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Phasic characteristic of elementary Ca^{2+} release sites underlies quantal responses to IP_3

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Ca^{2+} liberation by inositol 1,4,5-trisphosphate (IP_3) is 'quantal', in that low [IP_3] causes only partial Ca^{2+} release, but further increasing [IP_3] evokes more release. This characteristic allows cells to generate graded Ca^{2+} signals, but is unexpected, given the regenerative nature of Ca^{2+}-induced Ca^{2+} release through IP_3 receptors. Two models have been proposed to resolve this paradox: (i) all-or-none Ca^{2+} release from heterogeneous stores that empty at varying [IP_3]; and (ii) phasic liberation from homogeneously sensitive stores. To discriminate between these hypotheses, we imaged subcellular Ca^{2+} puffs evoked by IP_3 in *Xenopus* oocytes where release sites were functionally uncoupled using EGTA. Puffs were little changed by 300 μM intracellular EGTA, but sites operated autonomously and did not propagate waves. Photoreleased IP_3 generated flurries of puffs—different to the prolonged Ca^{2+} elevation following waves in control cells—and individual sites responded repeatedly to successive increments of [IP_3]. These data support the second hypothesis while refuting the first, and suggest that local Ca^{2+} signals exhibit rapid adaptation, different to the slower inhibition following global Ca^{2+} waves.

Keywords: Ca^{2+} signaling/inositol trisphosphate/quantal Ca^{2+} release/*Xenopus* oocyte

Introduction

The liberation of Ca^{2+} from intracellular stores into the cytosol through channels gated by inositol 1,4,5-trisphosphate (IP_3) mediates the initial Ca^{2+} mobilization step during transduction of signals initiated by many neurotransmitters and hormones (Berridge, 1993). Numerous observations from permeabilized cell preparations and single cells have shown that Ca^{2+} is released from intracellular stores in proportion to the strength of stimulation, so that a sustained elevation of [IP_3] at submaximal levels causes only a transient, partial release of Ca^{2+}, whereas further increments of [IP_3] evoke additional pulses of Ca^{2+} liberation (Muallem *et al*., 1989; Meyer and Stryer, 1990; Bootman, 1994a,b; Bootman *et al*., 1994; Parys *et al*., 1996). This characteristic was termed 'quantal' Ca^{2+} release, because it was thought to arise from the abrupt, all-or-none emptying of discrete intracellular stores that respond at differing threshold concentrations of IP_3 (Muallem *et al*., 1989). However, that hypothesis remains unproven, and the mechanism underlying quantal liberation is unclear (for reviews see Taylor, 1992; Bootman, 1994a; Parys *et al*., 1996; Koizumi *et al*., 1999). In particular, the graded nature of Ca^{2+} release is difficult to reconcile with the well-established stimulatory effect of cytosolic Ca^{2+} on IP_3 receptor (IP_3R) activity (Iino, 1990; Bezprozvanny *et al*., 1991; Finch *et al*., 1991), which would be expected to lead to regenerative, all-or-none responses through Ca^{2+}-induced Ca^{2+} release (CICR). Two major models have been proposed to resolve this discrepancy: (i) all-or-none release from stores exhibiting heterogeneous sensitivities to IP_3, as discussed above (Muallem *et al*., 1989; Bootman *et al*., 1992; Cheek *et al*., 1994; Beecroft and Taylor, 1997); and (ii) a 'steady-state' model, involving intracellular stores with similar sensitivities to IP_3 that each release Ca^{2+} in a phasic or adaptive manner in response to stepwise increments of [IP_3] (Irvine, 1990; Missiaen *et al*., 1991, 1992, 1999; Nunn and Taylor, 1992; Tanimura and Turner, 1996; van de Put and Elliott, 1996; Koizumi *et al*., 1999).

A possible means to discriminate between these models is provided by recent improvements in single cell imaging techniques, which have revealed that IP_3 evokes Ca^{2+} liberation as elementary packets ('Ca^{2+} puffs'), arising at discrete subcellular sites comprised of a few tens of IP_3R (Parker and Yao, 1991; Parker *et al*., 1996; Berridge, 1997; Bootman *et al*., 1997; Swillens *et al*., 1999). Puffs are generated at low concentrations of IP_3 by the concerted opening of several channels within a cluster, whereas at higher concentrations, neighboring sites become functionally coupled by Ca^{2+} diffusion and CICR so as to support global Ca^{2+} waves that propagate in a saltatory manner throughout the cell (Parker and Yao, 1991; Lechleiter and Clapham, 1992; Parker *et al*., 1996; Berridge, 1997; Bootman *et al*., 1997; Callamaras *et al*., 1998; Marchant *et al*., 1999).

The puff sites are believed to be formed from clusters of IP_3R distributed on a contiguous endoplasmic reticulum (ER) and, although not physically discrete, they represent functionally discrete subcellular Ca^{2+} stores (Parker *et al*., 1996; Berridge, 1997). We were, therefore, interested to study the responses of individual puff sites to determine whether they exhibit all-or-none or phasic Ca^{2+} release in response to incremental steps of [IP_3]. Our earlier attempts to do this were frustrated, however, because separable puffs could be observed only over a narrow range of IP_3 concentrations (Sun *et al*., 1998). Because of the steep dose dependence for activation of puffs, a certain threshold [IP_3] was required to evoke any events, whereas concentrations less than twice as great evoked propagating Ca^{2+} waves (Callamaras *et al*., 1998). To circumvent this problem, the present experiments were done by imaging

