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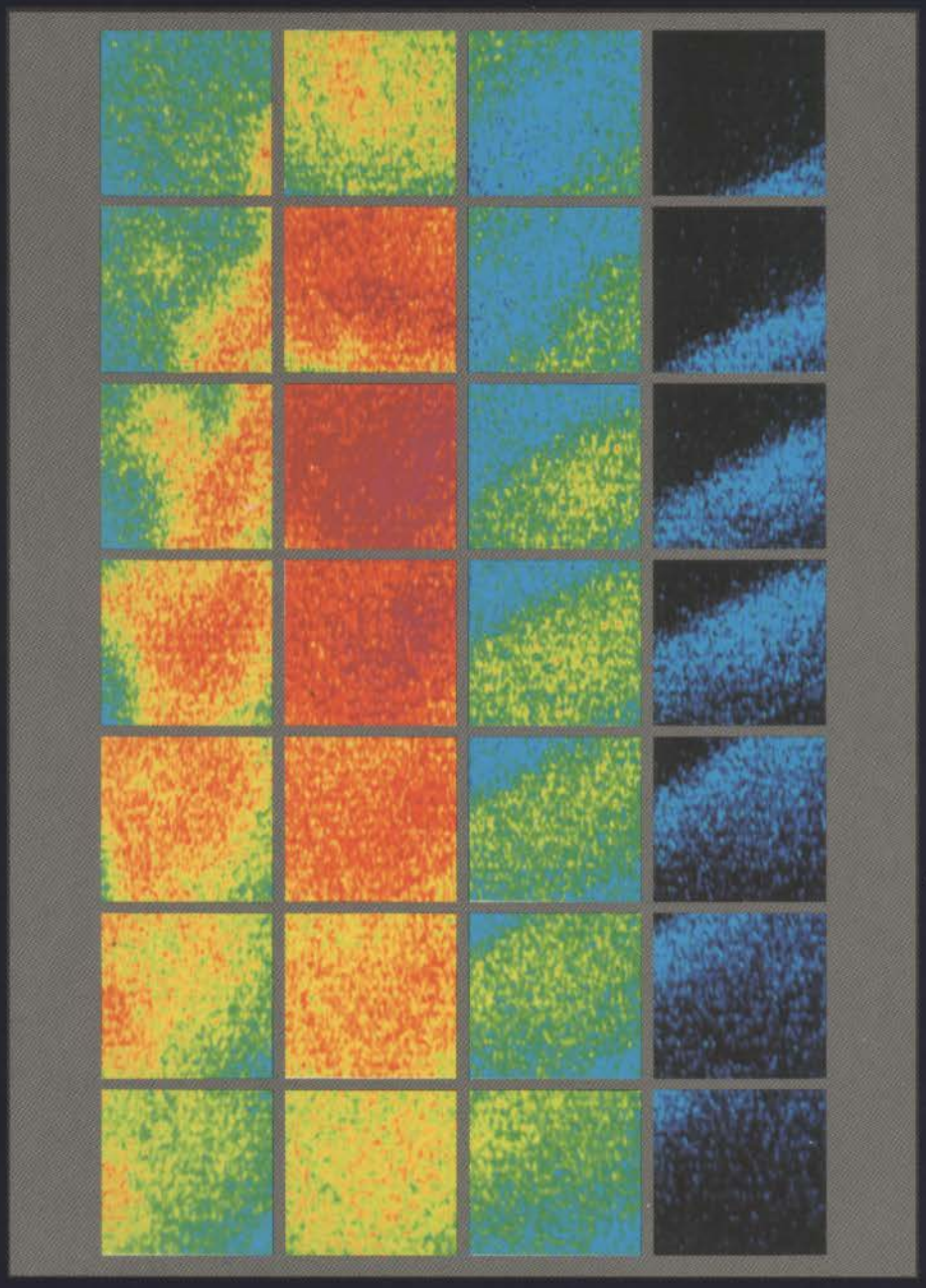

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Ca^{2+} influx modulation of temporal and spatial patterns of inositol trisphosphate-mediated Ca^{2+} liberation in *Xenopus* oocytes

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1. Inositol 1,4,5-trisphosphate ($InsP_3$) functions as a second messenger by liberating intracellular Ca^{2+} and by promoting influx of extracellular Ca^{2+}. We examined the effects of Ca^{2+} influx on the temporal and spatial patterns of intracellular Ca^{2+} liberation in *Xenopus* oocytes by fluorescence imaging of cytosolic free Ca^{2+} together with voltage clamp recording of Ca^{2+}-activated Cl^- currents. Oocytes were injected with a poorly metabolized $InsP_3$ analogue (3-F-$InsP_3$; see Introduction) to induce sustained activation of $InsP_3$ signalling, and Ca^{2+} influx was controlled by applying voltage steps to change the driving force for Ca^{2+} entry.

2. Positive- and negative-going potential steps (corresponding, respectively, to decreases and increases in Ca^{2+} influx) evoked damped oscillatory Cl^- currents, accompanied by cyclical changes in cytosolic free Ca^{2+}. The source of this Ca^{2+} was intracellular, since oscillations persisted when Ca^{2+} entry was suppressed by removing extracellular Ca^{2+} or by polarization close to the Ca^{2+} equilibrium potential.

