Ca^{2+} transients associated with openings of inositol trisphosphate-gated channels in *Xenopus* oocytes

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1. The mechanisms underlying inositol 1,4,5-trisphosphate ($InsP_3$)-induced Ca^{2+} liberation were studied in *Xenopus* oocytes by using scanning and stationary-point confocal fluorescence microscopy to record Ca^{2+} signals evoked by photorelease of $InsP_3$ from a caged precursor.

2. Fluorescence measurements from confocal images showed that increasing [$InsP_3$] evoked three distinct modes of Ca^{2+} liberation: a diffuse 'pacemaker' signal, localized transient puffs, and propagating waves. Peak free Ca^{2+} concentrations during waves and puffs (respectively, 2–5 μM and 100–200 nM) varied only slightly with [$InsP_3$], whereas the pacemaker amplitude varied over a wider range (at least 1–30 nM Ca^{2+}).

3. The improved resolution provided by confocal point recording revealed discontinuous Ca^{2+} 'blips' during pacemaker release. These events were resolved only at particular locations and had time courses similar to the puffs (rise, ~50 ms; decay, a few hundred milliseconds) but with amplitudes one-fifth or less of puff amplitudes.

4. We conclude that blips may arise through opening of single $InsP_3$-gated channels, whereas puffs reflect the concerted opening of several clustered channels due to local regenerative feedback by Ca^{2+}.

The second messenger inositol 1,4,5-trisphosphate ($InsP_3$) functions by releasing Ca^{2+} ions from intracellular stores (Berridge, 1993), and in several cell types release has been shown to occur from multiple discrete subcellular sites (Parker & Yao, 1991; Kasai, Li & Miyashita, 1993; Yagodin, Holtzclaw, Sheppard & Russell, 1994). This characteristic is clearly demonstrated in *Xenopus* oocytes by the appearance of Ca^{2+} 'puffs': brief all-or-none pulses of Ca^{2+} that occur randomly and remain localized to within a few micrometres of their sites of origin (Parker & Yao, 1991; Yao, Choi & Parker, 1995). Puffs are evoked at low intracellular concentrations of $InsP_3$, whereas higher concentrations evoke propagating Ca^{2+} waves. Both are regenerative responses, which appear to arise through the ability of liberated Ca^{2+} ions to act as a co-agonist on $InsP_3$ receptors (Bezprozvanny, Watras & Ehrlich, 1991; Finch, Turner & Goldin, 1991; Iino & Endo, 1992; Yao & Parker, 1992) and thereby trigger further Ca^{2+} release that may either remain restricted to a single site as a puff (Yao *et al.* 1995), or propagate as a wave across successive sites (Lechleiter & Clapham, 1992). However, although the all-or-none puffs represent a 'quantal' unit of $InsP_3$-mediated Ca^{2+} liberation, the amount of Ca^{2+} involved appears too great to be liberated through a single $InsP_3$ receptor channel, suggesting that puffs instead reflect the concerted opening of several tightly clustered channels (Yao *et al.* 1995). By using an improved technique for recording Ca^{2+} signals from femtolitre volumes within an intact oocyte we now show the existence of smaller Ca^{2+} 'blips' – discontinuous Ca^{2+} signals which may reflect the activity of single channels and reveal a further unitary level in the organization of $InsP_3$-mediated Ca^{2+} signalling.

METHODS

Experiments were done on defolliculated immature oocytes obtained by surgical removal from albino *Xenopus laevis*. Frogs were anaesthetized by immersion in a 0·17% aqueous solution of MS-222 (3-aminobenzoic acid ethyl ester) for 15 min, and were allowed to recover after the operation. Procedures for preparation of oocytes and intracellular injections were as described (Parker, 1992). In brief, oocytes were loaded by intracellular microinjection 0·5–2 h before recording with calcium indicator (Calcium Green-1, Calcium Green-5N or rhod-2; all obtained from Molecular Probes) and caged inositol 1,4,5-trisphosphate (*myo*-inositol 1,4,5-trisphosphate, $P^{4(5)}$-1-(2-nitrophenyl)ethyl ester; Calbiochem) to respective final intracellular concentrations of about 40 and 5 μM. Free Ca^{2+} concentrations were calculated assuming respective affinities for Calcium Green-1 and -5N of 250 nM and 12 μM (Yao *et al.* 1995). For the experiments in Fig. 1, oocytes were imaged

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using a video-rate confocal microscope (Odyssey, Noran Instruments, Middleton, WI, USA) coupled to an Olympus IMT2 inverted microscope equipped with an epi-fluorescence system modified to produce flashes of UV photolysis light (Yao *et al.* 1995). Traces were derived using the brightness-over-time function of the MetaMorph software package (Universal Imaging, West Chester, PA, USA) to measure average fluorescence intensity (F) over a defined square region (usually 3 × 3 μm^2) of the image.

