between functional release units must be sufficient that the amount of Ca^{2+} that diffuses from one site to the next has a low probability of triggering that site by

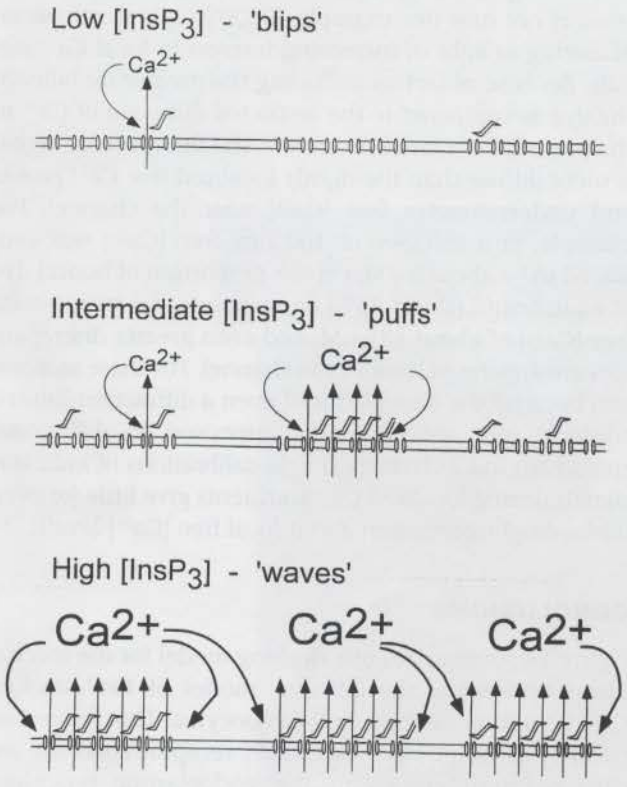


Fig. 14 Scheme illustrating putative activity of $InsP_3$ receptor/channels and clusters of channels in the presence of increasing concentrations of $InsP_3$ giving rise to blips (**A**), puffs (**B**) and waves (**C**). Diagrams represent the clustered arrangement of $InsP_3$ receptors in a continuous endoplasmic reticulum and the regenerative feedback of liberated Ca^{2+} ions on varying numbers of receptors which bind $InsP_3$.

CICR. Thus, the granularity of puff sites in the oocyte (mean spacing ~5 μm) is relatively coarse as compared to the radial spread of Ca^{2+} puffs, and at low [$InsP_3$] each site generally behaves independently. In other cells the spacings between sites can be very different, raising the possibility that the spatial arrangement of Ca^{2+} release channels may be 'tailored' together with other factors (including diffusivity, buffering and sequestration of Ca^{2+}, as well as amount and duration of Ca^{2+} flux during release events) to achieve either autonomous elementary events (puffs and sparks) or coupled release (waves) under appropriate conditions. For example, sparks in cardiac myocytes originate at sites about 1.8 μm apart, corresponding to locations of T-tubules [38]. Puff sites this close together in the oocyte show tightly coupled behavior (Fig. 8) yet, except during waves in Ca^{2+}-overloaded myocytes, sparks at adjacent sites occur independently [15], The difference presumably lies in the more restricted spatial spread of sparks (a little over 1 μm; [15]) as compared to puffs, due to their more rapid kinetics and faster Ca^{2+} resequestration in the myocyte.

A potentially important consequence of the quantized nature of Ca^{2+} liberation through both $InsP_3$ and ryanodine receptors is that it provides a mechanism to explain how Ca^{2+} liberation may be graded with stimulus intensity, despite the regenerative nature of CICR which might other wise lead to all-or-none responses [17,33,39,40]. Thus, an apparently smoothly graded cellular Ca^{2+} response would result from progressive recruitment of independent puffs or sparks, which each contribute a roughly fixed amount of Ca^{2+}. In the oocyte this mechanism holds at low [$InsP_3$], where the frequencies of blips and puffs increase steeply with increasing concentration. Further, because release sites all appear to have similar sensitivities to $InsP_3$, the recruitment of sites with increasing [$InsP_3$] arises because each autonomous site has the same higher probability of being activated, rather than because populations of sites with progressively lower sensitivities begin to respond. However, it remains unclear what (if any) signal may be transduced by an increase in puff frequency. Although locally high Ca^{2+} elevations around puff sites may induce biochemical responses [36], puffs fail to evoke detectable electrical signals in the form of activation of Ca^{2+}-dependent Cl^- current [41]. This is different to the situation in smooth muscle, where sparks evoke transient currents from closely associated sarcolemmal K^+ channels [42], probably because puff sites are situated more distantly (a few μm from the surface membrane) [26]. The most obvious consequence arising from the organization of Ca^{2+} release at discrete sites in the oocyte for electrical signaling is that Cl^- currents are evoked only by concentrations of Ca^{2+}-mobilizing agonists [43] or intracellular $InsP_3$ [37] above a well defined threshold at which waves become able to propagate. Even though the peak Ca^{2+} level during waves then shows relatively little dependence upon [$InsP_3$], Cl^- currents are still able to encode information about stimulus strength in two ways; 'digitally' as progressive increases in wave frequency [26], and as analogue increases of current amplitude because the rise time of Ca^{2+} spikes increases progressively with increasing [$InsP_3$] [27] and the Cl^- current signals preferentially rate of rise of [Ca^{2+}] rather than absolute [Ca^{2+}] [44].