3. Fluorescence recordings from localized (*ca* 5 μm) spots on the oocyte showed repetitive Ca^{2+} spikes. Their frequency increased at more negative potentials, but they became smaller and superimposed on a sustained 'pedestal' of Ca^{2+}. Spike periods ranged from about 50 s at +20 mV to 4 s at potentials between −60 and −120 mV. Ca^{2+} spike frequency decreased after removing extracellular Ca^{2+}, but the spike amplitude was not reduced and low frequency spikes continued for at least 30 min in the absence of extracellular Ca^{2+}.

4. Membrane current oscillations decayed in amplitude following voltage steps, while locally recorded Ca^{2+} spikes did not. This probably arose because Ca^{2+} release was initially synchronous across the cell, leading to large Ca^{2+}-activated Cl^- currents, but the currents then diminished as different areas of the cell began to release Ca^{2+} asynchronously.

5. Fluorescence imaging revealed that Ca^{2+} liberation in 3-F-$InsP_3$-loaded oocytes occurred as transient localized puffs and as propagating waves. Polarization to more negative potentials increased the frequency of puffs and the number of sites at which they were seen, and enhanced their ability to initiate waves. The frequency and velocity of Ca^{2+} waves increased at more negative potentials. When the potential was returned to more positive levels, repetitive Ca^{2+} spikes at first occurred synchronously across the recording area, but this synchronization was gradually lost and Ca^{2+} waves began at several foci.

6. We conclude that influx of extracellular Ca^{2+} regulates the temporal and spatial patterns of Ca^{2+} liberation from $InsP_3$-sensitive intracellular stores, probably as a result of dual excitatory and inhibitory actions of cytosolic Ca^{2+} on the $InsP_3$ receptor. This process imparts a voltage sensitivity to the $InsP_3$ pathway, which may be important for encoding of signals as frequency of Ca^{2+} spiking and for rapid synchronization of Ca^{2+} signalling over long distances.

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Inositol 1,4,5-trisphosphate ($InsP_3$) serves as an intracellular messenger in many types of excitable and inexcitable cells, mediating responses to diverse neurotransmitters, hormones and growth factors (Berridge, 1993). It is formed in response to agonist binding to cell surface receptors and acts on intracellular stores to liberate sequestered Ca^{2+} ions into the cytosol. This $InsP_3$-mediated Ca^{2+} liberation shows complex properties, including the generation of repetitive Ca^{2+} spikes and propagating Ca^{2+} waves (Meyer & Stryer, 1991; Meyer, 1991; Berridge, 1993), leading to the proposal that these Ca^{2+} signals may operate as a frequency-encoded mechanism whereby analog changes in agonist concentration result in changes in spike frequency (Berridge, 1988, 1993; Meyer, 1991). Moreover, an additional action of $InsP_3$ is to promote the influx of extracellular Ca^{2+} across the plasma membrane (Irvine, 1992; Berridge, 1993). Several results indicate that the actions of Ca^{2+} ions derived from intracellular and extracellular sources are not simply additive, and that the entry of extracellular Ca^{2+} may also act by modulating the release of Ca^{2+} from $InsP_3$-sensitive stores. For example, reductions in extracellular Ca^{2+} concentration reduce the frequency of Ca^{2+} spiking in a variety of cells (Jacob, Merritt, Hallam & Rink, 1988; Kawanishi, Blank, Harootunian, Smith & Tsien, 1989; Kurtz & Penner, 1989; Hajjar & Bonventre, 1991; Zhao, Loessberg, Sachs & Muallem, 1992), and a rapid increase in Ca^{2+} influx evokes transient liberation of intracellular Ca^{2+} (Yao & Parker, 1993). Such interaction was originally proposed to arise because Ca^{2+} influx serves to re-supply the $InsP_3$-sensitive intracellular stores (Kawanishi *et al.* 1989), but an alternative explanation is suggested by recent findings that cytosolic Ca^{2+} can both facilitate and inhibit the opening of the $InsP_3$-gated Ca^{2+} channels that mediate Ca^{2+} release from intracellular stores (Iino, 1990; Parker & Ivorra, 1990*a*; Finch, Turner & Goldin, 1991; Bezprozvanny, Watras & Erlich, 1991; Yao & Parker, 1992).

To study further the effects of Ca^{2+} influx on intracellular Ca^{2+} liberation, we monitored cytosolic Ca^{2+} levels in *Xenopus* oocytes by the use of the fluorescent indicator calcium green-5N, and by voltage clamp recording of Ca^{2+}-activated Cl^- membrane currents (Miledi & Parker, 1984). Oocytes were injected with a poorly metabolized $InsP_3$ analogue, 3-F-$InsP_3$ (3-deoxy-3-fluoro-D-*myo*-inositol 1,4,5-trisphosphate; Kozikowsky, Fauq, Askoy, Seewald & Powis, 1990) to induce a sustained increase in plasma membrane Ca^{2+} permeability, and changes in Ca^{2+} influx were imposed by clamping the cell at different potentials to alter the electrical driving force for Ca^{2+} entry. Using this approach we had previously shown that sudden increases in Ca^{2+} influx evoke a transient release of Ca^{2+} from intracellular stores by activating an $InsP_3$-dependent Ca^{2+}-induced Ca^{2+} release mechanism (Yao & Parker, 1993). However, during those experiments we also noticed that increases and decreases in Ca^{2+} influx evoked oscillations in Ca^{2+}-activated current, which resembled currents seen earlier in agonist-activated oocytes (Parker, Gundersen & Miledi, 1985), and suggested a more complex interaction between Ca^{2+} influx and its release from intracellular stores. The studies described here were prompted by these observations and concern the effects of Ca^{2+} influx on the Ca^{2+} spikes and waves generated by Ca^{2+} liberation from intracellular stores. We show that step increases and decreases in influx promote synchronous intracellular Ca^{2+} liberation across wide areas of the oocyte, and that the steady-state rate of Ca^{2+} influx modulates temporal (spike frequency) and spatial (Ca^{2+} wave velocity) aspects of intracellular Ca^{2+} liberation.