Confocal point fluorescence recordings (Figs 2–4) were made by focusing a laser beam (547 nm He–Ne laser for rhod-2 excitation; 488 nm argon ion laser for Calcium Green-1) through a ×40 oil immersion objective (Nikon; numerical aperture, 1·3) to a diffraction-limited spot about 5 μm into the oocyte, at which depth InsP_3-sensitive release sites are concentrated (Yao *et al.* 1995). The laser power was attenuated to <20 μW to avoid photodamage. Fluorescence emission at wavelengths >515 nm (with 488 nm excitation) and >560 nm (547 nm excitation) was detected by a low noise PIN photodiode, using an Axopatch-1B amplifier (Axon Instruments) to measure photodiode current, and signals were low-pass filtered at 50 or 100 Hz. The small size of the photodiode (active area of 0·02 mm^2, equivalent to 2·6 μm^2 at the specimen) served as the confocal aperture (Wilson, 1995) and by scanning through a 0·3 μm fluorescent bead the resolution of the confocal detection was estimated (full width at half-intensity) to be about 3 μm axially and 0·8 μm laterally. The confocal spot system was interfaced through the camera port on the inverted microscope, which could be rapidly switched to allow imaging of the same region of the cell via the Noran Odyssey confocal scanner coupled through the trinocular phototube. This point confocal system is identical in principle to systems we have previously described (Parker & Ivorra, 1993; Ilyin & Parker, 1994), but the present version provided better sensitivity, primarily through the use of a photodiode with a quantum efficiency greater than that of the photomultipliers employed previously. Because the blips we describe here represent changes in fluorescence intensity of only a few per cent, a concern was whether they may have arisen artefactually through fluctuations in laser output. To check for this, a separate photodiode was usually employed to monitor laser power, and records were discarded if fluctuations were observed. In addition, long control records were always obtained prior to stimulation.

RESULTS

Three modes of InsP_3-evoked Ca^{2+} liberation

Figure 1*A* illustrates different spatio-temporal patterns of Ca^{2+} liberation evoked by increasing concentrations of InsP_3. Traces show fluorescence of the high-affinity Ca^{2+} indicator dye Calcium Green-1, monitored from a 3 × 3 μm region of an oocyte imaged using a video-rate scanning confocal microscope (Yao *et al.* 1995) in response to photorelease of InsP_3 from a caged precursor by flashes of UV light uniformly irradiating a wide (100 μm diameter spot) area around the measuring region. Brief flashes (25 and 33 ms traces in Fig. 1*A*) evoked small elevations of Ca^{2+} throughout the stimulated region ('pacemaker' signals), which increased in a graded, though non-linear, fashion

