Activation and co-ordination of InsP_3-mediated elementary Ca^{2+} events during global Ca^{2+} signals in *Xenopus* oocytes

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1. The activation of elementary calcium release events ('puffs') and their co-ordination to generate calcium waves was studied in *Xenopus* oocytes by confocal linescan imaging together with photorelease of inositol 1,4,5-trisphosphate (InsP_3) from a caged precursor.

2. Weak photolysis flashes evoked no responses or isolated calcium puffs, whereas flashes of increasing strength evoked more frequent puffs, often occurring in flurries as abortive waves, and then a near-simultaneous calcium liberation originating at multiple sites. The numbers of sites activated increased initially as about the fourth power of photoreleased [InsP_3].

3. Following repeated, identical photolysis flashes, puffs arose after stochastically varying latencies of a few hundred milliseconds to several seconds. The cumulative number of events initially increased as about the third power of time. No rise in free [Ca^{2+}] was detected preceding the puffs, suggesting that this co-operativity arises through binding of multiple InsP_3 molecules, rather than through calcium feedback.

4. The mean latency to onset of calcium liberation shortened as about the square of the flash strength, and the dispersion in latencies between events reduced correspondingly.

5. Weak stimuli often evoked coupled puffs involving adjacent sites, and stronger flashes evoked saltatory calcium waves, propagating with non-constant velocity. During waves, [Ca^{2+}] rose slowly between puff sites, but more abruptly at active sites following an initial diffusive rise in calcium.

6. Initial rates of rise of local [Ca^{2+}] at release sites were similar during puffs and release induced by much (>10-fold) greater [InsP_3]. In contrast, macroscopic calcium measurements averaged over the scan line showed a graded dependence of rate of calcium liberation upon [InsP_3], due to recruitment of additional sites and decreasing dispersion in activation latencies.

7. We conclude that the initiation of calcium liberation depends co-operatively upon [InsP_3] whereas the subsequent regenerative increase in calcium flux depends upon local calcium feedback and is largely independent of [InsP_3]. Wave propagation is consistent with the diffusive spread of calcium evoking regenerative liberation at heterogeneous discrete sites, the sensitivity of which is primed by InsP_3.

The second messenger inositol 1,4,5-trisphosphate (InsP_3) functions primarily by liberating calcium ions from intracellular stores, thereby causing elevations in cytosolic free calcium concentration that are frequently observed as repetitive waves propagating throughout the cell (Berridge, 1993). Recent experiments have suggested that these waves may arise through recruitment of functionally discrete intracellular release units, which have been proposed to comprise the elementary building blocks for global calcium signals (Lipp & Niggli, 1996; Parker, Choi & Yao, 1996*a*; Berridge, 1997). The first visualization of such elementary calcium signals was obtained in *Xenopus* oocytes, in which stimulation by low concentrations of InsP_3 evoked transient, localized calcium elevations (calcium 'puffs'), whereas higher concentrations evoked propagating waves (Parker & Yao, 1991; Yao, Choi & Parker, 1995). Similar findings have subsequently been obtained in a variety of other excitable and non-excitable cell types, including HeLa cells (Bootman, Niggli, Berridge & Lipp, 1997*b*), rat RBL tumour cells (Horne & Meyer, 1997), glia (Yagodin, Holtzclaw, Sheppard & Russell, 1994) and PC12 cells (Reber & Schindelholz, 1996), suggesting that a hierarchical arrangement of elementary units may be a ubiquitous feature of InsP_3-mediated calcium signalling.

The appearance of calcium signals as either localized, transient elevations or actively propagating waves is thought to arise through two key properties of InsP_3-evoked calcium liberation. Firstly, opening of the integral release channel associated with the InsP_3 receptor (InsP_3R) is promoted by moderate elevations of cytosolic calcium as well as by InsP_3 (Iino, 1990; Finch, Turner & Goldin, 1991; Bezprozvanny, Watras & Ehrlich, 1991). This positive feedback imparts a regenerative characteristic to calcium liberation giving rise to a mechanism of calcium-induced calcium release (CICR). Secondly, the InsP_3R appear to be distributed in clusters, forming discrete functional release sites spaced a few micrometres apart (Yagodin *et al.* 1994; Yao *et al.* 1995; Bootman *et al.* 1997*b*; Horne & Meyer, 1997). Depending upon the concentration of InsP_3, which is a major factor in determining the sensitivity of the system, CICR may either be restricted to individual sites or involve successive triggering of multiple sites mediated by diffusion of calcium ions in the cytosol. Thus, calcium puffs probably arise through calcium feedback recruiting several InsP_3R within a tight cluster (Yao *et al.* 1995; Bootman *et al.* 1997*b*; Sun, Callamaras, Marchant & Parker, 1998), but the puff will remain localized if neighbouring receptor clusters are insufficiently sensitive to respond to calcium diffusing across the much larger distances between release sites. At higher concentrations of InsP_3, however, such triggering is enabled, turning the cytoplasm into an excitable medium capable of supporting propagating waves (Lechleiter & Clapham, 1992).

In the preceding paper (Sun *et al.* 1998), we used a high-resolution linescan confocal microscope to examine mechanisms underlying the generation of individual elementary calcium events (puffs and blips) evoked in *Xenopus* oocytes by photorelease of constant, low concentrations of InsP_3. Here we extend these studies on a more global scale within the cell by exploring the recruitment of puffs by increasing concentrations of InsP_3 and their co-ordination to generate calcium waves.

METHODS

Experiments were done on defolliculated immature oocytes from *Xenopus laevis*, using linescan confocal microscopy to image intracellular calcium signals evoked by flash photolysis of caged InsP_3. Experimental procedures were as described in the preceding paper (Sun *et al.* 1998). Further details regarding preparation of oocytes and intracellular loading with calcium indicator and caged InsP_3 are as described previously in Callamaras & Parker (1998), and construction of the confocal microscope and photolysis system is as described in Parker, Callamaras & Wier (1997).

RESULTS

Patterns of calcium liberation with increasing photorelease of InsP_3

Figure 1 shows calcium images obtained by scanning along a fixed line within the oocyte while photoreleasing increasing amounts of InsP_3 by photolysis flashes of progressively increasing strength. In each frame, distance along the scan line runs vertically, and time runs from left to right. Fluorescence of the calcium indicator dye Oregon Green 488 BAPTA-1 is shown at each pixel along the scan as a ratio (F/F_0) relative to that at the same pixel along the scan before stimulation, and increasing calcium levels (increasing fluorescence) are depicted by increasingly 'warm' colours. Photolysis flashes were delivered when marked by the arrows, with intervals of 60 s between trials to allow for recovery from inhibition (Parker & Ivorra, 1990).