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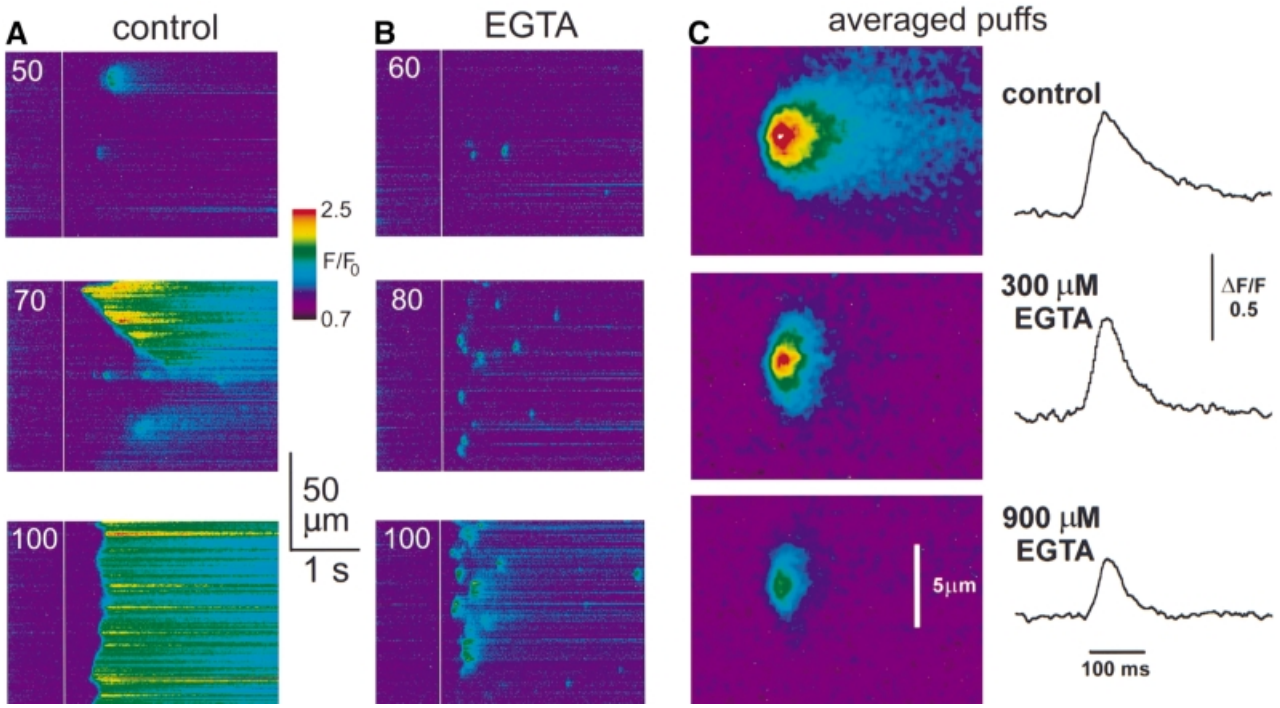

Fig. 1. Functional uncoupling of IP_3-sensitive Ca^{2+} release sites following intracellular injection of EGTA. Panels show linescan images of Ca^{2+}-dependent fluorescence, with distance along the scan line depicted vertically and time running from left to right. Increasing fluorescence ratio ($\Delta F/F$) (increasing free [Ca^{2+}]) is denoted by increasingly 'warm' colors (as indicated by the color bar). (**A**) Records showing responses to photolysis flashes of varying duration (indicated in ms) delivered at the times indicated by vertical white lines in the images. (**B**) Comparable responses evoked in the same oocyte after injecting EGTA to a final intracellular concentration of 300 μM, together with 150 μM Ca^{2+}. Results are representative of findings in 17 oocytes (three donor frogs). (**C**) Images show averages of puffs in a single cell before injecting EGTA, and after progressively loading EGTA to final intracellular concentrations of 300 and 900 μM. Each frame is an average of eight events, aligned to their peaks in space and time. Traces show the time course of fluorescence signals measured from three pixel (0.4 μm) regions centered on the averaged puff images.

Ca^{2+} signals in *Xenopus* oocytes that were loaded with the Ca^{2+} buffer EGTA. Owing to its slow on-rate for Ca^{2+} binding (20 $\mu M^{-1}s^{-1}$), EGTA inhibits Ca^{2+} diffusion over micrometer distances between release sites (Roberts, 1994; Horne and Meyer, 1997; Song *et al*., 1998; Kidd *et al*., 1999), while sparing short-range Ca^{2+} feedback between IP_3 receptors clustered within <200 nm at individual puff sites (Sun *et al*., 1998; Swillens *et al*., 1999). This resulted in a functional uncoupling between release sites, such that the properties of individual puffs were little changed, but release sites continued to operate autonomously and did not propagate Ca^{2+} waves even at high [IP_3].

Our main findings were that uncoupled release sites generated puffs only transiently following a step increase of [IP_3] but—in marked contrast to the pronounced inhibition of Ca^{2+} liberation that persists for tens of seconds following Ca^{2+} waves (Parker and Ivorra, 1990; Ilyin and Parker, 1994)—were again able to evoke puffs following further stepwise increases of [IP_3]. The summated activity of numerous subcellular sites thus mimicked a quantal release process. Furthermore, individual sites exhibited phasic responses to stepwise increments of IP_3, consistent with a 'steady-state' model in which quantal responses result from phasic release of Ca^{2+} from intracellular stores displaying similar sensitivities to IP_3.

Results

Functional uncoupling of release sites by EGTA

Experiments were carried out using immature *Xenopus* oocytes, imaged using a linescan confocal microscope to monitor fluorescence of the Ca^{2+} indicator Oregon Green 488 BAPTA 1 (OG-1) along a 100 μm scan line focused in the animal hemisphere at a depth of 3–5 μm below the cell surface (Callamaras and Parker, 1999). Flashes of UV light from a mercury arc lamp were focused as a 200 μm spot around the scan line to allow uniform photolysis of caged IP_3 loaded into the cell. The flash duration and intensity, and thus the extent of photorelease, were regulated by an electronic shutter and neutral density filters (Callamaras and Parker, 1998).

In control cells (before loading with EGTA), brief photolysis flashes evoked transient Ca^{2+} puffs arising at discrete sites, whereas progressively longer flashes evoked propagating Ca^{2+} waves and then nearly simultaneous Ca^{2+} release from multiple sites along the scan line (Figure 1A; Callamaras *et al*., 1998). The range of IP_3 concentrations over which discrete puffs were observed was narrow, and waves were usually initiated by flashes ~50% stronger than that required to evoke one or two puffs along the scan line. These spatio-temporal patterns of Ca^{2+} signals were dramatically altered after intracellular loading of EGTA. In the presence of EGTA the same range of