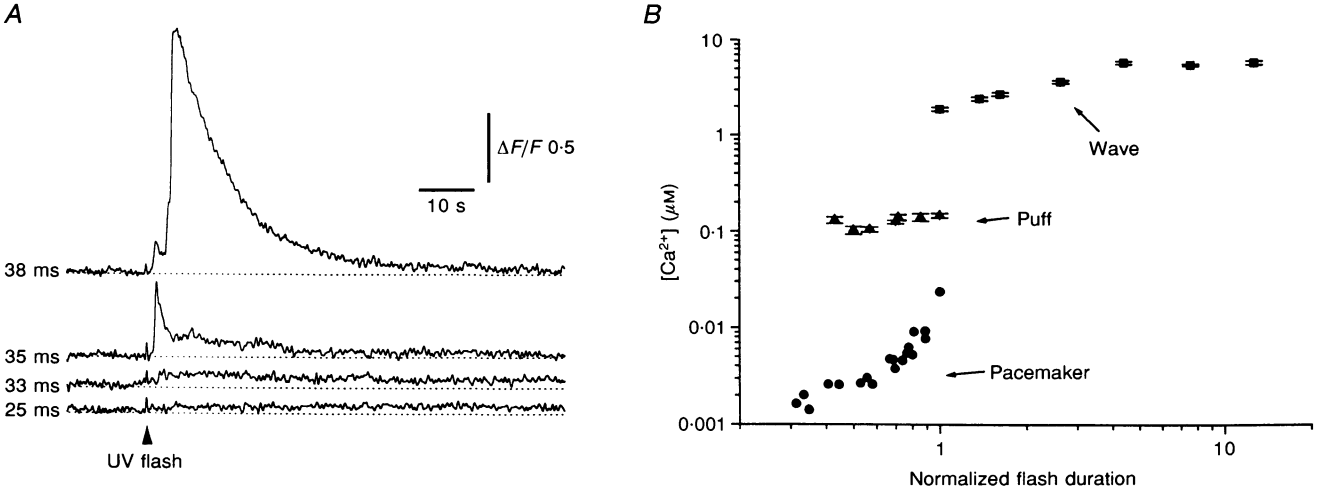

Figure 1. Pacemaker, puffs and waves of Ca^{2+} evoked by increasing photorelease of InsP_3

A, traces show Ca^{2+}-dependent fluorescence signals averaged over a 3 × 3 μm region of an oocyte in response to photolysis flashes of increasing duration (indicated in ms next to each trace). Flashes of 25 and 33 ms duration evoked increasing pacemaker Ca^{2+} signals, the 35 ms flash evoked a puff followed by pacemaker, and the 38 ms flash gave a Ca^{2+} wave following an initial puff. The oocyte was loaded to final intracellular concentrations of about 50 μM Calcium Green-1 and 5 μM caged InsP_3. Fluorescence signals are expressed as fractional increases above the resting level ($\Delta F/F$). Traces are representative of results in >20 oocytes. *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 to the threshold for evoking a wave in each experiment. Measurements of pacemaker and puffs were obtained using Calcium Green-1, whereas waves were measured in other oocytes loaded with the lower-affinity indicator Calcium Green-5N to avoid saturation at high free $[Ca^{2+}]$. Pacemaker data show individual measurements from 3 oocytes, whereas points for puffs and waves indicate means ± S.E.M. from 3 or more oocytes.

with increasing photorelease of $InsP_3$ (Fig. 1*B*) and lasted a few tens of seconds. Slightly stronger stimuli (e.g. 35 ms trace in Fig. 1*A*) then evoked random, transient puffs of Ca^{2+}, which were apparent only at specific loci and were superimposed on a higher level of pacemaker Ca^{2+}. Finally, stimuli above a certain threshold evoked a propagating Ca^{2+} wave, evident in the 38 ms trace in Fig. 1*A* as a large transient spike following an initial puff.

$InsP_3$ thus liberates Ca^{2+} in at least three different modes, activated at differing concentrations of $InsP_3$ (cf. Parker & Yao, 1991). The resulting free Ca^{2+} changes are plotted in Fig. 1*B* as a function of flash intensity normalized to the threshold required to just evoke waves (roughly 50 nM $InsP_3$; Yao *et al.* 1995). However, it must be remembered that these values represent a spatial average of Ca^{2+} throughout the measuring volume and that localized free Ca^{2+} levels at release sites may be vastly greater. From Fig. 1*B* it is apparent that the pacemaker signal amplitude increased more than tenfold with increasing photorelease of $InsP_3$, in contrast to the puffs and waves which, although increasing in frequency (Parker & Ivorra, 1993; Yao *et al.* 1995), showed only small increases in amplitude.

Discontinuous Ca^{2+} signals during pacemaker release

Despite the apparently smoothly graded nature of the pacemaker signal, we wondered if it might arise through the summation of discontinuous underlying elementary Ca^{2+} release events, which were individually too small to resolve in confocal images. To look for such events, we used confocal microfluorimetry (Parker & Ivorra, 1993; Ilyin & Parker, 1994) to monitor Ca^{2+} signals with high resolution from fixed, minute (a few femtolitre) volumes within a much