The weakest flashes tested sometimes evoked only single puffs during the recording period (Fig. 1*B*), or in other cases failed to evoke any detectable response (Fig. 1*A*). Even small increases in stimulus strength, however, were sufficient to evoke many puffs which, on average, arose with shorter mean latencies following the flash (Fig. 1*C*). In some instances (e.g. as marked by the asterisk in Fig. 1*C*) it appeared that a puff at one site triggered an adjacent puff at a neighbouring site after a short delay, and with a further increase in stimulus strength this behaviour became more prominent, leading to the appearance of flurries of puffs during abortive waves (Fig. 1*D*).

A further increase in flash strength (Fig. 1*E*) caused a more synchronous calcium liberation along the whole scan line. This 'wave' threshold was sharply defined, and provided a convenient means by which to normalize flash strengths between different oocytes. The wavefront in the linescan images displayed an irregular profile, suggesting that release began independently at a few sites along the line, and that calcium liberated at those sites then triggered further release from adjacent sites. Following the initial release, calcium levels declined more slowly than after individual puffs, and discrete release events were visible on the falling phase of the wave.

Finally, stimuli with strengths several times greater than the wave threshold evoked an abrupt release along the entire scan with short latency (Fig. 1*F* and *G*). The wavefront still displayed a serrated appearance, indicative of release originating at multiple discrete sites, but the dispersion in latencies (i.e. spread between the earliest and latest responding sites) was slight (90 ms). It is likely, therefore, that calcium release originated independently at most sites before it could be triggered by calcium diffusing from a neighbouring site that activated with shorter latency.

Latencies of puffs

As is evident in Fig. 1, puffs tended to occur within a few seconds of the photolysis flash following latencies that, even with a constant flash strength, varied randomly between different sites and between responses to successive flashes at a given site. The frequency of occurrence of puffs during successive 250 ms time bins following repeated photolysis flashes of constant strength (about 70% of the wave threshold) is plotted in Fig. 2*A*, and Fig. 2*B* shows the cumulative numbers of puffs observed at increasing times, pooling measurements from fourteen puff sites in response to twenty-one repeated stimuli. No puffs were observed

with latencies shorter than about 250 ms. The probability of observing puffs then increased to a maximum after 1–1·5 s, before declining again over a few seconds. The decline at longer intervals was probably determined largely by diffusion of InsP_3 away from its restricted (approximately 100 μm spot) site of photorelease, together with its metabolic degradation (Parker & Ivorra, 1992). At intervals less than about 1 s, when the decline of [InsP_3] would be relatively slight, the cumulative number of puffs increased as an approximately third-power relationship with time (smooth curve, Fig. 2*B*), a relationship similar to that observed between steady-state puff frequency and [InsP_3] (Yao *et al.* 1995). Because the kinetics of photorelease of InsP_3 are rapid on this timescale (McCray & Trentham, 1989), this suggests that triggering of puffs does not simply depend upon a 1:1 binding of InsP_3 to receptors, but instead involves a co-operative process.

One possibility is that puffs may be preceded by a diffuse liberation of 'pacemaker' calcium, leading to an increasing probability of channel opening as the free [Ca^{2+}] rises. To test this idea, we monitored fluorescence at release sites in selected records where puffs arose following long latencies. No rise in the calcium signal could be detected for over 1 s after the photolysis flash, even though puffs arose at other sites with maximal frequency during this time (Fig. 2*C*). The co-operativity in puff activation probably arises, therefore, from a requirement for InsP_3 binding to several sites on an individual receptor to cause channel opening (Meyer, Wensel & Stryer, 1990; Marchant & Taylor, 1997), or from a requirement for several receptors within a cluster to become primed by binding InsP_3 to support the generation of a puff.

Dependence of mean latency to calcium release on [InsP_3]

Despite the variability in latencies of individual events at a given flash strength, it was evident that the mean latency shortened dramatically with increasing photorelease of InsP_3, decreasing from values of a few seconds with stimuli evoking single puffs (Fig. 1*B*) to less than 100 ms with strong flashes evoking abrupt calcium waves (Fig. 1*F* and *G*). Figure 3*A* shows examples of distributions of latencies observed at four different flash strengths, and further demonstrates that the dispersion in latencies (i.e. the spread