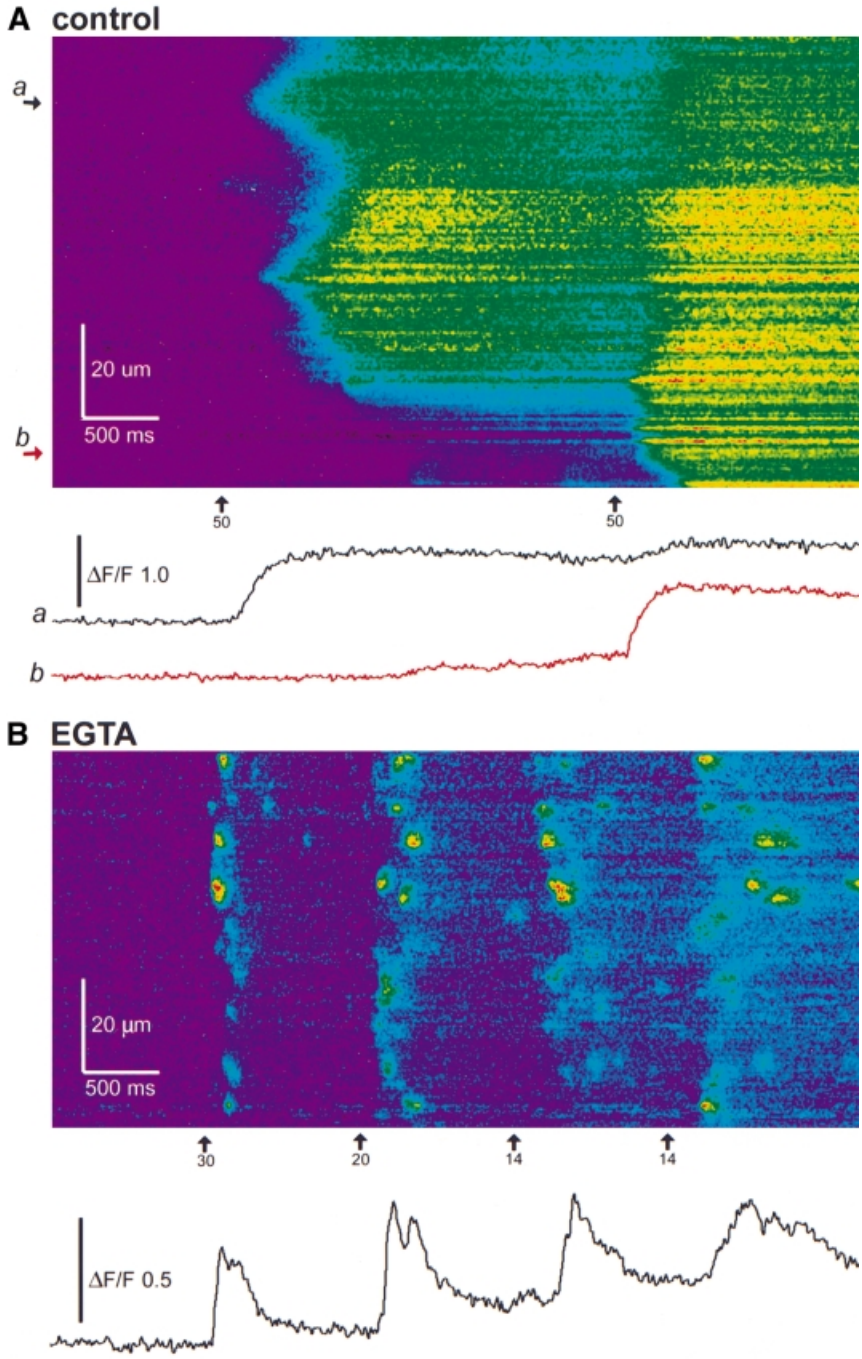

Fig. 2. Responses to repeated photolysis flashes before and after loading with EGTA. (**A**) Linescan image shows calcium signals in a control oocyte in response to two identical photolysis flashes (50 ms duration), delivered when marked by the arrows. The first flash evoked calcium waves originating at two sites ('V' patterns), which propagated across all but the lower region of the scan line. The second flash evoked little additional calcium release in regions that responded to the first flash, but triggered a wave in the region that failed to respond. The traces show fluorescence profiles (averaged over 22 pixels) measured at the two sites (a and b) indicated on the image. (**B**) Responses in a different oocyte, loaded with 300 µM EGTA plus 150 µM Ca^{2+}. The cell was stimulated with four repeated photolysis flashes of decreasing durations, as indicated in ms. The trace shows fluorescence monitored from a 20 µm region near the center of the image.

flash durations evoked only puffs. These arose with greater frequency and shorter latency with increasing [IP_3], but remained discrete and did not support propagating waves (Figure 1B).

Similar results were obtained when EGTA was injected either alone (four oocytes) or together with sufficient Ca^{2+} (10 mM EGTA + 5 mM $CaCl_2$; 19 oocytes) to buffer free [Ca^{2+}] in the injection solution to a level (~150 nM) comparable to or higher than the basal intracellular concentration. Thus, the uncoupling between release sites is unlikely to have arisen because the binding of Ca^{2+} ions to EGTA led to a depletion of store Ca^{2+} or to a reduction of cytosolic free [Ca^{2+}]. Furthermore, buffers have little effect on Ca^{2+} pumps in the ER or plasma