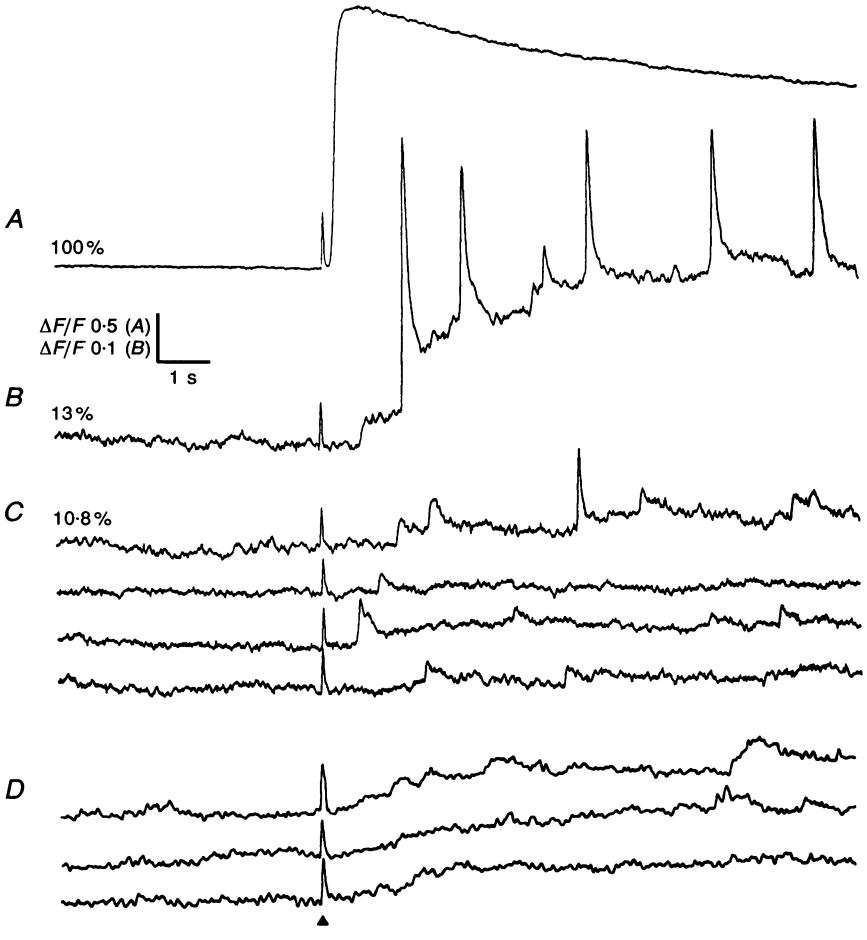

Figure 2. Ca^{2+} wave, puffs and blips recorded from a virtual point source by confocal microfluorimetry

Traces show rhod-2 fluorescence monitored from a fixed confocal spot in response to photolysis flashes (25 ms duration; delivered at arrowhead) of varying intensities which irradiated an area 80 μm in diameter around the recording spot. *A*, Ca^{2+} wave evoked by a strong flash applied at the arrowhead. Note that recording gain is 5 times lower than for other traces. *B*, a flash 13% the strength of that in *A* evoked pacemaker Ca^{2+} with superimposed puffs and smaller Ca^{2+} blips. *C*, four repeated flashes with intensities 10·8% of that in *A* evoked only small pacemaker signals, together with discrete Ca^{2+} blips. *D*, traces from a different, non-focal recording site in the same oocyte, illustrating pacemaker signals with poorly resolved fluctuations. Flash intensity was the same as in *C*.