METHODS

Preparation of oocytes and electrophysiological recording

Experiments were done on isolated oocytes of *Xenopus laevis*, obtained from albino frogs to avoid problems during optical recording and stimulation encountered with normally pigmented oocytes. Procedures for preparation of oocytes, intracellular injection and voltage clamp recording were as described previously (Sumikawa, Parker & Miledi, 1989; Parker, 1992; Yao & Parker, 1993). Briefly, oocytes were loaded with 100–200 fmol 3-F-$InsP_3$ (3-deoxy-3-fluoro-D-*myo*-inositol 1,4,5-trisphosphate), to give a final intracellular concentration of roughly 100–200 nM (assuming a cytosolic volume of 1 μl). After allowing about 30 min to allow for diffusion of 3-F-$InsP_3$ throughout the large (1 mm diameter cell), oocytes were placed in the recording chamber and voltage clamped using a conventional two-microelectrode system. Membrane current was monitored by a virtual ground circuit, and records of Ca^{2+}-activated Cl^- current were stored on floppy disk by a digital oscilloscope for subsequent analysis. During recording, oocytes were continually superfused with frog Ringer solution (composition, mM: NaCl, 120; KCl, 2; $CaCl_2$, 1·8; Hepes, 5; at pH about 7·0) at room temperature. Ca^{2+}-free solution was made by omitting $CaCl_2$ and adding 1 mM EGTA together with 5 mM $MgCl_2$.

In many experiments, the electrical driving force for Ca^{2+} entry into the oocyte was varied by clamping the cell to different potentials, so that influx through the $InsP_3$-mediated pathway would be enhanced at more negative potentials (Parker & Miledi, 1987; Yao & Parker, 1993; Girard & Clapham, 1993). However, a possible complication with this approach is that depolarizing steps may activate voltage-gated Ca^{2+} channels in the oocyte (Miledi, 1982; Miledi & Parker, 1984), resulting in a net increase in Ca^{2+} entry despite the reduced driving force. In practice this was not a problem, since native oocytes possess only small numbers of Ca^{2+} channels (Miledi & Parker, 1984; Yao & Parker 1993). Furthermore, these Ca^{2+} channels inactivate within a few hundred milliseconds (Leonard, Nargeot, Snutch & Lester, 1987), so that any influx of Ca^{2+} would terminate rapidly in comparison with the time scale of the present recordings. Fluorescence recordings thus showed a decrease of intracellular free Ca^{2+} following depolarizing steps rather than an immediate transient increase, as would be expected if there were appreciable Ca^{2+} entry through voltage-gated channels (e.g. Figs 5*A* and 9*A*).

Fluorescence monitoring of intracellular free Ca^{2+}

Experiments to monitor intracellular Ca^{2+} were done using calcium green-5N as the fluorescent probe of intracellular Ca^{2+}. Oocytes were injected with about 10 nl of a solution containing both 15 μM 3-F-$InsP_3$ and 5 mM calcium green-5N. This newly

available dye allowed good fluorescence signals to be obtained with levels of dye loading (final intracellular concentration about 50 μM) that caused little change in oscillatory membrane current responses in oocytes loaded with 3-F-InsP_3. In contrast, preliminary experiments using fluo-3 and fura-2 showed that current oscillations were reduced or abolished at intracellular concentrations as low as 20 μM. Measurements of calcium green-5N fluorescence in solutions buffered to various free Ca^{2+} concentrations (Calcium Calibration Buffer Kit C3722, Molecular Probes Inc., Eugene, OR, USA; including 100 mM KCl and 1 mM $MgCl_2$) showed a 30-fold increase on going from zero to saturating free Ca^{2+} levels, with a K_d of about 10–12 μM. This latter value is in good agreement with that most recently reported by the manufacturer (Bioprobes 17; Molecular Probes Inc.), but is almost three times higher than originally reported (3·3 μM; Molecular Probes Handbook 1992–1994). The roughly 30-fold lower affinity of calcium green-5N as compared to fluo-3 and fura-2 may thus account for its relative lack of interference with Ca^{2+}-activated membrane current responses. Because calcium green-5N does not show shifts in excitation or emission spectra on binding Ca^{2+}, it was not possible to use ratio measurements to calibrate fluorescence signals in terms of absolute free Ca^{2+} concentrations. Fluorescence measurements are therefore expressed simply as fractional changes in fluorescence (F) above the resting baseline ($\Delta F/F$). Changes in fluorescence are, however, expected to give a nearly linear measure of relative changes in free Ca^{2+} concentration, since the resting free Ca^{2+} level in the oocyte (< 100 nM; Y. Yao & I. Parker, unpublished data) is very low in comparison with the K_d of the dye, and peak values of $\Delta F/F$ during Ca^{2+} spikes were about 1, which is small compared to the 15-fold increase in fluorescence expected from increasing free Ca^{2+} from 100 nM to a saturating concentration.