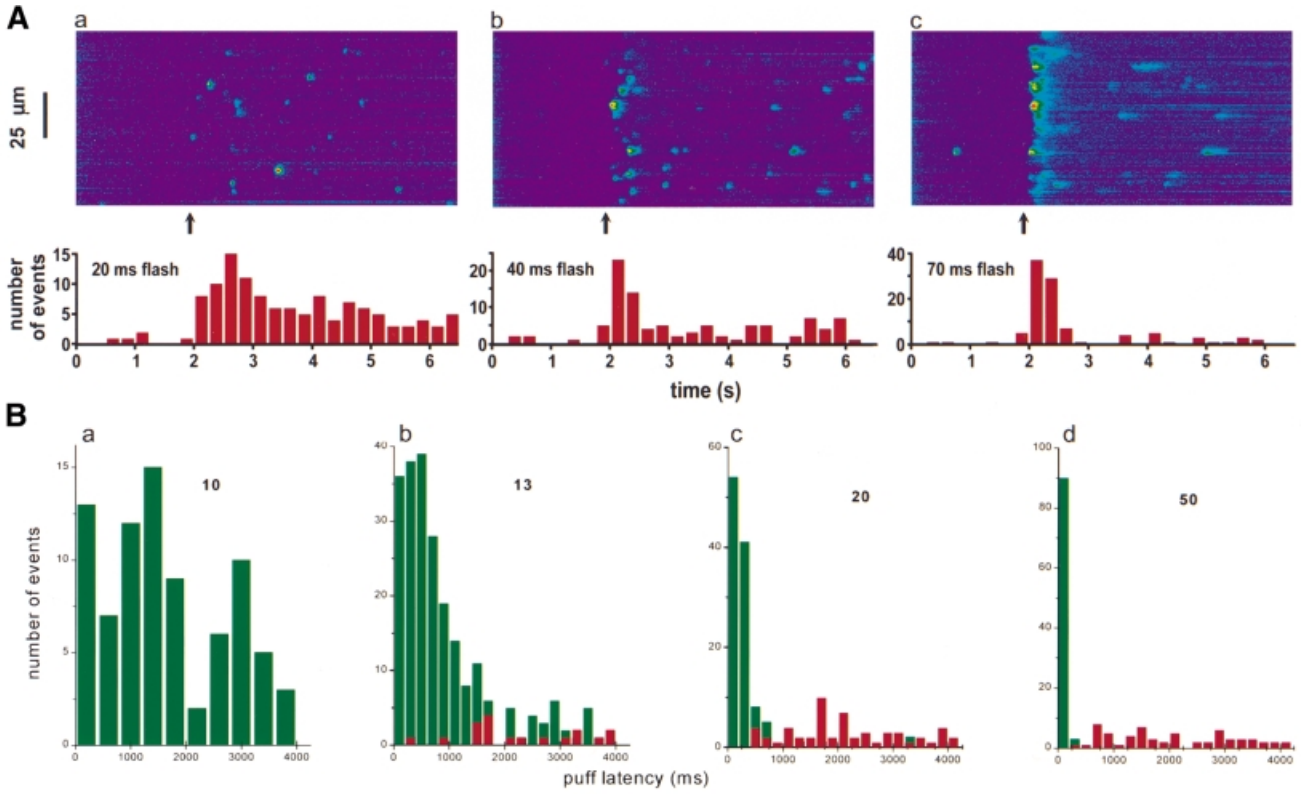

Fig. 3. Ca^{2+} release sites exhibit phasic responses following step increases in $[IP_3]$. (**A**) Linescan images illustrate puffs evoked by photolysis flashes of varying durations delivered at the times indicated by the arrows. Histograms show the corresponding frequencies of puff occurrence as a function of time after the flashes. Data were obtained from a fixed scan line in a single oocyte, and numbers of events were measured in 250 ms time bins by counting all puffs occurring along the scan line in response to eight, four and five repeated trials in a, b and c, respectively. Similar distributions were observed in two additional oocytes. (**B**) Latency distributions measured in another oocyte, plotting separately the occurrence of the first (green) and subsequent (red) puffs at each release site following flashes of varying duration (indicated in ms).

membrane (Zhang and Muallem, 1992; Mogami *et al*., 1998). Instead, the long (2 µm) length constant for EGTA buffering that results from its slow on-rate for Ca^{2+} binding (Horne and Meyer, 1997; Song *et al*., 1998; Kidd *et al*., 1999) is consistent with inhibition of Ca^{2+} diffusing between release sites spaced 2–3 µm apart (Callamaras *et al*., 1998) while sparing short-range Ca^{2+} feedback between IP_3 receptors clustered within <200 nm at individual puff sites (Yao *et al*., 1995; Berridge, 1997; Sun *et al*., 1998; Swillens *et al*., 1999). All subsequent experiments were performed using 300 µM intracellular EGTA (+150 µM Ca^{2+}), as this effectively uncoupled sites without substantially affecting the size or kinetics of puffs (control: $\Delta F/F$ 0.815 ± 0.041, half decay time 135 ± 10 ms, n = 59 events; EGTA: $\Delta F/F$ 0.69 ± 0.026, half decay time 113 ± 7 ms, n = 90 events; paired measurements in five oocytes). Higher concentrations of EGTA resulted in greater reductions in puff amplitude (Figure 1C).

Responses to incremental steps of $[IP_3]$

Our main object was to study Ca^{2+} release from individual sites in response to stepwise increases of $[IP_3]$ generated by successive photolysis flashes. Figure 2A illustrates representative responses in a control cell (not loaded with EGTA) evoked by two identical photolysis flashes, delivered at an interval of 2.5 s. The first flash triggered calcium waves that propagated across most of the scan line. A second flash subsequently evoked only a very slight further increase in $[Ca^{2+}]$ throughout this region, although a large response was elicited in a region that was not invaded by the initial wave. The inhibition of Ca^{2+} response following the first wave did not result because of saturation of the indicator dye, because we previously demonstrated a similar inhibition by use of a low-affinity indicator (Ilyin and Parker, 1994) and by recording endogenous Ca^{2+}-activated Cl^- currents in the oocyte (Parker and Ivorra, 1990).

A dramatically different pattern of Ca^{2+} responses was observed after intracellular loading of EGTA (Figure 2B). An initial flash evoked a transient flurry of puffs at numerous sites along the scan line, which largely ceased within a few hundred milliseconds and the overall Ca^{2+} level declined rapidly toward the baseline. A second flash at an interval of 1 s then evoked another transient flurry of puffs. Indeed, the sensitivity at this time was greater than for the first flash and, in the experiment illustrated, we shortened the duration of the second flash to two-thirds that of the first, so as to evoke a response of similar magnitude. Additional responses were then evoked by repeated flashes of further reduced duration. The summated activity of multiple release sites, indicated by the lower trace in Figure 2B, thus displayed a succession of Ca^{2+} transients in response to repeated photorelease of IP_3, closely analogous to the quantal release phenomenon described in other systems (reviewed in the Introduction).