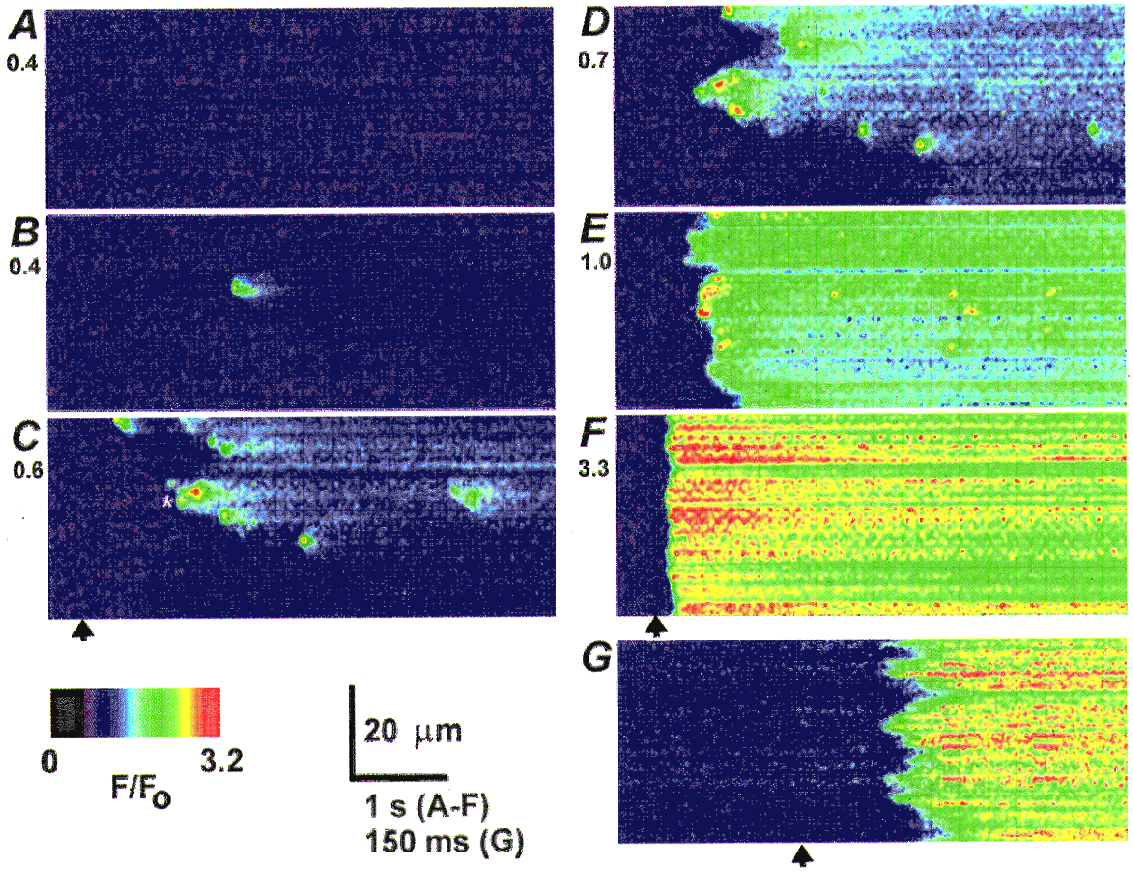

Figure 1. Patterns of calcium liberation along a fixed scan line evoked by increasing photorelease of InsP_3

Linescan images were acquired at a scan rate of 1·5 ms per line, and a nominal resolution of 0·2 μm per pixel. Responses were evoked by photolysis flashes of varying strengths, indicated next to each frame relative to the flash strength that just evoked a calcium wave. Arrows mark the onset of the flashes. The image in *G* shows the same response as in *F* on an expanded timescale.

between shortest and longest latency sites) decreases markedly with increasing flash strength.

Mean latencies, measured in four oocytes, are plotted as filled circles in Fig. 3*B* as a function of normalized flash strength. Using stationary-spot confocal recording, we had previously found a linear reciprocal relationship between latency and photorelease of $InsP_3$ for stimuli above the wave threshold (Parker, Yao & Ilyin, 1996*b*), and those data have been replotted as open squares in Fig. 3*B*. The ability to resolve release events by linescan imaging now allowed measurements over a much wider range (>100-fold) of [$InsP_3$], and revealed that the mean latency to calcium release shortened as about the second power of the flash strength. Further, experimental limitations are likely, if

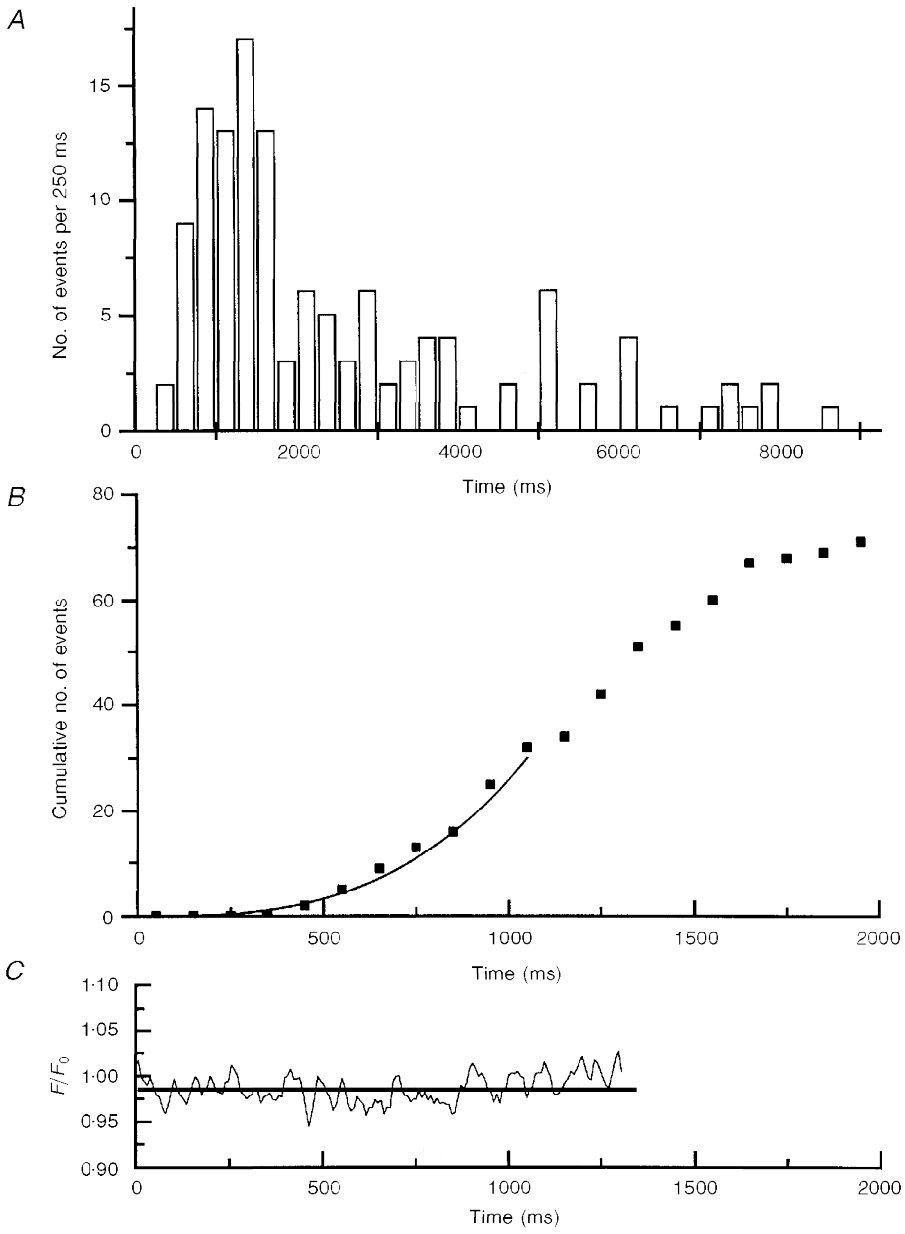

Figure 2. Latencies of puffs following the photolysis flash and absence of calcium rise preceding puffs

A, numbers of puffs observed during consecutive 250 ms periods following photolysis flashes. Data were derived from 21 trials at the same location, with a total of 14 discrete sites responding along the linescan. *B*, cumulative total of puffs as a function of time, derived from the same data as *A*, and shown on an expanded time scale. The curve is a third power relationship fitted to the initial part of the data. *C* shows that puffs are not preceded by any detectable calcium rise. The trace shows an average of fluorescence monitored from 9 sites that showed puffs beginning after latencies longer than 2 s. Note that there was no detectable increase in fluorescence for about 1·3 s after the flash, although the majority of puffs at other sites had occurred by this time.

anything, to underestimate the latencies with weak stimuli and thus underestimate the steepness of this relationship. Recordings were made for only 6 s following photolysis flashes, and the flash would not result in a maintained step of [InsP_3], but rather a transient decaying over several seconds (Parker & Ivorra, 1992). Thus, any puffs occurring after the end of the record would have been missed, and decaying [InsP_3] levels would tend to decrease the likelihood of observing puffs at increasingly long latencies.