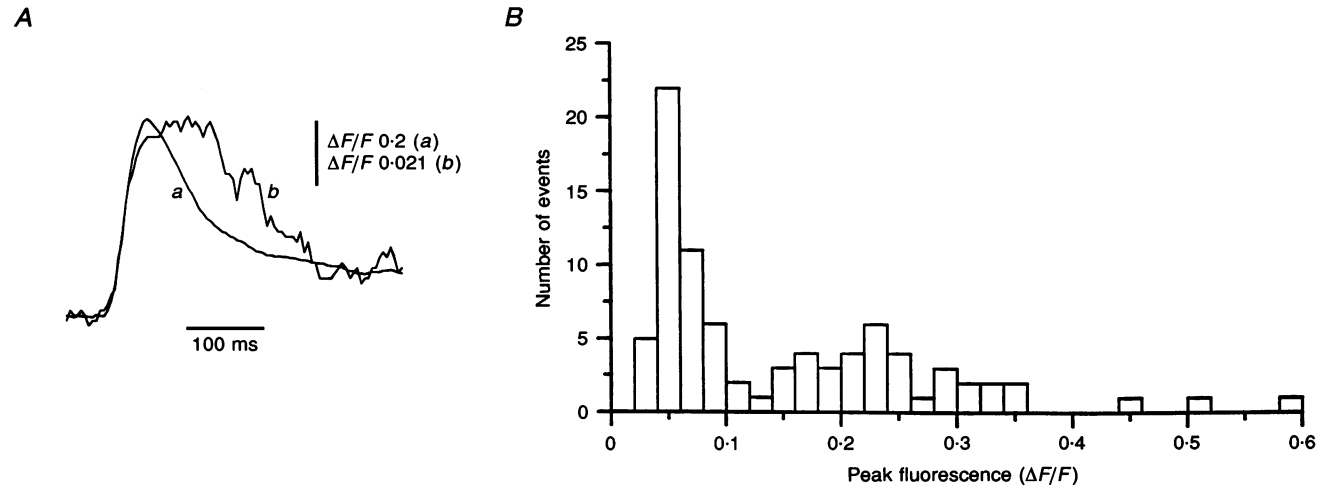

Figure 3. Ca^{2+} blips do not represent small or attenuated puffs

A, rise times of puffs and blips are similar. Traces show a puff (smooth trace *a*) and blip (noisy trace *b*), recorded at the same site as Fig. 2*B* and *C*, scaled to the same peak size and aligned so as to facilitate comparison of their rising phases. *B*, distribution of peak amplitudes of 85 events (puffs and blips) with rise times <70 ms in records from 6 oocytes loaded with rhod-2.

larger region of the oocyte that was exposed to photolysis flashes (Fig. 2). Flashes of appropriate strength invariably evoked a slow pacemaker signal, but the improved signal-to-noise ratio available with the present system revealed that Ca^{2+} rose in an irregular fashion, suggesting a discontinuous release process. Recordings were made at many randomly located spots, a majority of which showed fluctuations of Ca^{2+} that were rounded and not well resolved (e.g. Fig. 2*D*). However, a small proportion of sites showed discrete Ca^{2+} 'blips', unitary events which occurred at apparently random times after the photolysis flash and showed rise times of <100 ms followed by decay over a few hundred milliseconds (Fig. 2*C*). Weak stimuli evoked only infrequent blips, whereas slightly stronger stimuli

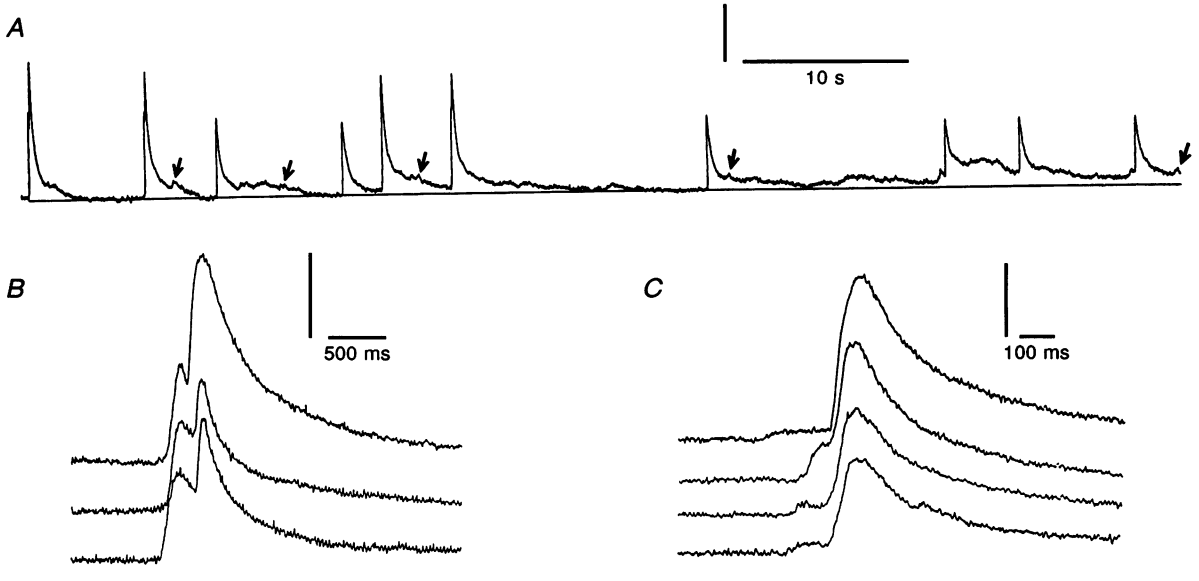

Figure 4. Ca^{2+} puffs recorded in oocytes loaded with 3-F-InsP_3

Traces show confocal spot recordings of Calcium Green-1 fluorescence in oocytes loaded with 3-F-InsP_3 to final intracellular concentrations of about 30 nM. *A*, continuous record from a single site shown on a slow time scale to illustrate Ca^{2+} tails and blips following puffs (examples arrowed). *B*, examples of double puffs occurring at short intervals. Lower two traces show the first two puffs in *A* on an enlarged time scale; upper event is from a different oocyte. *C*, examples of puffs preceded by small Ca^{2+} events. Calibration bars correspond to $\Delta F/F$ of 0·2.