Two systems were used for optical recording. Initial experiments were done using a conventional epifluorescence microscope fitted with an intensified CCD camera, as previously described (Parker & Yao, 1991; Yao & Parker, 1993). Subsequently, this was replaced by a 'real time' laser scanned confocal unit (Odyssey; Noran Instruments, Middleton, WI, USA) interfaced to an Olympus IMT2 inverted microscope fitted with a ×40 oil immersion objective lens (Nikon; NA = 1·3). Excitation was by the 488 nm line of an argon laser, and emission was monitored at wavelengths > 515 nM. With a confocal slit of 25 μm and using the ×40 objective lens, fluorescence was monitored from a confocal 'slice' about 2–3 μm thick, focused at a depth of 5–10 μm into the oocyte. Similar results were obtained with both the conventional and confocal systems, although the confocal microscope provided records with better resolution. However, much of the improvement with the confocal system was probably due to factors such as the higher excitation energy available and the increased aperture of the objective lens used, rather than to the confocal effect *per se*. Because of the turbidity of the oocyte cytoplasm, fluorescence recordings are in any case limited to a depth of about 20 μm inward from the cell membrane. Video signals from the camera and confocal microscope were recorded on VHS tape for subsequent processing and analysis using Image 1 and MetaMorph packages (Universal Imaging Corp., West Chester, PN, USA).

Materials

DM-Nitrophen and 3-F-InsP_3 were obtained from Calbiochem (La Jolla, CA, USA), and calcium green-5N was from Molecular Probes Inc. All other reagents were from Sigma.

RESULTS

Oscillatory Ca^{2+}-activated currents in oocytes loaded with 3-F-InsP_3

Oocytes were loaded about 30 min before recording with 3-F-InsP_3, so as to induce a long-lasting (several hours) activation of intracellular Ca^{2+} liberation and increase of plasma membrane permeability to Ca^{2+} (Yao & Parker, 1993). Voltage clamp steps were then imposed to change the membrane potential, and hence the electrochemical driving force for Ca^{2+} entry. In 3-F-InsP_3-loaded oocytes, voltage steps in both positive and negative directions (corresponding, respectively, to decreases and increases in driving force for Ca^{2+} entry) evoked series of regular, decaying oscillations in membrane current (Fig. 1*A*), which resembled responses to voltage steps in oocytes where InsP_3 signalling was activated by agonists (Parker *et al.* 1985).

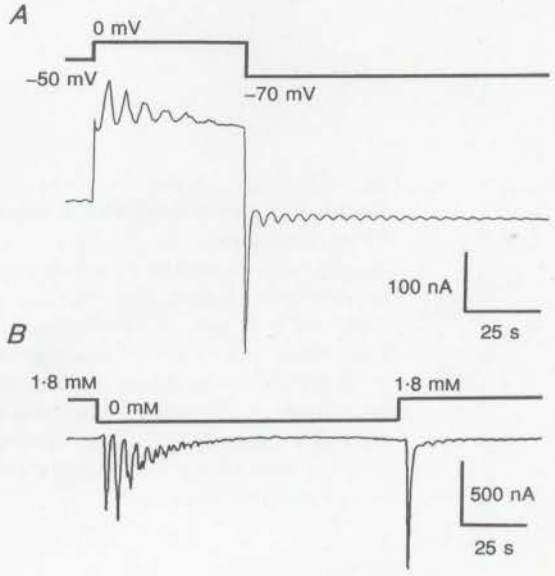

Figure 1. Damped oscillations in membrane current evoked by increases and decreases in Ca^{2+} influx
A, membrane currents in an oocyte bathed in normal Ringer solution, in which Ca^{2+} influx was altered by changing the clamp potential from −50 to 0 mV and finally returning to −70 mV. In this, and other figures, outward membrane currents correspond to upward deflections. Note that Ca^{2+}-activated Cl^- currents are outwardly directed at 0 mV, since this voltage is positive to the Cl^- equilibrium potential (about −25 mV), whereas at −70 mV the currents are inward. *B*, current oscillations evoked in an oocyte clamped at a fixed potential of −60 mV by changing from normal (1·8 mM Ca^{2+}) to Ca^{2+}-free Ringer solution and then back to normal solution.

Control (non-injected) oocytes failed to show oscillations, whereas all 3-F-InsP_3-loaded oocytes examined ($n = 35$) showed oscillations following positive-going steps (to 0 or +20 mV from holding potentials between −40 and −140 mV). Negative-going steps evoked transient inward currents (T_{in} current in Parker *et al.* 1985) in all thirty-five oocytes, but only seventeen of these showed subsequent oscillations like those in Fig. 1*A*. In those cases where oscillations were seen on hyperpolarization, the frequency was higher than that elicited following depolarizing steps (Fig. 1*A*; and see later).