Increase in number of sites responding with increasing [InsP_3]

For stimuli below the wave threshold, linescan images showed an increasing number of sites responding to progressively stronger flashes (Fig. 1*A–D*). Above the wave threshold, the images presented an irregular sawtooth appearance, indicating that calcium liberation began at multiple sites following slightly varying latencies (Fig. 1*E–G*). The numbers of sites showing calcium liberation following a given flash were estimated from the appearance of discrete puffs, or as initiation sites on the wavefront. Measurements of mean numbers of sites responding per scan line (50 μm) are plotted in Fig. 4*A* as a function of normalized flash strength. Few puffs were evoked by flashes less than about one-half the wave threshold, and the numbers of sites responding then increased extremely steeply to a maximum with flashes equal to or greater than the wave threshold (Fig. 4*A*). The Hill slope derived by plotting the initial part of this relationship on double logarithmic co-ordinates (Fig. 4*B*) was 4·2 and a further experiment gave a value of 4·9, indicating a high degree of co-operativity in the activation of release events. While it is likely that part of this co-operativity arises through a requirement for binding of InsP_3 to multiple sites on the InsP_3R, the observed slope may be increased further by calcium ions released during a preceding puff facilitating release at adjacent sites.

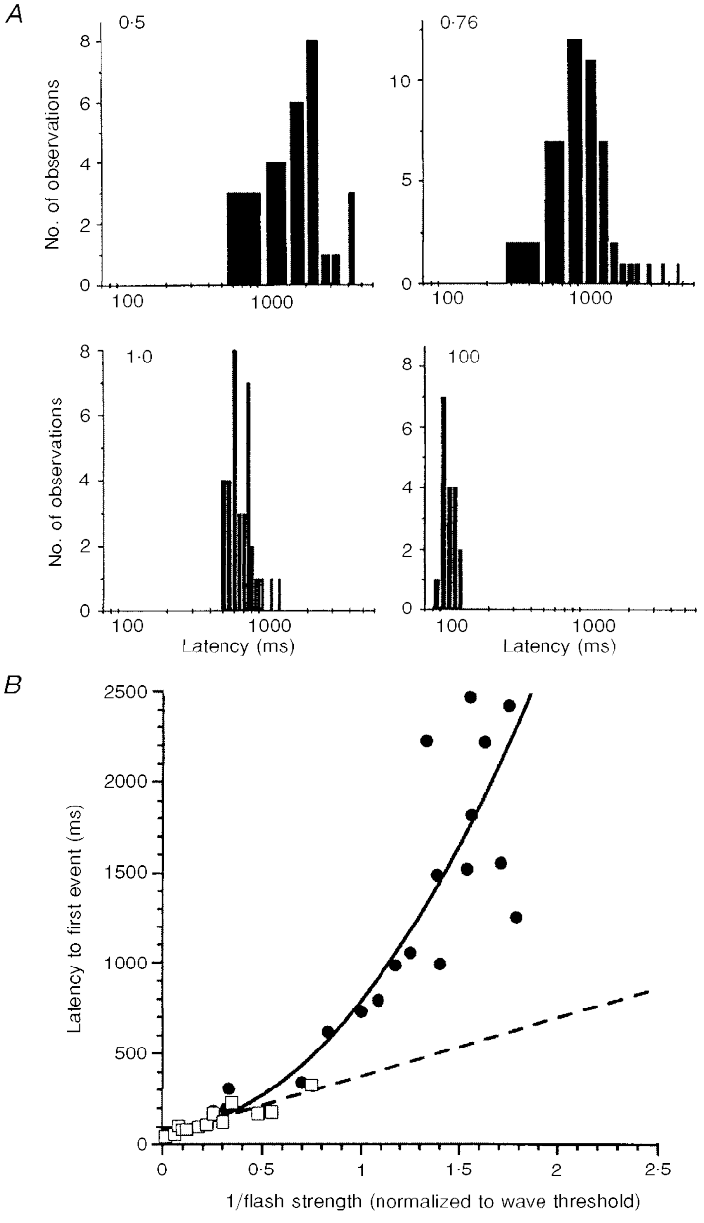

Figure 3. Mean latency to onset of calcium liberation shortens with increasing photorelease of InsP_3

A, semi-logarithmic plots show distributions of latencies to onset of calcium liberation. Measurements were obtained from images recorded at a single scan line in response to photolysis flashes of the strengths indicated (normalized to wave threshold). *B*, filled symbols (●) show mean latencies to onset of calcium liberation from four linescan recording experiments (different oocytes). Each point is a mean of between 4 and 20 individual events. Both the duration and intensity of the photolysis flashes were varied, and data are plotted on a reciprocal scale of flash strength normalized relative to that required to just evoke a calcium wave in each oocyte. Open symbols (□) are replotted from Parker *et al.* (1996), and show measurements of response latencies obtained by stationary-spot confocal recording. The curve is a second-power function fitted to the data, and intercepts the *y*-axis at a minimal latency of 80 ms.