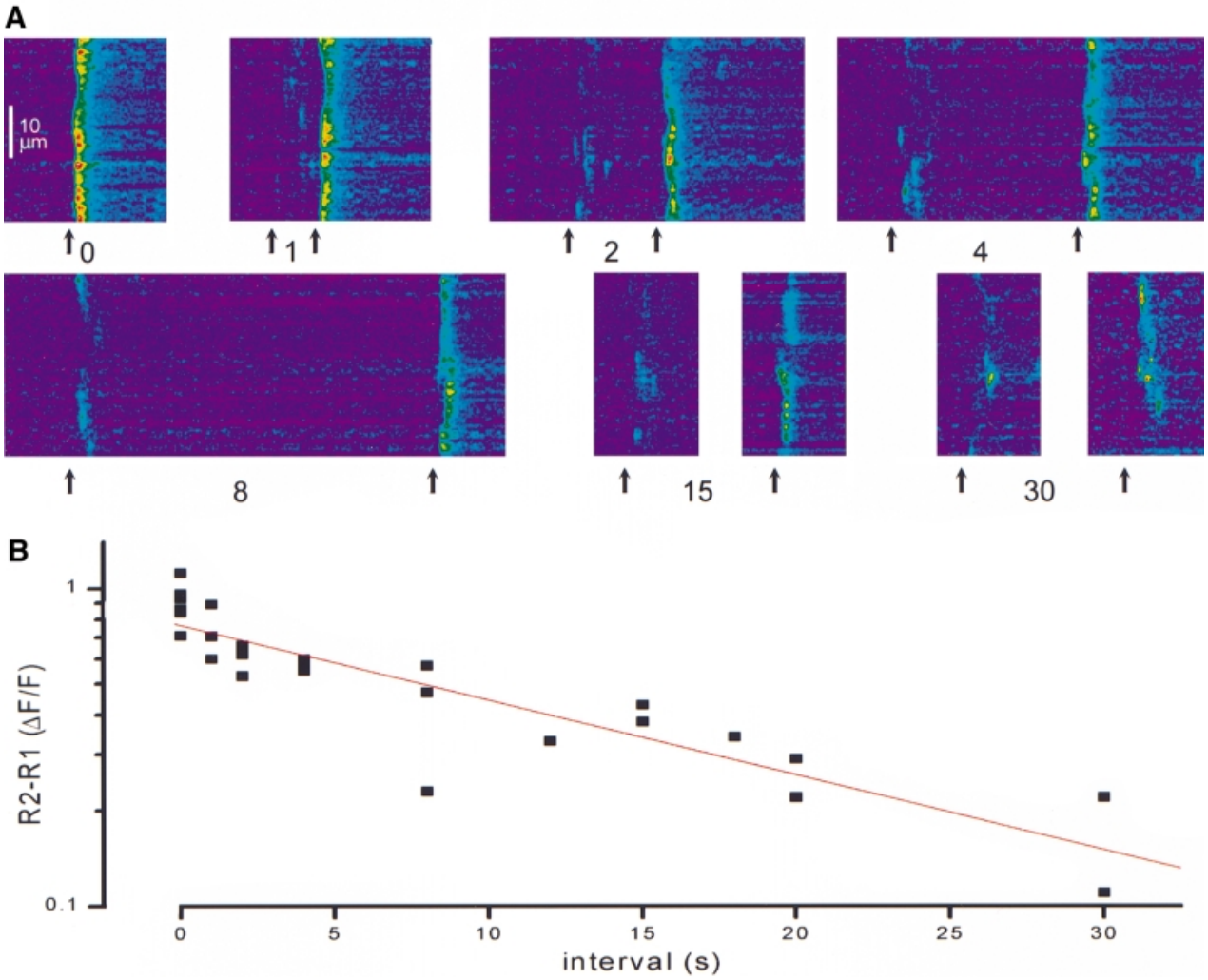

Fig. 4. Estimation of decay of [IP_3] following photorelease, using paired-pulse facilitation. (**A**) Linescan images show Ca^{2+} responses evoked by paired photolysis flashes, delivered when marked by the arrows at varying inter-pulse intervals (indicated in s). All photolysis flashes were of a fixed duration (20 ms), chosen so that a single flash evoked only a very slight response. Periods of 90 s were allowed between each trial. To conserve space, records are omitted during the time between flashes at longer intervals (15 and 30 s). (**B**) Measurements from images like those in (A), obtained in two oocytes, showing the decay of facilitation of the second response as a function of interval between the flashes. Data were obtained by integrating the Ca^{2+} signal along the entire scan line, and are plotted as the peak fluorescence ratio change evoked by the second flash minus that evoked by the first flash in that trial.

Puffs arise transiently following increasing steps of [IP_3]

To analyze the kinetics of Ca^{2+} release at individual sites in more detail, we first measured the occurrence of puffs generated by single photolysis flashes in the presence of EGTA (Figure 3). Weak photorelease evoked puffs that arose with a relatively long mean latency, and were observed at a gradually declining rate for several seconds after the flash (Figure 3Aa). When the flash duration was lengthened progressively, the mean latencies of the puffs shortened, and they became increasingly bunched together as brief flurries shortly after the flash, although further puffs (often with distinctively prolonged time courses) continued at a low frequency for several seconds (Figure 3Ab and c). Figure 3B shows latency distributions measured separately for the initial puff generated at each site (green) and for subsequent puffs generated at those sites following each flash (red). With the weakest flashes tested, individual sites usually responded only once, and few or no instances were seen of repeated puffs at a given site (Figure 3Ba). Progressively longer flashes evoked increasing numbers of subsequent puffs, and this 'tail' of events persisted with little diminution for several seconds, despite the marked shortening in mean latency of the initial puffs (Figure 3Bb–d).

A trivial explanation for the transient occurrence of puffs following stronger flashes could be that the photolysis flashes generated a rapidly declining spike of [IP_3], rather than a relatively sustained step increase. Several observations indicate that this was not the case. First, the more sustained occurrence of puffs following weak stimuli (Figure 3Aa and Ba) indicates that IP_3 remained present for several seconds. Secondly, metabolic degradation of IP_3 in the oocyte is slow (half-life 60 s; Sims and Allbritton, 1998). Finally, we estimated the decline of [IP_3] under our experimental conditions using a