ACKNOWLEDGEMENTS

We thank Noran Instruments Inc. for loan of an Odyssey XL confocal microscope system used to obtain Figures 3 and 4, and Dr Sandra Spence (Noran Instruments) for excellent technical assistance. Work in the author's laboratory was supported by NIH grant GM48071.

REFERENCES

1. Berridge M.J. Inositol trisphosphate and calcium signalling. *Nature* 1993; **361**: 315–325.
2. Dascal N. The use of *Xenopus* oocytes for the study of ion channels. *CRC Crit Rev Biochem* 1987; **22**: 317–387.
3. Parker I., Ivorra I. Caffeine inhibits inositol trisphosphate-mediated liberation of intracellular calcium in *Xenopus* oocytes. *J Physiol* 1991; **433**: 229–240.
4. DeLisle S., Welsh M.J. Inositol trisphosphate is required for the propagation of calcium waves in *Xenopus* oocytes. *J Biol Chem* 1992; **267**: 7963–7966.
5. Kusano K., Miledi R., Stinnakre J. Cholinergic and catecholaminergic receptors in the *Xenopus* oocyte membrane. *J Physiol* 1982; **328**: 143–170.
6. Parker I., Miledi R. Non-linearity and facilitation in phosphoinositide signalling studied by the use of caged inositol trisphosphate in *Xenopus* oocytes. *J Neurosci* 1989; **9**: 4068–4077.
7. Lechleiter J.D., Girard S., Peralta E., Clapham D.E. Spiral calcium wave propagation and annihilation in *Xenopus laevis* oocytes. *Science* 1991; **252**: 123–126.
8. Bezprozvanny I., Watras J., Ehrlich B.E. Bell-shaped calcium-response curves of Ins(1,4,5)P3- and calcium-gated channels from endoplasmic reticulum of cerebellum. *Nature* 1991; **351**: 751–754.
9. Finch E.A., Turner T.J., Goldin S.M. Calcium as a coagonist of inositol 1,4,5-trisphosphate-induced calcium release. *Science* 1991; **254**: 443–446.
10. Parker I., Ivorra I. Inhibition by Ca^{2+} of inositol trisphosphate-mediated Ca^{2+} liberation: a possible mechanism for oscillatory release of Ca^{2+}. *Proc Natl Acad Sci USA* 1991; **87**: 260–264.
11. Iino M., Endo M. Calcium dependent immediate feedback control of inositol 1,4,5-trisphosphate-induced Ca^{2+} release. *Nature* 1992; **360**: 76–78.
12. Parker I., Yao Y. Regenerative release of calcium from functionally discrete intracellular stores by inositol trisphosphate. *Proc R Soc Lond [Biol]* 1991; **246**: 269–274.
13. Yao Y., Choi J., Parker I. Quantal puffs of intracellular Ca^{2+} evoked by inositol trisphosphate in *Xenopus* oocytes. *J Physiol* 1995; **482**: 533–553.
14. Parker I., Yao Y. Ca^{2+} transients associated with openings of inositol trisphosphate-gated channels in *Xenopus* oocytes. *J Physiol* 1996; **491**: 653–658.
15. Cheng H., Lederer W.J., Cannell M.B. Calcium sparks: elementary events underlying excitation-contraction coupling in heart muscle. *Science* 1993; **262**: 740–743.
16. Thorn P., Laurie A.M., Smith P.M., Gallagher D.V., Petersen O.H. Local and global cytosolic Ca^{2+} oscillations in exocrine cells evoked by agonists and inositol trisphosphate. *Cell* 1993; **74**: 661–668.
17. Bootman M.D., Berridge M.J. The elemental principles of calcium signaling. *Cell* 1995; **83**: 675–678.
18. Cannell M.B., Cheng H., Lederer W.J. The control of calcium release in heart muscle. *Science* 1995; **268**: 1045–1048.
19. Minta A., Kao J.P.Y., Tsien R.Y. Fluorescent indicators for cytosolic calcium based on rhodamine and fluoresceine chromophores. *J Biol Chem* 1989; **264**: 8171–8178.
20. Parker I. Use of caged intracellular messengers for studying the inositol trisphosphate pathway. In: Boulton A.A., Baker G.B., Taylor C.W. eds *Neuromethods, vol. 20, Intracellular Messengers.* New Jersey: Humana, 1992; 369–393.
21. Yao Y., Parker I. Ca^{2+} influx modulation of temporal and spatial patterns of inositol trisphosphate-mediated Ca^{2+} liberation in *Xenopus* oocytes. *J Physiol* 1994; **476**: 17–28.