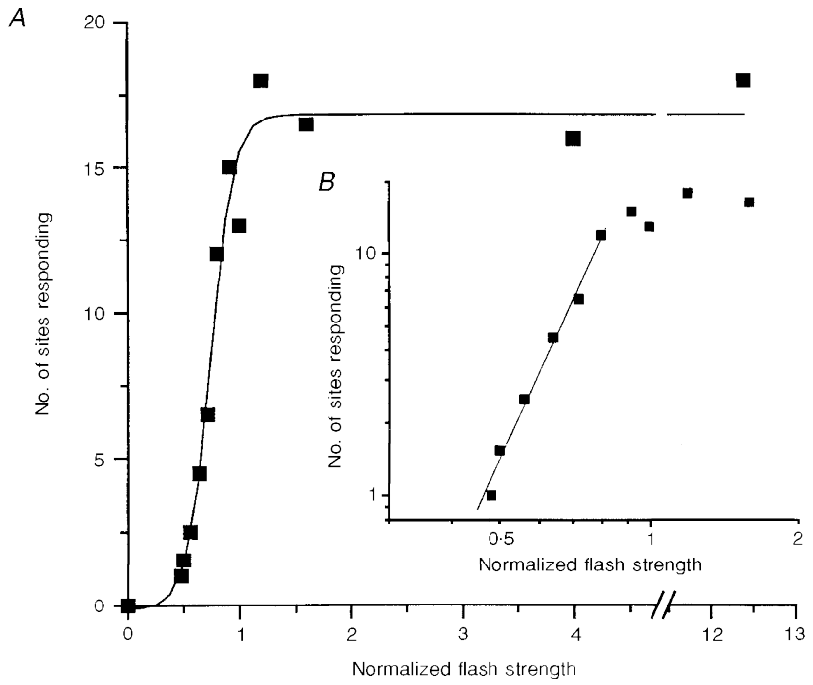

Figure 4. Numbers of release sites responding as a function of [InsP_3]
Measurements from the same experiment as in Fig. 1, showing the numbers of calcium release sites responding during 6 s recording periods following photolysis flashes of varying strengths. Points are means from 2 to 11 repeated trials, all at the same scan line, and flash strength is normalized relative to the threshold to evoke a wave. Data are shown on linear (*A*) and double logarithmic scales (*B*). The line drawn through the data at low flash strengths in *B* has a slope (Hill coefficient) of 4·2.

Puffs involving near-synchronous release at multiple sites

Many events were observed involving near-simultaneous calcium release from two closely neighbouring sites, even with flashes weaker than those evoking flurries of puffs such as in Fig. 1*C*. An example is shown in Fig. 5, where a puff originating at one site (i) evoked release at an adjacent site (ii) about 2·5 μm away, following a latency of about 40 ms. Several hundred milliseconds later, another puff arose at site (ii), but was unaccompanied by calcium release at site (i), demonstrating clearly that the initial event involved near-synchronous activation at two sites. Such multiple-site events were most prevalent in the animal hemisphere of oocytes, where they accounted for about one-

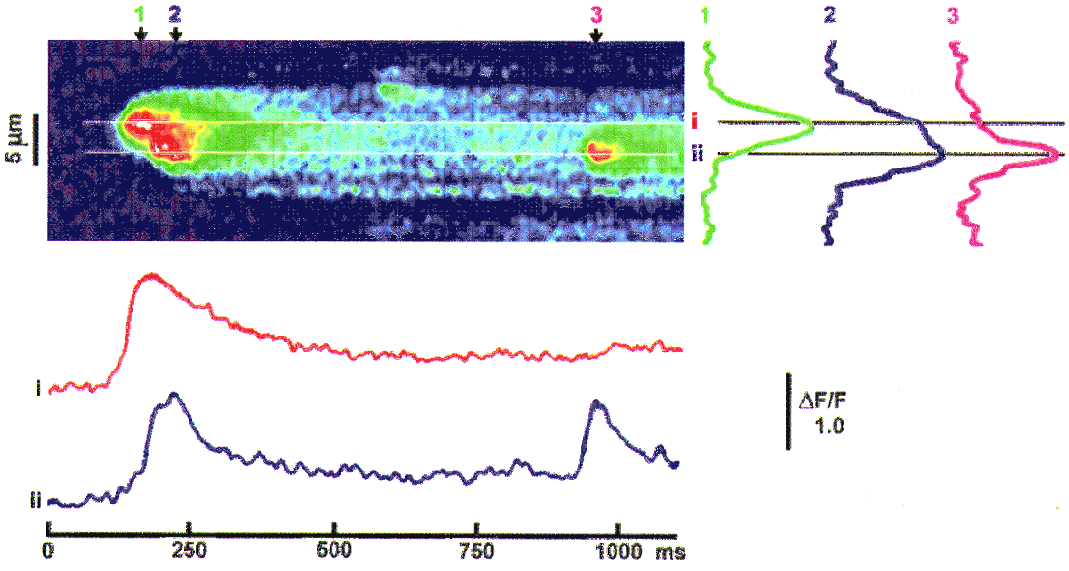

Figure 5. Puffs involving coupled calcium release at adjacent sites
The linescan image (acquired at a rate of 1·5 ms per line) shows an example where an initial puff involved activation at two adjacent sites (i and ii), and a subsequent puff involved only a single site (ii). Traces below the image show the time course of fluorescence signals monitored at the 2 sites marked by white lines in the image, and traces at the right show fluorescence profiles along the scan line at the respective times marked by the arrows.

half of all puffs, even at low concentrations of $InsP_3$ evoking low frequencies of puffs (I. Parker and N. Callamaras, unpublished data). It is unlikely, therefore, that multiple-site events represent the chance occurrence of puffs originating at nearly the same time from adjacent sites. Instead, calcium released at one site may serve to activate locally a closely neighbouring site.

Saltatory wave propagation

Calcium waves evoked by stimuli just above the wave threshold did not propagate in a smoothly continuous manner, but showed varying degrees of discontinuity (Figs 1 and 6*A*). The overall wave velocity was around 21 μm s^{-1} (indicated by white diagonal lines in Fig. 6*A*), similar to previously reported values in the oocyte (Lechleiter &