evoked larger puff signals, superimposed upon a pedestal of pacemaker Ca^{2+} and accompanied also by blip events (Fig. 2*B*). Finally, yet stronger stimuli evoked Ca^{2+} spikes with a peak fluorescence change roughly 50 times greater than the blips (Fig. 2*A*).

Ca^{2+} blips are distinct from puffs

An obvious concern was whether the small blip signals might arise through diffusion of Ca^{2+} ions from puffs occurring some distance from the confocal recording spot. Two observations indicated this was not the case. Firstly, weak flashes that evoked blips were found to evoke few or no puffs when the same region of the oocyte was imaged by scanning confocal microscopy. Secondly, blips and puffs at a given release site displayed similar rates of rise (Fig. 3*A*), whereas the blips would have been greatly slowed if they had arisen through diffusion of Ca^{2+} from a puff at a distant site. For example, line-scan fluorescence imaging showed that signals would be attenuated to about 20% at a displacement about 5 μm from a puff site and that their rise time would slow to about 300 ms (see Fig. 7*A* in Yao *et al.* 1995). In marked contrast, the mean rise time for eighteen blips at the site in Fig. 2*C* was 50·8 ± 10·6 ms (mean ± S.D.), virtually the same as for puffs at the same recording site (53 ± 9 ms; $n = 5$).

Finally, it seems that the blips represent a mode of Ca^{2+} release distinct from the puffs. Figure 3*B* shows the distribution of peak amplitudes of focal fluorescence events (blips and puffs), which were selected as having rise times less than about 70 ms so as to minimize inclusion of attenuated signals arising from Ca^{2+} diffusing from distant sources. The blips form a separate population, with a mean size about one-fifth that of the puffs, rather than representing a 'tail' of small puffs.

'Blip-like' events preceding and following puffs

The improved resolution available with the confocal spot recording also revealed new characteristics of the Ca^{2+} puffs. For this purpose, we injected oocytes with a poorly metabolized derivative of $InsP_3$, 3-F-$InsP_3$, so as to evoke puff activity sustained over many minutes (Yao *et al.* 1995), and positioned the confocal spot over puff sites previously identified by confocal imaging. One novel feature evident in Fig. 4*A* is that puffs were usually followed by a 'tail' of elevated Ca^{2+} lasting for several seconds, during which blips were often apparent, suggesting that puffs trigger a prolonged low level of Ca^{2+} liberation. Also, whereas we had noted a decline in the occurrence of puffs at short (< 8 s) intervals in previous low-resolution measurements (Yao *et al.* 1995) instances of 'double' puffs (two puffs occurring within < 250 ms; Fig. 4*B*) were now frequently seen (e.g. 3 out of 10 events in Fig. 4*A*), further indicating that Ca^{2+} released during a puff may evoke regenerative release from the same, or a closely adjacent, site. Finally, although many puffs appeared to begin abruptly from the baseline, others (Fig. 4*C*) were preceded by small elevations of Ca^{2+} which may have acted as triggering events.

DISCUSSION

We had previously reported that $InsP_3$ gives rise to three different modes of Ca^{2+} liberation in *Xenopus* oocytes: evoked by progressively increasing concentrations of $InsP_3$ as small, spatially uniform elevations of pacemaker Ca^{2+}, then as transient, localized Ca^{2+} puffs and finally as propagating Ca^{2+} waves (Parker & Yao, 1991; Yao *et al.* 1995). The major new result in the present paper is the finding that the pacemaker signal does not represent a continuous and smoothly graded release of calcium, but appears to arise through summation of localized Ca^{2+} transients ('blips'), which resemble the puffs but are several times smaller. Thus, $InsP_3$ liberates intracellular Ca^{2+} in a discontinuous fashion on at least three different scales, giving rise to blips, puffs and waves. What, then, are the relationships between these events, and how do they arise?