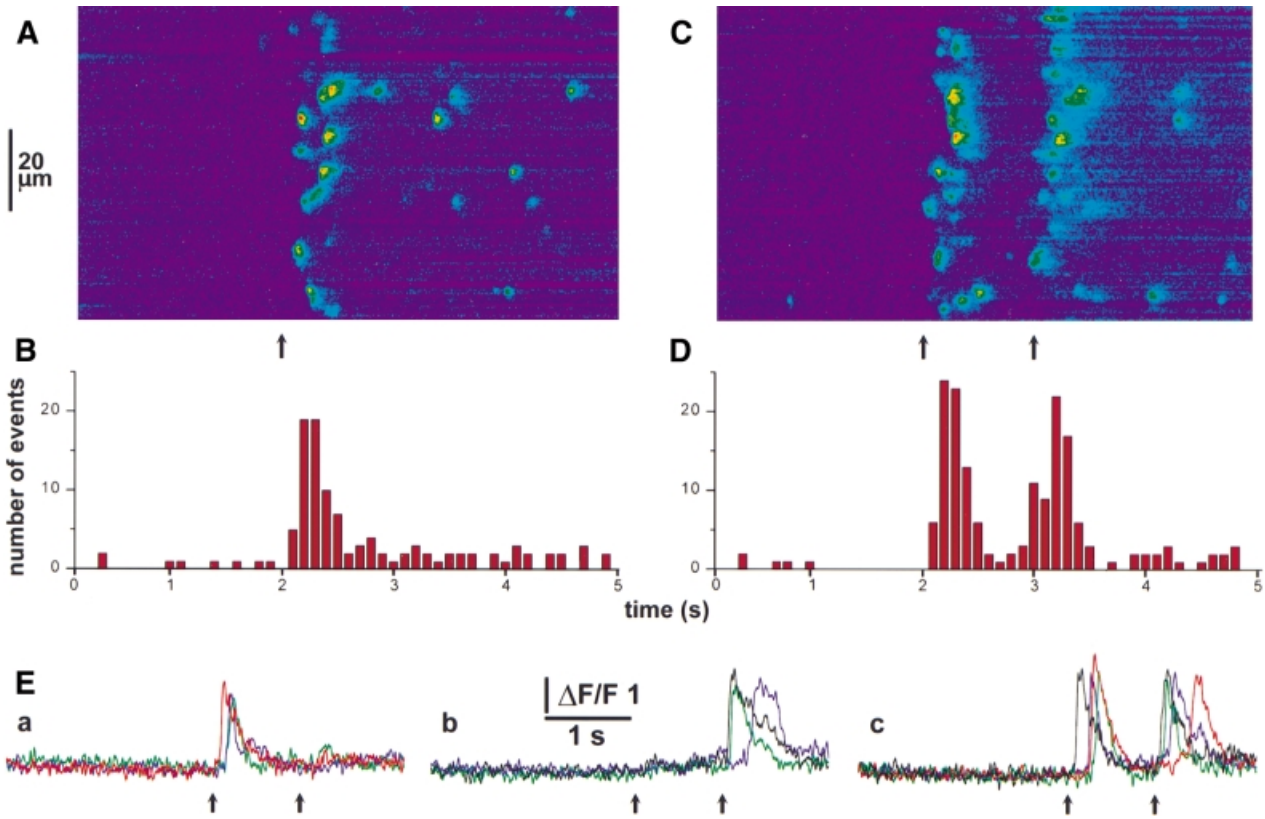

Fig. 5. Incremental responses of Ca^{2+} release sites in response to paired photolysis flashes. (**A** and **B**) Linescan image and latency histogram illustrating puffs evoked by a single photolysis flash (40 ms duration). (**C** and **D**) Corresponding records showing responses to paired flashes at an interval of 1 s. Data were obtained from the same scan line as in (A), and with an identical first flash: the duration of the second flash was 20 ms. Latency distributions were derived from seven trials in (B) and nine trials in (D). (**E**) Instances when given sites exhibited puffs in response to only the first or second flash (a and b) or to both flashes (c). Traces show fluorescence ratios measured from five pixel (0.65 µm) regions of the scan line, centered on release sites. Results are representative of trials at 65 linescan locations in 12 oocytes.

protocol based on the non-linear sensitivity of Ca^{2+} release to IP_3 (Parker and Ivorra, 1992). Pairs of identical photolysis flashes were delivered, with their duration adjusted so that the first flash evoked only a barely detectable response. Much larger responses were then evoked by the second flash of the pair, even at inter-flash intervals as long as 30 s (Figure 4A), presumably because residual IP_3 remaining after the first flash summated with that photoreleased by the second flash, to generate greater Ca^{2+} release (Parker and Ivorra, 1992). The decline of paired-pulse facilitation then gives a measure of the decline of $[IP_3]$, which, from the data of Figure 4B, occurred with a half-time of ~10 s. On the timescale of our experiments, therefore, the photolysis flashes can be considered to produce relatively sustained steps of $[IP_3]$, and the rapid decline of puff occurrence within a few hundred milliseconds following stronger flashes must represent a decrease in their probability of triggering despite the sustained elevation of $[IP_3]$.

Phasic responses of individual release sites

The repeated transient Ca^{2+} signals to successive photolysis flashes illustrated in Figure 2B might have arisen either because individual sites generated repeated responses, or because new sites were recruited while those that responded to a previous flash remained refractory and failed to respond again. To discriminate between these possibilities we recorded several trials at fixed linescan positions, so that the activity of specific sites could be monitored in response to 10 or more trials (limited by local laser damage to the cell). Figure 5 shows results representative of experiments in a total of 12 oocytes. The oocyte was first stimulated by a single flash, using a duration chosen to evoke a flurry of puffs that largely ceased within ~500 ms (Figure 5A and B). Paired flashes were then delivered at an interval of 1 s, with the duration of the second flash reduced by 50%, so that similar responses were evoked by both stimuli. The second flash again evoked a transient flurry of puffs, beginning shortly after the flash at a time when events during the first response had largely ceased (Figure 5C and D). Most importantly, many sites generated puffs in response to both flashes (Figure 5Ec), although in some instances a particular site responded to only one or other flash (Figure 5Ea and b). This variability in patterns of response probably arose simply from the statistical likelihood of puff triggering. Neither the probability of occurrence, nor the size of puff evoked by the second flash depended significantly upon the response of that site to the first flash. Measurements of repeated puffs evoked at given sites showed that the likelihood of observing a puff after the second flash was not significantly different whether or not

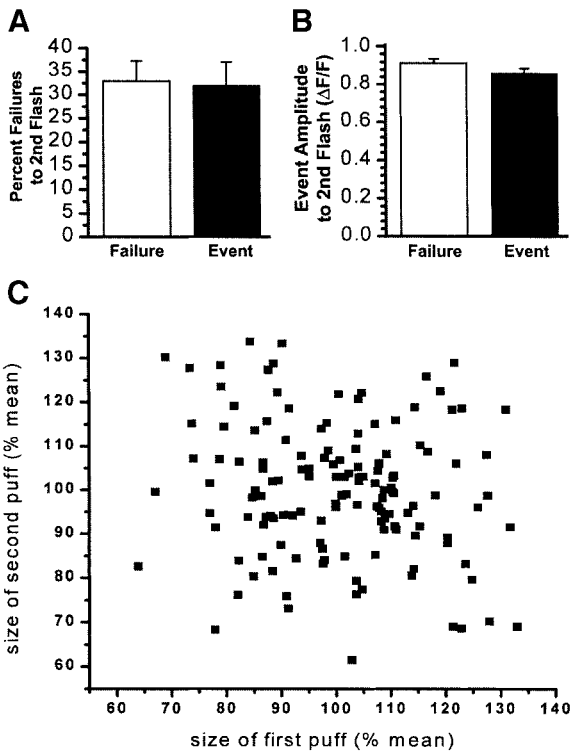

Fig. 6. The probability of occurrence and amplitude of puffs in response to the second flash are independent of whether a particular site generated a puff in response to the first flash. (**A**) Percentage of times that sites failed to show puffs in response to a second flash, as a function of whether or not each site gave a puff to the first flash. (**B**) Amplitudes of puffs generated by a second flash, as a function of whether or not that site responded to the first flash. Data in (A) and (B) are from nine repeated trials (paired flashes) at a single scan line encompassing 16 discrete release sites. Error bars indicate ± 1 SEM. Paired flashes were delivered at a 1 s interval, with respective durations of 30 and 20 ms. Puffs were evoked by the first flash in 38% (54/144) of instances. (**C**) Scatter plot showing measurements of the amplitude (F/F_o) of second puffs as a function of the size of the first puff during individual trials at given sites. Data are included only from instances when puffs were evoked by both flashes, and are normalized as a percentage of the mean puff size averaged over repeated trials at each site.

that site had given a puff to the first flash (Figure 6A), and the mean amplitude of puffs evoked by the second flash was similarly unchanged whether or not they were preceded by a puff in response to the first flash (Figure 6B). Furthermore, there was little or no correlation between the size of the second puff and that of the preceding puff at the same site (Figure 6C).