Two experiments indicated that the current oscillations arose as a result of voltage-dependent changes in Ca^{2+} influx rather than from potential changes *per se*. Firstly, similar oscillations were evoked when oocytes were clamped at a fixed membrane potential (−60 mV), and step changes in Ca^{2+} influx were imposed by rapidly removing and replacing free Ca^{2+} ions in the bathing solution (Fig. 1*B*). As was the case with voltage steps, reduction of Ca^{2+} influx consistently evoked large oscillations (14 oocytes) but although increases in influx consistently evoked transient spikes of current, subsequent oscillations were apparent in only seven oocytes. Secondly, voltage steps like those in Fig. 1*A* failed to evoke appreciable oscillatory currents in oocytes ($n = 11$) bathed in Ca^{2+}-free solution.

Xenopus oocytes possess large numbers of Ca^{2+}-activated Cl^- membrane channels, which mediate membrane current responses to many stimuli that elevate cytosolic free Ca^{2+} levels (Miledi & Parker, 1984). To confirm that the oscillatory currents also arose through opening of these channels in response to cyclical changes in cytosolic Ca^{2+}, we determined the ionic basis of the currents by measuring their reversal potential. After holding at −100 mV, the membrane potential was stepped to various more positive levels, resulting in current oscillations that reversed direction at about −25 mV (Fig. 2*A*), close to the Cl^- equilibrium potential in the oocyte (Kusano, Miledi & Stinnakre, 1982). Furthermore, fluorescence measurements of cyclical changes in intracellular Ca^{2+} accompanying the current oscillations (see later) strongly support the idea that the current oscillations arose through opening of Ca^{2+}-activated Cl^- channels.

Current oscillations arise through cyclical liberation of Ca^{2+} from intracellular stores

In principle, the changes in cytosolic Ca^{2+} underlying the oscillatory currents could arise because of cyclical liberation of Ca^{2+} from intracellular stores, or because of cyclical changes in Ca^{2+} permeability of the plasma membrane. To discriminate between these possibilities we tested whether current oscillations could be evoked under various conditions where Ca^{2+} influx was largely suppressed. The results provide strong support for an intracellular source of Ca^{2+}. (i) Large oscillatory currents were evoked when extracellular free Ca^{2+} was reduced to very low levels in a solution containing 1 mM EGTA and no added Ca^{2+} (Fig. 1*B*). (ii) Polarization of oocytes to very positive potentials approaching the Ca^{2+} equilibrium potential (*ca* +150 mV assuming 60 nM intracellular free Ca^{2+}) still evoked current oscillations (Fig. 2*B*), even though the

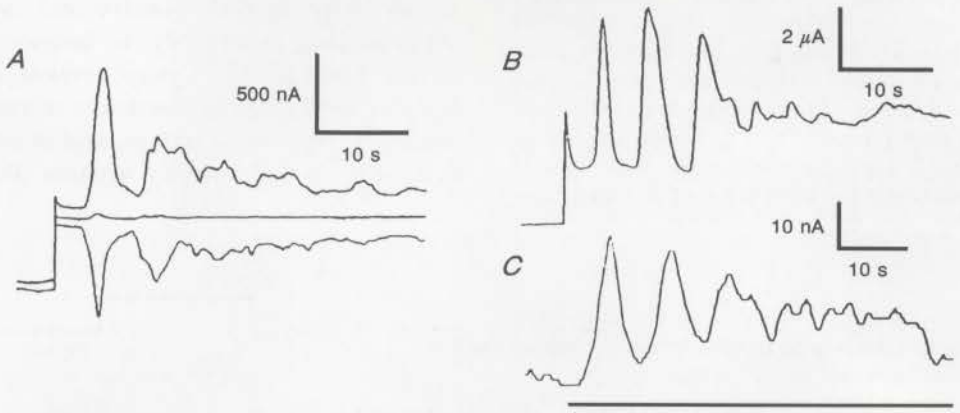

Figure 2. Current oscillations arise because cyclical liberation of intracellular Ca^{2+} activates a Cl^- conductance

A, current oscillations reversed direction close to the Cl^- equilibrium potential (about −25 mV). Superimposed traces show currents evoked by stepping from −100 mV to (from top to bottom) −14, −24 and −34 mV. *B*, oscillations were still evoked when Ca^{2+} influx was diminished by strong polarization near the Ca^{2+} equilibrium potential. Trace shows currents evoked by a step from −60 to +100 mV. *C*, oscillations evoked in an oocyte bathed in Ca^{2+}-free solution by photorelease of intracellular Ca^{2+} from a caged precursor. The oocyte was loaded with 3-F-InsP_3 together with DM-nitrophen ('caged Ca^{2+}'; final intracellular concentration about 1 mM, injected as a 30 mM solution together with 1·5 mM Ca^{2+}) and exposed to UV light (100 W Hg arc lamp with UG5 and ND 1·2 filters) for the duration of the bar.

electrochemical gradient for Ca^{2+} influx would be greatly diminished. (iii) The oscillatory currents could be mimicked by using photorelease from a caged precursor (DM-Nitrophen; Kaplan & Ellis-Davies, 1988) to elevate cytosolic Ca^{2+} levels, even when Ca^{2+} influx was abolished by bathing the oocyte in Ca^{2+}-free solution (Fig. 2*C*).