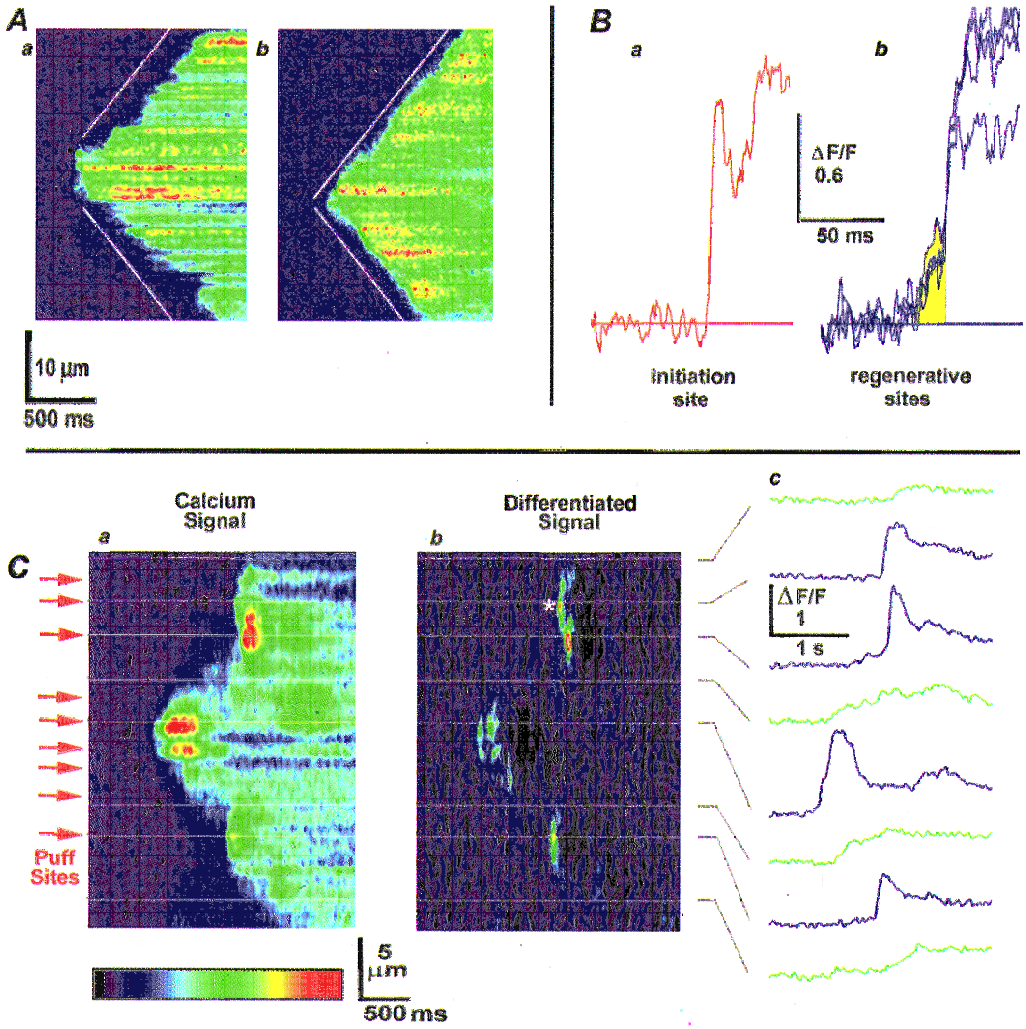

Figure 6. Saltatory calcium wave propagation

A, examples in different oocytes of calcium waves evoked by photolysis flashes just stronger than the wave threshold. The image in *a* shows a markedly stepped propagation profile, while that in *b* is more continuous. Diagonal white lines correspond to propagation velocities of 21 μm s^{-1}. *B*, traces show kinetics of fluorescence signals monitored locally (0·5 μm window) from presumptive active release sites in the image in *Aa*. The single trace in *Ba* illustrates the abrupt rise of calcium during a puff that triggered the wave. Superimposed traces in *b* show records from 4 release sites along the wavefront, and illustrate that a slow rise in calcium (marked in yellow) preceded the abrupt, regenerative release of calcium. In *C*, panel *a* is a calcium ratio image illustrating, on an enlarged distance scale, a further example of a saltatory calcium wave. Arrows at the left mark the locations of sites where puffs were observed following other photolysis flashes (of the same strength as that which evoked the wave). The image in *b* was derived from the calcium image (*a*) by calculating the differential of the fluorescence ratio with respect to time for each pixel along the scan line. 'Warmer' colours correspond to rapid rates of calcium increase, and thus more clearly locate sites of active calcium release. Data were smoothed by a 5 × 5 pixel low-pass filter. Traces in *c* show the time courses of fluorescence signals (averaged over 3 pixels) measured at locations along the scan line as indicated by the thin horizontal lines. Traces in blue correspond to active release sites, and traces in green to locations between release sites.

Clapham, 1992; Yao & Parker, 1994). Although the waves propagated rapidly across regions of a few micrometres, there were often steps where propagation stalled for tens of milliseconds (Fig. 6*Aa* and *Ca*). This saltatory propagation is thought to arise through sequential triggering of CICR at discrete release sites by calcium diffusing between these sites (Yagodin *et al.* 1994; Yao *et al.* 1995; Wang & Thompson, 1995; Berridge, 1997; Bootman *et al.* 1997*b*). In agreement, recordings of local fluorescence signals at release sites activated during wave propagation showed an initial slow elevation preceding an abrupt rise (Fig. 6*Bb*), consistent with the triggering of regenerative calcium liberation by a rise in free $[Ca^{2+}]$ resulting from calcium diffusing from neighbouring active sites. In contrast, however, fluorescence at sites where waves were initiated rose abruptly from the baseline (Fig. 6*Ba*).

Figure 6*C* presents an analysis of the regenerative sites underlying saltatory wave propagation, in which regions of active calcium liberation (Fig. 6*Cb*) were highlighted by forming the time derivative of the fluorescence image (Fig. 6*Ca*) so as to emphasize regions of rapid calcium increase. The arrows at the left mark the positions of puff sites identified along the same scan line in response to other photolysis flashes that failed to evoke waves. In general, the locations of the puff sites correspond well to sites of active release during the wave, though it appeared that one puff site (second from the bottom) may have failed to be triggered during the wave. Profiles of fluorescence as a function of time at selected active sites (Fig. 6*Cc*, blue traces) showed rapidly rising calcium transients, whereas intermediate regions displayed smaller and more slowly rising calcium signals arising through passive diffusion of calcium (green traces). A final point of interest is that the wave 'leapfrogged' at one release site (marked by the asterisk in Fig. 6*Cb*) where calcium liberation began earlier than at a neighbouring site closer to the initiation point of the wave.