Individual release sites thus responded in a phasic manner to successive steps of $[IP_3]$, and we found no evidence that they became inactivated or refractory even when the inter-flash interval was shortened so that a second puff was evoked almost immediately after a first. Figure 7 illustrates responses evoked by paired flashes at an interval of 400 ms, which was the shortest time that still allowed a clear separation of puffs arising from each flash. A single flash evoked puffs that were largely grouped within a period of ~400 ms (Figure 7Aa), and delivery of the second flash then evoked further events arising with a clearly separable distribution of latencies (Figure 7Ab). In many instances, second puffs began before the Ca^{2+} signal to the first puff had declined to the resting level.

Discussion

Quantal Ca^{2+} liberation

Our results demonstrate that individual subcellular Ca^{2+} release sites exhibit incremental responses to IP_3, in that the probability of generating Ca^{2+} release events (puffs) was raised only transiently following a step increase of $[IP_3]$, but was again transiently increased following a further elevation of $[IP_3]$. Macroscopic Ca^{2+} signals, summated over numerous subcellular release sites, were thus analogous to the quantal Ca^{2+} liberation originally described in permeabilized cell preparations (Muallem *et al*., 1989), whereby submaximal $[IP_3]$ evoked transient release of only a fraction of the store content.

Quantal responses have been proposed to involve all-or-none emptying of discrete stores displaying heterogeneous sensitivities to IP_3 (Muallem *et al*., 1989; Bootman *et al*., 1992; Cheek *et al*., 1994; Beecroft and Taylor, 1997), so that a low dose of IP_3 empties only the most sensitive stores whereas further increments activate other stores with progressively lower sensitivities. This was not the case here, because individual elementary release sites usually responded in succession to repeated steps of $[IP_3]$. Although instances were observed where a particular site gave a puff to only a first, or only a second step of IP_3, these probably arose merely because of the stochastic characteristic of puff triggering and, on average, Ca^{2+} release to a second step was not influenced by whether that site had responded to the first step. The results, instead, favor a steady-state model (Irvine, 1990; Missiaen *et al*., 1991; Nunn and Taylor, 1992; Tanimura and Turner, 1996; van de Put and Elliott, 1996; Koizumi *et al*., 1999; Missiaen *et al*., 1999), in which quantal responses result from rapid attenuation of Ca^{2+} release from discrete release sites that have roughly similar sensitivities to IP_3. A further point is that the amplitude of a second puff was little diminished even when it arose within a few hundred milliseconds of a preceding event, indicating that the luminal Ca^{2+} level was not appreciably depleted. Ca^{2+} re-uptake from the cytosol is probably too slow to have refilled the store during the inter-puff interval (half-time ~1 s; Yao *et al*., 1995), suggesting either that puffs liberate only a small fraction of the store content, or that local depletion of luminal Ca^{2+} at a puff site is rapidly replenished by diffusion of Ca^{2+} from neighboring connected regions of the ER (Mogami *et al*., 1997).

The mechanisms involved in the termination of Ca^{2+} release during puffs, and its re-triggering by an incremental step of $[IP_3]$ remain unclear. One possibility is that a puff involves activation of only a fraction of the IP_3 receptors clustered at a release site, which then become refractory so that a puff evoked by a second step of $[IP_3]$ recruits other receptors that were not activated during the initial event. This is difficult to reconcile, however, with observations that puffs evoked by a second stimulus were, on average, undiminished by the appearance of a preceding puff, and that individual release sites responded in rapid succession to several stepwise increments in $[IP_3]$. Recent modeling studies (Swillens *et al*., 1999) indicate that a release site may contain ~25 IP_3Rs, of which five to 10 are activated during a typical puff; numbers that are expected to result in a marked attrition of receptors available for activation during successive events. A more