Fluorescence monitoring of intracellular Ca^{2+} spikes and waves

Although recordings of Ca^{2+}-activated Cl^- current provided evidence that changes in Ca^{2+} influx evoked oscillatory liberation of intracellular Ca^{2+}, further interpretation was difficult for two reasons. One is that the current reflects the summation of Ca^{2+} signals throughout the whole oocyte, whereas Ca^{2+} release can occur independently and asynchronously at many different sites within the cell (Parker & Ivorra, 1990*b*; Lechleiter, Girard, Peralta & Clapham, 1991; Parker & Yao, 1991; Lechleiter & Clapham, 1992). Also, the amplitude of the Cl^- current appears to reflect the rate of rise of cytosolic Ca^{2+}, rather than its absolute level (Parker & Yao, 1992; Parker & Ivorra, 1993). To circumvent these limitations we used a fluorescent Ca^{2+} probe, calcium green-5N, to image the underlying patterns of Ca^{2+} liberation.

Imaging of oocytes loaded with calcium green-5N together with 3-F-InsP_3 revealed complex patterns of subcellular Ca^{2+} release, including localized 'puffs' (Parker & Yao, 1991) and propagating circular, spiral and planar waves (Lechleiter *et al.* 1991; Parker & Yao, 1991; Lechleiter & Clapham, 1992; DeLisle & Welsh, 1992). Recordings of fluorescence from localized (about 5 μm) spots or squares thus showed series of spikes due to repetitive release of Ca^{2+}.

Ca^{2+} spike frequency varies with voltage and extracellular Ca^{2+} concentration

Figure 3*A* shows representative records of Ca^{2+} spikes recorded in one oocyte in response to changes in clamp potential. When the oocyte was held at 0 mV, spikes occurred with a period of about 26 s as successive Ca^{2+} waves swept across the measuring site, but at −20 mV the interspike interval shortened to about 10·8 s. Further polarization to more negative potentials caused a progressive shortening of spike period to 7·3 and 5·4 s at potentials of −40 and −60 mV. However, the spikes became superimposed on a steady 'pedestal' of Ca^{2+}, and their peak-to-trough excursions became smaller. Indeed, in the oocyte illustrated, spikes could no longer be detected at potentials of −80 and −100 mV and, instead, there was only a sustained elevation of intracellular Ca^{2+} during the polarizing pulses.

All twenty-five oocytes examined showed an increase in Ca^{2+} spike frequency with polarization to more negative potentials, but their sensitivity varied considerably. In some cases only a slight increase in frequency was observed on stepping from +20 to −120 mV, whereas in other cases the spike frequency was already maximal at about −30 mV and further polarization resulted only in a sustained

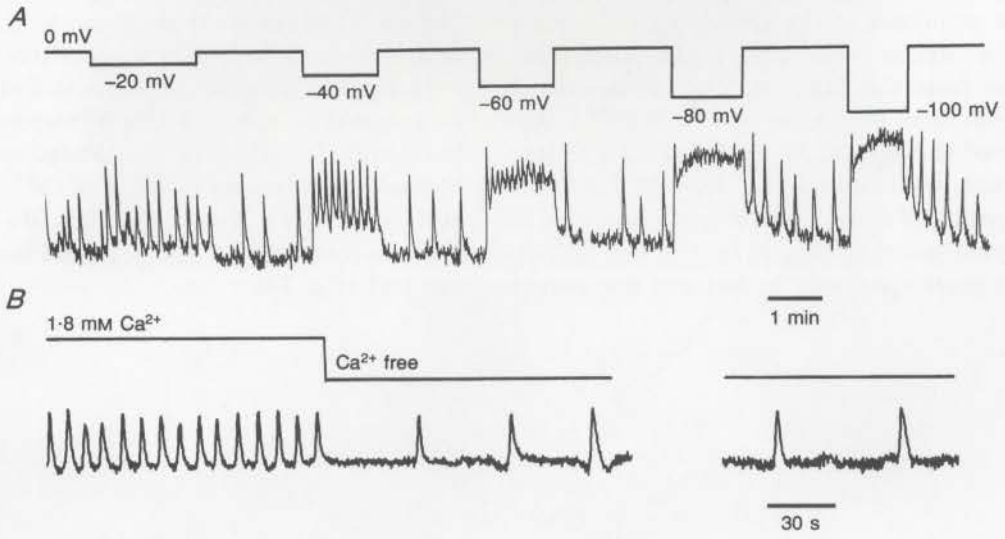

Figure 3. Frequency of intracellular Ca^{2+} spikes varies with membrane potential (*A*) and extracellular Ca^{2+} concentration (*B*)

In both frames, the lower traces show Ca^{2+}-dependent fluorescence signals monitored from localized (about 5 μm) spots on the oocyte surface. *A*, the oocyte was bathed in Ringer solution including 1·8 mM Ca^{2+}, and the clamp potential was stepped to the various voltages indicated in the upper trace from a holding potential of 0 mV. *B*, record from a different oocyte, clamped at a fixed potential of −20 mV, showing decreased spike frequency following removal of extracellular Ca^{2+}. The two spikes shown on the right were recorded after continuous superfusion with Ca^{2+}-free solution for 20 min.

increase in Ca^{2+} on which spikes could no longer be discerned. Figure 4 shows measurements of spike frequency in five oocytes as a function of voltage. The graph provides an indication of the range of frequencies observed and of the variation between oocytes in steepness of the voltage dependence. Oocytes that were loaded with greater amounts of 3-F-InsP_3 showed a greater increase in spike frequency with hyperpolarization, probably because of an increased permeability of the plasma membrane to Ca^{2+}. Also, the sensitivity of a given oocyte to voltage changes gradually reduced over several hours, probably because of slow metabolism of 3-F-InsP_3.