Kinetics of calcium signals evoked by varying [InsP_3]

Figure 7*A* shows calcium signals monitored over a localized region (3 pixels; 0·6 μm) centred on a given release site in response to a weak photolysis flash that evoked an isolated puff (lower trace) and to a much (15-fold) stronger flash that evoked a near-simultaneous calcium release at multiple sites (upper trace). The puff began abruptly following a latency of 1·5 s, with the fluorescence then rising rapidly to a peak value similar to that during synchronous release, and subsequently declining almost to the baseline within about 1 s. In contrast, calcium liberation began with a much shorter latency (about 100 ms) following the strong flash, but the fluorescence then remained high for many seconds. Even though saturation of the indicator (Oregon Green BAPTA-1; affinity, 170 μM) probably distorted the time course and magnitude of calcium signals evoked by high concentrations of InsP_3, comparison with signals recorded

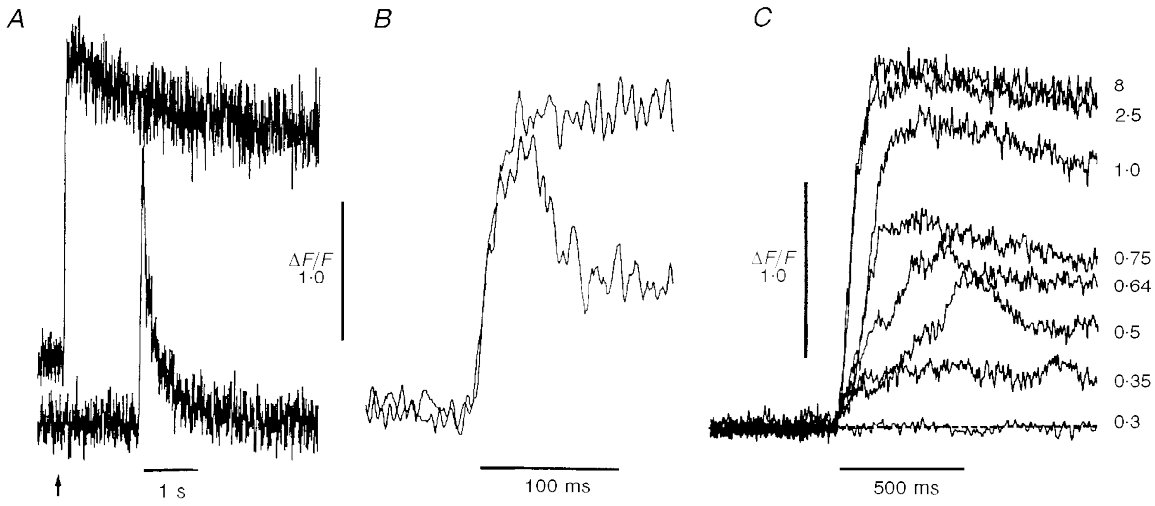

Figure 7. Kinetics of calcium signals during puffs and waves

A, traces show kinetics of fluorescence signals during a puff (lower trace) and abrupt wave (upper trace), both measured at the same calcium release site over a 3 pixel (0·6 μm) window at a scan rate of 1·5 ms per line. The puff was evoked following a flash with a strength of 0·6 relative to the wave threshold, whereas the wave was evoked by a subsequent flash with a strength 8 times the wave threshold. The arrow marks the timing of the flashes, and the traces are displaced vertically for clarity. *B*, the same records shown on an expanded time scale and after aligning their rising phases, to facilitate comparison of their kinetics. *C*, macroscopic calcium signals in response to photolysis flashes of varying strength, obtained by averaging fluorescence across the entire 50 μm scan line. The traces were aligned in time to superimpose the initial rise in calcium in each instance, and numbers at the right indicate flash strengths relative to the wave threshold. The weakest flash (0·3) evoked no detectable release events, and this trace is shown with the same timing, relative to the photolysis flash, as the record with a flash strength of 0·35.

using low-affinity dyes (Parker *et al.* 1996*b*) still reveals the puff to have a much more rapid decay. This probably arises largely from diffusion of calcium. Calcium ions liberated from an isolated point source during a puff will diffuse rapidly into the bulk of the cytosol, whereas such gradients for diffusion will be greatly minimized during near-simultaneous release from many sites distributed throughout a wide region. In addition, a sustained liberation of calcium at high [InsP_3] may further tend to maintain an elevated level of cytosolic free calcium (Parker *et al.* 1996*b*).

The initial rates of rise of fluorescence during a puff and an abrupt wave recorded at the same site were virtually identical (Fig. 7*B*), despite the 15-fold greater photorelease of InsP_3 used to evoke the wave. The mean rate of rise of fluorescence ($\Delta F/F_0$ ms^{-1}) was 53·6 ± 5·4 (means ± S.E.M., 10 events) for puffs evoked by flashes of a strength 0·3 times wave threshold, as compared with 61·8 ± 2·4 at fourteen sites during waves evoked by flashes 2·5–8 times threshold. Furthermore, the initial rise in fluorescence would be undistorted by problems of dye saturation. Thus, although the initiation of calcium liberation shows a strong dependence upon [InsP_3], other factors, including the kinetics of calcium feedback on InsP_3R, appear to be rate limiting for the activation of *microscopic* calcium flux from an individual release site once this process is initiated. On the other hand, the *macroscopic* rise in calcium, averaged over an extensive cellular region, shows a strong dependence upon [InsP_3] for both kinetics and magnitude. Figure 7*C* illustrates fluorescence traces in response to flashes of varying strength derived by averaging the fluorescence signal across the 50 μm length of the scan line. As the flash strength was increased, the amplitude and rate of rise of the signals increased progressively, both because calcium release was initiated at more sites along the line, and because release events occurred more synchronously.

DISCUSSION

Hierarchy of calcium signalling

Calcium imaging studies in *Xenopus* oocytes (Parker & Yao, 1991, 1996; Yao *et al.* 1995; Parker *et al.* 1996*a*) and other cells (Yagodin *et al.* 1994; Thorn, 1996; Bootman *et al.* 1997*b*; Horne & Meyer, 1997) have shown that the cytoplasm may act as an excitable medium, composed of discrete calcium release units that are functionally linked through calcium diffusion and CICR at InsP_3R (reviewed by Bootman & Berridge, 1995; Lipp & Niggli, 1996; Berridge, 1997). InsP_3-mediated calcium release in the oocyte has been described at several different scales and has been conceptually organized as a hierarchy of fundamental, elementary and global events (blips, puffs and waves, respectively) sequentially recruited in a step-wise fashion with increasing levels of InsP_3. Results obtained with improved calcium imaging techniques in this and the preceding paper (Sun *et al.* 1998) support the notion that global calcium signals are generated by recruitment of calcium release from discrete units. However, transitions between different levels of the hierarchy are less distinct than originally envisaged. It appears that localized events display a wide and continuous variation in magnitude (Sun *et al.* 1998) rather than comprising two populations (blips and puffs) of differing, quantized size. Furthermore, responses to increasing concentrations of InsP_3 do not show an abrupt transition from discrete, autonomous release events to globally propagating waves. At intermediate concentrations of InsP_3, interactions between closely spaced release units ($\leqslant 3$ μm) may yield localized events involving two sites, or abortive waves travelling distances of tens of micrometres.