When originally considering the puffs (Yao *et al.* 1995) we wondered if they might arise through the opening of single $InsP_3$-gated channels, but rejected this idea as the amount of Ca^{2+} involved (a few attomoles) was equivalent to currents (11–23 pA for 50 ms) greater than would be expected through single oocyte $InsP_3$ receptor channels (< 1 pA: Stehno-Bittel, Luckhoff & Clapham, 1995). Thus, it seemed that the puffs must involve the concerted opening of several closely clustered channels. The observation of blips lends strong support to this argument by showing that the puffs do not represent an irreducible minimum unit of calcium release. Instead, an attractive possibility is that a blip arises through the opening of a single channel, and that a puff represents some multiple of blips. From the size of the puff relative to the likely single channel Ca^{2+} flux we estimated that perhaps ten or a few tens of channels may be involved (Yao *et al.* 1995), whereas the mean size of the blip appears about one-fifth that of the puff. Although there is some discrepancy between these values if a blip represents a single channel opening, the measurements on which they are based have considerable uncertainties. In particular, integration of the fluorescence puff signal in three dimensions is subject to large errors, little is known of Ca^{2+} buffers in the oocyte, and the mean size of the blips may be overestimated by failure to resolve small events below the noise floor. Indeed, an alternative calculation, based on integration throughout the volume of the confocal spot of the predicted distribution of Ca^{2+}-bound indicator around the mouth of an open Ca^{2+} channel, suggested that a current of < 0·1 pA would be sufficient to account for the blip signal (Y. Yao, unpublished data).

If the blips do represent single channel events, it might be expected that the stochastic nature of channel gating would give rise to an exponential distribution of open times, and hence an exponential distribution of blip amplitudes. In contrast, a roughly Gaussian distribution was observed (Fig. 3*B*). However, because the blips were close to the limit of resolution it may be that many small events escaped detection, so that only the 'tail' of an underlying exponential

distribution was observed. Alternatively, feedback processes like those terminating release during puffs and waves may act so that gating of single channels is regulated by the local Ca^{2+} level, such that the open time and Ca^{2+} flux during each event is roughly constant.

The distinct modes of Ca^{2+} liberation as blips, puffs and waves may be accounted for by the combined effects of the clustering of several $InsP_3$ receptors within release sites a few micrometres apart (Yao *et al.* 1995), together with the ability of cytosolic Ca^{2+} to promote opening of $InsP_3$ receptor channels (see Introduction). At low concentrations of $InsP_3$, when only a small fraction of receptors are occupied (and hence are susceptible to opening by Ca^{2+}), the flux of Ca^{2+} through a single channel may fail to activate surrounding channels within a cluster, and give rise only to a blip. As the $InsP_3$ concentration is raised the average distance between bound receptors would decrease, so that Ca^{2+} released through one channel may then trigger regenerative activation of closely neighbouring channels (e.g. Fig. 4*C*), yet release may remain localized as a puff because of the greater distances (a few micrometres) between clusters. Finally, at yet higher $InsP_3$ levels the proportion of bound receptors within each cluster becomes sufficient that a puff at one site has a high probability of triggering adjacent sites, giving rise to a propagating Ca^{2+} wave.

Many properties of $InsP_3$-mediated signalling, including the complex and highly non-linear dose dependence of Ca^{2+} liberation, appear therefore to be determined by the spatial arrangement of receptors within the cell, as well as by the inherent properties of the receptors. A closely analogous situation is also seen with Ca^{2+} liberation through ryanodine receptor channels, which display a similar potentiation of their opening by Ca^{2+}. Cardiac muscle cells show transient, localized Ca^{2+} 'sparks' at rest (Cheng, Lederer & Cannell, 1993) and in response to depolarization (Lopez-Lopez, Shacklock, Balke & Wier, 1994). Although sparks were originally proposed to result from the opening of single ryanodine receptors (Cheng *et al.* 1993), it is now thought more likely that concerted opening of a small group of receptors is involved (Cannell, Cheng & Lederer, 1994), making the sparks analogous to puffs rather than to blips. Improved optical techniques are thus beginning to reveal new levels of Ca^{2+} signalling in several cell types, and the high-resolution confocal microfluorimeter described here provides a relatively simple and inexpensive means of characterizing further these elementary events, as well as being potentially applicable to other ligand- and voltage-gated Ca^{2+} channels.

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