The effects of voltage on spike frequency arose from changes in Ca^{2+} influx rather than from the voltage change *per se*, since a slowing of spike frequency was observed when Ca^{2+} was removed from the bathing solution with the oocyte clamped at a fixed potential (Fig. 3*B*). The slowing in frequency occurred within one cycle of the spiking after removal of extracellular Ca^{2+}, but spikes then continued at roughly constant frequency and undiminished amplitude for at least 30 min while the cell was bathed in Ca^{2+}-free solution.

Damping of current oscillations reflects loss of synchronization of Ca^{2+} spikes

The membrane current oscillations initiated following positive- and negative-going voltage steps invariably decayed in amplitude, so that they were very small or undetectable after about six cycles (see, for example, Figs 1*A* and 2). We could envisage two explanations for this. One is that the amplitude of the underlying Ca^{2+} spikes declined with a similar time course. An alternative possibility arose from the fact that the voltage clamp monitored current from the entire oocyte. If Ca^{2+} spikes were synchronized throughout the cell following a voltage step, large currents would initially be expected due to the synchronous opening of many Cl^- channels. However, as spikes at different locations began to fall out of step synchronization would gradually be lost and the current oscillations would decline in amplitude, even though Ca^{2+} spikes at a given location remained unchanged in size.

Figure 5 shows simultaneous records of membrane current and locally recorded Ca^{2+} signals, demonstrating that the damping of the current oscillations was not accompanied by corresponding diminution in amplitude of Ca^{2+} spikes. Following both positive- and negative-going steps the current oscillations decayed within about 20 s, even though local Ca^{2+} spikes declined little, or even grew in amplitude during this time.

Ca^{2+} influx increases frequency of Ca^{2+} puffs and promotes wave propagation

When clamped at +20 mV to minimize Ca^{2+} influx, some oocytes did not show propagating Ca^{2+} waves but, instead, Ca^{2+} release occurred as transient 'puffs' that remained localized to within a few micrometres of their origin. This pattern of release probably resulted because these oocytes were loaded with relatively little 3-F-InsP_3, since progressively increasing levels of InsP_3 lead first to the generation of localized puffs and then to propagating waves (Parker & Yao, 1991). The effects of changes in membrane potential on Ca^{2+} puffs are illustrated by the Ca^{2+} images shown in Fig. 6, together with measurements of fluorescence recorded from two localized spots within the image field (Fig. 7). These results are representative of findings in a total of nine oocytes which showed puffs when clamped at +20 mV.

When the oocyte was clamped at +20 mV, Ca^{2+} release occurred only as localized puffs, two of which are shown in Fig. 6*A*. Fluorescence measurements in Fig. 7 were made from two sites which showed repetitive puffs. These sites were about 15 μm apart, so that a puff at one site was seen as a transient spike in the corresponding fluorescence trace while the other trace registered little or no increase because of the limited diffusion of Ca^{2+}. The frequency of puffs was low at +20 mV. During a 70 s recording a single puff was recorded at one site (*a*), and the other (*b*) showed two puffs (Fig. 7*A*).

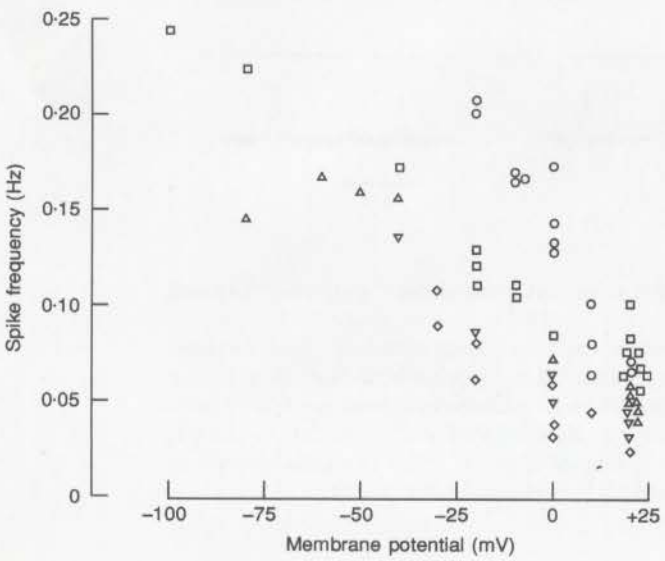

Figure 4. Variation in Ca^{2+} spike frequency as a function of membrane potential
Data are from 5 oocytes (denoted by different symbols), all bathed in normal Ringer solution containing 1·8 mM Ca^{2+}.