22. Pawley J.B. *Handbook of Biological Confocal Microscopy.* New York: Plenum, 1995.
23. Kozikowski A.P., Fauq A.H., Askoy I.A., Seewald M.J., Powis G. Synthesis of the first optically pure fluorinated inositol 1,4,5-trisphosphate of *myo*-inositol. Stereochemistry and its effect on Ca^{2+} release in Swiss 3T3 cells. *J Am Chem Soc* 1990; **112**: 7403–7404.
24. Yao Y., Parker I. Inositol trisphosphate-mediated Ca^{2+} influx into *Xenopus* oocytes triggers Ca^{2+} liberation from intracellular stores. *J Physiol* 1993; **468**: 275–296.
25. Parker I., Ivorra I. Characteristics of chloride currents evoked by photorelease of inositol trisphosphate in *Xenopus* oocytes. *Am J Physiol* 1992; **263**: C154–C165.
26. Parker I., Ivorra I. Confocal microfluorimetry of Ca^{2+} signals evoked in *Xenopus* oocytes by photoreleased inositol trisphosphate. *J Physiol* 1991; **461**: 133–165.
27. Parker I., Yao Y., Ilyin V. Fast kinetics of calcium liberation induced in *Xenopus* oocytes by photoreleased inositol trisphosphate. *Biophys J* 1996; **70**: 222–237.
28. Callamaras N., Parker I. Inositol 1,4,5-trisphosphate receptors in *Xenopus laevis* oocytes: localization and modulation by Ca^{2+}. *Cell Calcium* 1994; **15**: 60–72.
29. Stehno-Bittel L., Luckhoff A., Clapham D.A. Calcium release from the nucleus by $InsP_3$ receptor channels. *Neuron* 1995; **14**: 163–167.
30. Meyer T., Holowka D., Stryer L. Highly cooperative opening of calcium channels by inositol 1,4,5-trisphosphate. *Science* 1988; **240**: 653–656.
31. Parys J.B., Sernett S.W., DeLisle S., Snyder P.M., Welsh M.J., Campbell K.P. Isolation, characterization and localization of the inositol 1,4,5-trisphosphate receptor protein in *Xenopus laevis* oocytes. *J Biol Chem 1992;* **267**: 18776–18782.
32. Kume S., Muto A., Aruga J. et al. The *Xenopus* IP_3 receptor: structure, function and localization in oocytes and eggs. *Cell* 1993; **73**: 555–570.
33. Muallem S., Pandol S.J., Beeker T.G. Hormone-evoked calcium release from intracellular stores is a quantal process. *J Biol Chem* 1989; **264**: 205–212.
34. Taylor C.W., Potter B.V.L. The size of inositol 1,4,5-trisphosphate sensitive stores depends on inositol 1,4,5-trisphosphate concentration. *Biochem J* 1990; **266**: 189–194.
35. Bootman M.D. Quantal Ca^{2+} release from $InsP_3$-sensitive intracellular Ca^{2+} stores. *Mol Cell Endocrinol* 1994; **98**: 157–166.
36. Rizzuto R., Brini M., Murgia M., Pozzan T. Mitochondrial Ca^{2+} homeostasis in intact cells. *Science* 1993; **262**: 744–747.
37. Roberts W.M. Localization of calcium signals by a mobile calcium buffer in frog saccular hair cells. *J Neurosci* 1994; **14**: 3246–3262.
38. Shacklock P.S., Wier W.G., Balke C.W. Local calcium transients (Ca^{2+} sparks) originate at transverse tubules in at heart cells. *J Physiol* 1995; **487**: 601–608.
39. Stern M.D. Theory of excitation-contraction coupling in cardiac muscle. *Biophys J* 1992; **63**: 497–515.
40. Lopez-Lopez J.R., Shacklock P.S., Balke C.W., Wier W.G. Local calcium transients triggered by single L-type calcium channel currents in cardiac cells. *Science* 1995; **268**: 1042–1045.
41. Miledi R., Parker I. Chloride current induced by injection of calcium into *Xenopus* oocytes. *J Physiol* 1984; **357**: 173–183.
42. Nelson M.T., Cheng H., Rubart M. et al. Relaxation of arterial smooth muscle by calcium sparks. *Science* 1995; **270**: 633–637.
43. Parker I., Sumikawa K., Miledi R. Activation of a common effector system by different brain neurotransmitter receptors in *Xenopus* oocytes. *Proc R Soc Lond [Biol]* 1987; **232**: 59–70.
44. Parker I., Yao Y. Relation between intracellular Ca^{2+} signals and Ca^{2+}-activated Cl^- current in *Xenopus* oocytes. *Cell Calcium* 1994; **15**: 276–288.