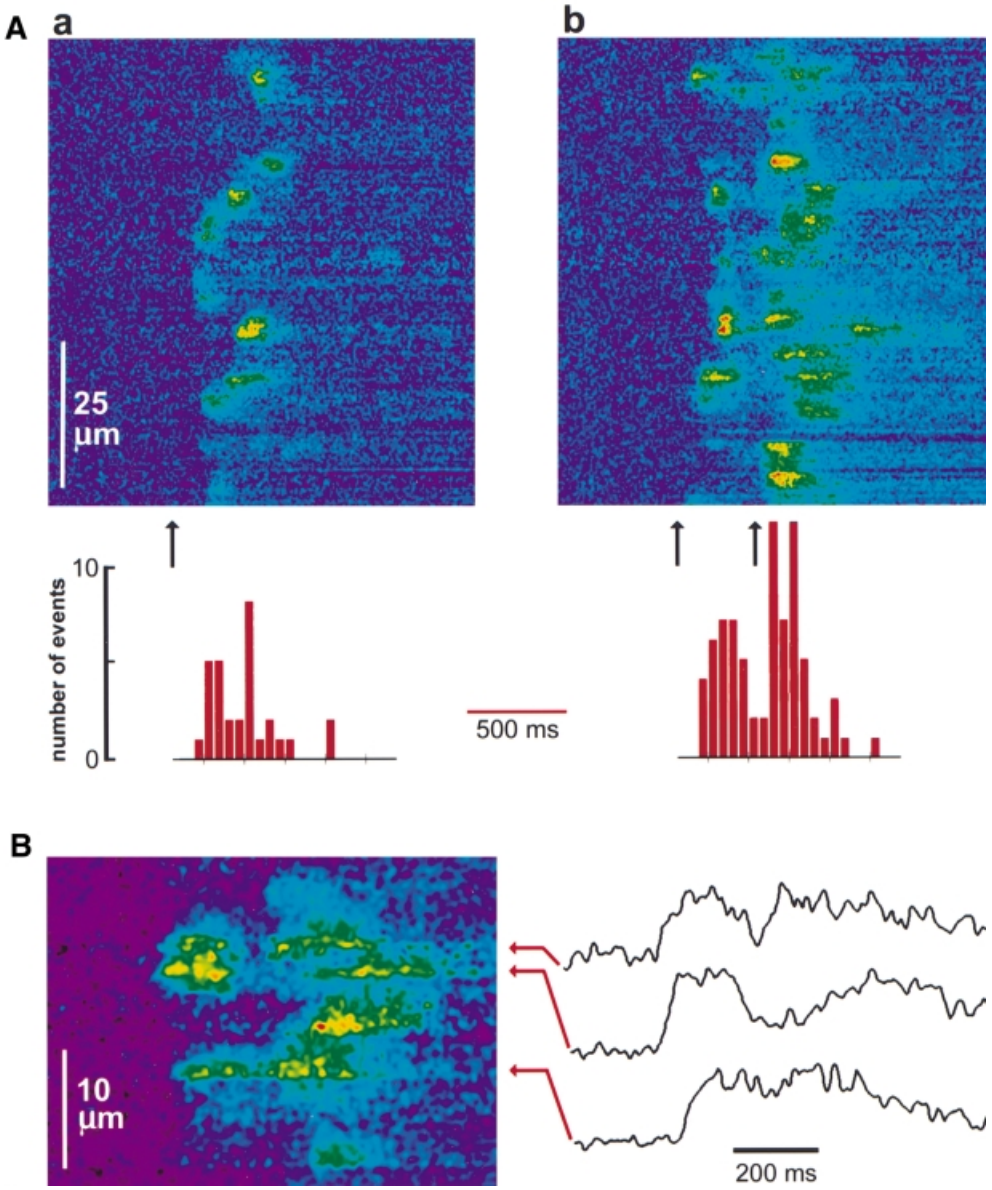

Fig. 7. Incremental responses of puff sites at short intervals. (**A**) Linescan images and puff latency distributions showing responses to a single flash of 30 ms duration (a), and to paired flashes with durations of 30 and 20 ms delivered at an interval of 400 ms (b). Flashes were delivered when marked by the arrows. Latency distributions were derived from three trials in each instance, in a single oocyte. (**B**) Examples of second puffs evoked at short latencies following initial events, shown on expanded time and distance scales. Traces on the right show fluorescence measurements from 0.5 µm regions at the sites marked by the red arrows.

attractive possibility is that IP_3Rs exhibit an adaptive sensitivity to IP_3, analogous to that proposed for the homologous ryanodine receptors. The mechanisms underlying this adaptation remain unclear, but may involve intrinsic receptor adaptation (Gyorke and Fill, 1993; Hajnóczky and Thomas, 1994; Stern, 1996), modulation of receptor sensitivity by luminal $[Ca^{2+}]$ (Nunn and Taylor, 1992; Tanimura and Turner, 1996; Koizumi *et al*., 1999; Missiaen *et al*., 1999), or reversal of Ca^{2+}-dependent inhibition at higher $[IP_3]$ (Bootman *et al*., 1995; Mak *et al*., 1998).

Effect of buffers on local and global Ca^{2+} signals

The incremental behavior of 'uncoupled' puff sites described here is markedly different from the strong inhibition that follows Ca^{2+} waves generated in control conditions (without EGTA), in which responses to $[IP_3]$ are strongly suppressed for several seconds (Parker and Ivorra, 1990; Ilyin and Parker, 1994). Termination of IP_3-evoked Ca^{2+} liberation and its subsequent recovery thus appear to involve at least two distinct processes of rapid adaptation and slower inhibition, which are likely to be important during local and global modes of cellular Ca^{2+} signaling, respectively (Yao *et al*., 1995; Berridge, 1997; Bootman *et al*., 1997; Marchant *et al*., 1999). Local signaling by Ca^{2+} puffs provides a means for cells to respond in a continuously graded manner to increasing stimuli, whereas Ca^{2+} waves represent a form of 'digital' signaling whereby stimulus strength is encoded by the frequency of all-or-none Ca^{2+} spikes (Parker *et al*., 1996;

Berridge, 1997). In the *Xenopus* oocyte the range of [IP_3] over which puffs are triggered is narrow, probably reflecting the primary function of the IP_3–Ca^{2+} pathway to generate a Ca^{2+} wave during egg fertilization (Nucitelli *et al*., 1993). The profound switch in signaling modalities following introduction of EGTA raises the possibility that other cell types may express endogenous Ca^{2+} buffers with specific kinetics and affinities so as to 'tailor' the spatio-temporal dynamics of Ca^{2+} signaling to their particular needs.

Materials and methods

Experiments were performed on immature oocytes obtained from *Xenopus laevis* as described previously (Callamaras *et al*., 1998; Sun *et al*., 1998). Frogs were anesthetized by immersion in a 0.15% aqueous solution of MS-222 (3-aminobenzoic acid ethyl ester) for 15 min, and small pieces of ovary removed by surgery. Epithelial layers were removed from oocytes either manually or by collagenase treatment (Sigma Type I, used at 1 mg/ml for 1 h). Oocytes were microinjected 1 h prior to recording with OG-1 together with caged IP_3 [*myo*-inositol 1,4,5-trisphosphate, $P^{4(5)}$-1-(2-nitrophenyl) ethyl ester], to final intracellular concentrations of ~40 and 5 μM, respectively. Subsequent injections of EGTA were made after obtaining control responses, and a period of >5 min was allowed after injection before images were obtained again. The injection solution (pH 7.0; set by KOH) contained 10 mM EGTA, either alone or together with 5 mM $CaCl_2$, and oocytes were usually loaded with 30 nl so as to achieve a final cytosolic concentration of ~300 μM EGTA. All recordings were made at room temperature, imaging in the animal hemisphere of oocytes bathed in Ringer's solution (120 mM NaCl, 2 mM KCl, 1.8 mM $CaCl_2$, 5 mM HEPES pH 7.2). OG-1 and caged IP_3 were obtained from Molecular Probes Inc. (Eugene, OR). All other reagents were from Sigma Chemical Co. (St Louis, MO).

Linescan confocal Ca^{2+} images were obtained using a custom-built scanner (Callamaras and Parker, 1999) and depict fluorescence monitored along a 100 μm line (760 pixels) scanned every 4 ms and focused at the level of the pigment granules in the oocyte. Fluorescence is expressed as a pseudoratio ($\Delta F/F$) of the intensity at each pixel relative to that at the same pixel before stimulation. IP_3 was photoreleased by delivering flashes of UV light, focused uniformly throughout a 200 μm diameter spot surrounding the imaging scan line (Callamaras and Parker, 1998). Varying flash durations were used to control (in a linear manner) the amount of photoreleased IP_3. Because each flash consumed a negligible fraction of the available caged IP_3 (Callamaras and Parker, 1998), numerous consistent responses were evoked by repeated flashes. Intervals of >60 s were allowed between trials.

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