Activation of InsP_3 receptors

Global cellular calcium responses evoked by InsP_3 in the oocyte show a high degree of co-operativity, in that responses begin abruptly following long latencies and show a non-linear dependence upon [InsP_3] (Parker & Miledi, 1989; Miledi & Parker, 1989). It remains unclear, however, whether this arises through co-operative activation of receptors by InsP_3 alone, or by positive feedback from cytosolic [Ca^{2+}]. Distinguishing between these possibilities in intact cells has been difficult, since InsP_3R activation inevitably increases cytosolic [Ca^{2+}]; and the same limitation applies to other experimental systems, including permeabilized cells and microsomal preparations, unless free [Ca^{2+}] is well buffered. Numerous reports in the literature provide conflicting conclusions regarding the degree of co-operativity (see Marchant & Taylor (1997) for references). The precise control of intracellular [InsP_3] together with resolution of elementary calcium signals offers important advantages toward resolving this question. Firstly, information is derived from release units that involve activation of only individual or small numbers of InsP_3R, rather than from large and possibly heterogeneous populations. Secondly, puffs begin abruptly following long quiescent periods when cytosolic [Ca^{2+}] remains at the resting level, so that measurements of the latent period give information regarding InsP_3 binding in the absence of feedback regulation by calcium.

Three findings indicate an inherent co-operativity for InsP_3 action: (i) the numbers of puffs observed following photorelease of InsP_3 increase initially as the third power of time (Fig. 2*B*); (ii) the mean latency to activation of calcium liberation decreases as approximately the square of the flash strength (Fig. 3*B*); and (iii) the numbers of release sites activated vary as about the fourth power of the flash strength for weak stimuli (Fig. 4*B*). These observations could not have arisen artifactually through non-linearity in the photolysis of caged InsP_3, because the formation of product is a linear function of flash strength (Parker & Ivorra, 1992). The degree of co-operativity indicated by the various approaches, however, varies considerably and each is subject to errors. Thus the fact that photolysis produces a decaying transient of [InsP_3], rather than a sustained step may tend to underestimate the co-operativity derived from

latency distributions, whereas calcium diffusion from neighbouring puffs may steepen the relationship between [InsP_3] and numbers of sites activated. Nonetheless, the evidence for co-operativity is strong, even if the data do not yet allow a precise quantification of its degree. In principle, co-operativity may arise through a requirement for a certain number of InsP_3 molecules to bind to sites on the InsP_3R, or for binding to a sufficient number of InsP_3R within a cluster to generate a detectable calcium signal. Given that the sensitivity of the confocal system appears sufficient to resolve calcium flux through single channels (Parker & Yao, 1996; Sun *et al.* 1998), the former explanation appears more likely, and is also consistent with the tetrameric structure of the InsP_3 receptor–channel complex.

In contrast to the marked dependence of latency to calcium liberation upon [InsP_3], the initial rates of calcium rise measured focally at release sites show little or no dependence upon [InsP_3]. We had previously suggested that activation of the InsP_3R involves a sequential, two-step process in which binding of InsP_3 molecules initially 'primes' the receptor by exposing facilitatory calcium sites, and subsequent binding of calcium to these unmasked sites then leads to channel opening (Parker *et al.* 1996; Marchant & Taylor, 1997). The priming step is slow in the case of low InsP_3 concentrations evoking puffs, thus giving rise to long-latency responses, whereas once a puff is triggered (by stochastic opening of a channel) the subsequent activation of calcium flux may be rate limited by the kinetics of calcium binding to the facilitatory sites and thus relatively independent of [InsP_3]. Resolution of this behaviour depends upon the ability to monitor calcium signals from a single release site. Macroscopic measurements (from a whole cell or even large subcellular regions encompassing several sites) show an apparently continuously graded relationship between [InsP_3] and the rate of rise of [Ca^{2+}], because increasing [InsP_3] activates increasing numbers of sites with a progressively smaller spread in their latencies.

Comparison with other cell types

The *Xenopus* oocyte has provided much crucial information regarding both macroscopic (Lechleiter & Clapham, 1992) and microscopic (Yao *et al.* 1995; Parker & Yao, 1996) aspects of InsP_3-evoked calcium release, but concerns have been raised as to whether other non-excitable cells of more typical size show the same functional architecture (Bootman *et al.* 1997*b*). The spatiotemporal patterns of calcium release in the oocyte, however, show striking similarities with recent results in HeLa cells (Bootman, Berridge & Lipp, 1997*a*; Bootman *et al.* 1997*b*), in which applications of low concentrations of calcium-mobilizing agonists cause generation of saltatory calcium waves, with rapid calcium liberation occurring at specific release sites spaced some 5–10 μm apart. Transient release events were resolved preceding the rising phase of calcium waves in HeLa cells (Bootman *et al.* 1997*b*) and were observed in isolation at low agonist concentrations (Bootman *et al.* 1997*a*). Similar to the oocyte, HeLa cells displayed events of widely differing magnitudes associated with estimated calcium currents of roughly 1 pA during blips and 25 pA during puffs (Bootman *et al.* 1997*a*). Furthermore, Bootman *et al.* (1997*b*) describe the recruitment of elementary calcium signals in HeLa cells to generate global calcium waves. Increasing activation towards the wave threshold was often, but not invariably associated with an increasing frequency of elementary events. In addition, the amplitude of events in some cells increased markedly, a result that differs from our earlier finding in the oocyte of only a slight dependence of puff amplitude upon InsP_3 concentration (Yao *et al.* 1995).

Glial cells also display saltatory calcium waves although the architecture of the calcium signalling machinery in these cells appears to be arranged more macroscopically than in oocytes or HeLa cells, with specialized regions of wave amplification of finite (1–5 μm) diameter spaced roughly 20 μm apart (Yagodin *et al.* 1994; Simpson & Russell, 1996). Finally, Horne & Meyer (1997) utilized a novel immobile calcium indicator to image transient, localized calcium release events in RBL cells in response to photoreleased InsP_3, under conditions where coupling between release domains was reduced by EGTA.

Despite the 10^5- or 10^6-fold difference in cell volume between a HeLa or RBL cell and the oocyte, the properties of the elementary events are remarkably consistent. Calcium fluxes at individual release sites are similar, probably involving concerted activation of several InsP_3R to generate a puff, and release sites are spaced similarly at distances of a few micrometres. The notion that InsP_3-mediated calcium signalling is composed of elementary events arising at specialized sites involving clusters of InsP_3R and other calcium-handling systems may, therefore, be a universal mechanism among very diverse cell types.

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