Polarization to more negative potentials caused two main changes in the pattern of Ca^{2+} release. Firstly, the frequency of puffs increased, and Ca^{2+} release was seen at additional sites where activity was not evident at +20 mV. For example, when clamped at −40 mV, site *b* in Fig. 7*B* showed five localized puffs within about 1 min (identified as spikes occurring at site *b* but not accompanied by spikes at site *a*). Also, the first frames of Fig. 6*B* show Ca^{2+} release beginning from three discrete sites within 3 s of stepping the potential to −60 mV. The second effect of polarization was that some, but not all, puffs failed to remain localized, but instead acted as foci for initiation of Ca^{2+} waves. This is illustrated in Fig. 6*B*, where Ca^{2+} signals from puff sites continued to enlarge to produce a propagating Ca^{2+} wave. The Ca^{2+} level then declined before subsequent Ca^{2+} waves began again from a site near the centre of the field (second row, Fig. 6*B*), leading to repetitive wave activity at high frequency (Fig. 7*C*).

Effects of Ca^{2+} influx on Ca^{2+} waves

As mentioned earlier, many oocytes showed Ca^{2+} waves when clamped at +20 mV. However, analysis of the effects of changes in Ca^{2+} influx was complicated because the wave patterns were usually complex and chaotic, consisting of planar, circular and spiral waves originating from multiple foci that collided with and annihilated each other (cf. Lechleiter *et al.* 1991; Lechleiter & Clapham, 1992). To simplify interpretation we selected records in which a regular series of nearly planar waves swept across the field of view. Figure 8 shows images of such Ca^{2+} waves, and measurements derived from the raw image data are presented in Fig. 9. The velocity of wave propagation was estimated by recording fluorescence at a series of spots spaced 21 μm apart along a line roughly perpendicular to the wave front (positions indicated in first frame of Fig. 8*A*). The slopes of lines joining the times of peak Ca^{2+} at each location then gave a measure of wave velocity (Fig. 9*B*).

Figure 8*A* shows a series of images at a holding potential of +20 mV, illustrating the propagation of a wave across the imaging field at a velocity of about 18 μm s^{-1}. The potential was then stepped to −80 mV, and Fig. 8*B* shows images of a subsequent wave a few seconds after the step. While the oocyte was held at −80 mV, the overall level of Ca^{2+} increased over several seconds, waves began to sweep over the recording field with increased frequency, and their velocity increased to over 60 μm s^{-1} (Figs 8*B* and 9*B*). When the potential was returned to +20 mV the planar wave pattern was lost for several tens of seconds, although cyclical fluctuations of Ca^{2+} continued (Fig. 9*A*). The peak Ca^{2+} levels during this time were higher than at any time during polarization to −80 mV, despite the reduced driving force for Ca^{2+} influx. Furthermore, during the first few cycles after returning to +20 mV, the Ca^{2+} level rose and fell nearly uniformly throughout the entire recording field (Fig. 8*C*) but over the next few cycles this synchronization was gradually lost and Ca^{2+} waves began at discrete foci (Fig. 8*D*). Eventually these foci disappeared, and after about 100 s the Ca^{2+} waves reverted to a pattern like that seen before the polarizing step.

The triggering of spatially synchronized spikes of Ca^{2+} release following depolarizing steps was apparent only when intracellular Ca^{2+} had first been raised to a high level, as in Fig. 9*A*. When the initial holding potential was made less negative, so as to produce a smaller elevation of the baseline Ca^{2+} level, depolarizing steps resulted a slowing of wave frequency, but did not synchronize Ca^{2+} release from different areas.

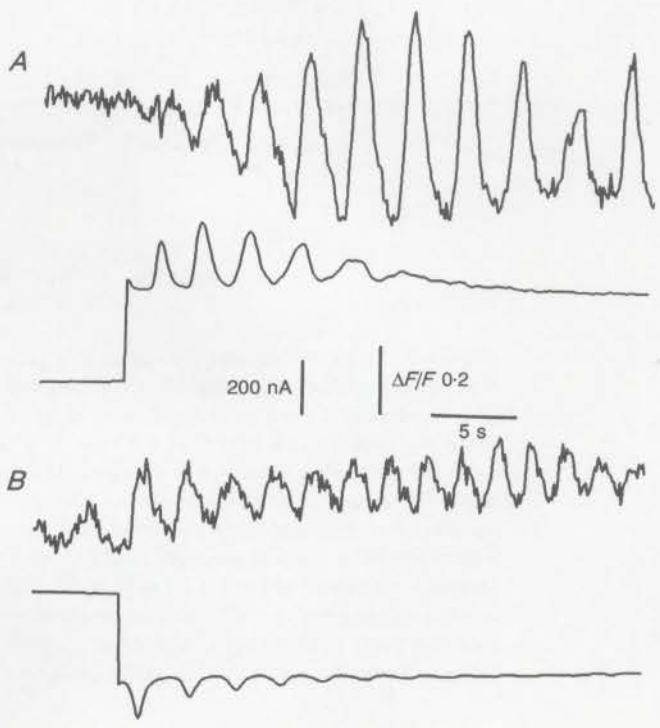

Figure 5. Whole-cell membrane current oscillations are damped, whereas locally recorded Ca^{2+} spikes persist with little diminution in amplitude
In each frame, the upper trace shows local Ca^{2+}-dependent fluorescence monitored from a 5 μm spot on the oocyte, and the lower trace shows clamp current. *A*, the clamp potential was stepped from −140 to +20 mV. *B*, step from +20 to −100 mV. Both records are from the same oocyte.

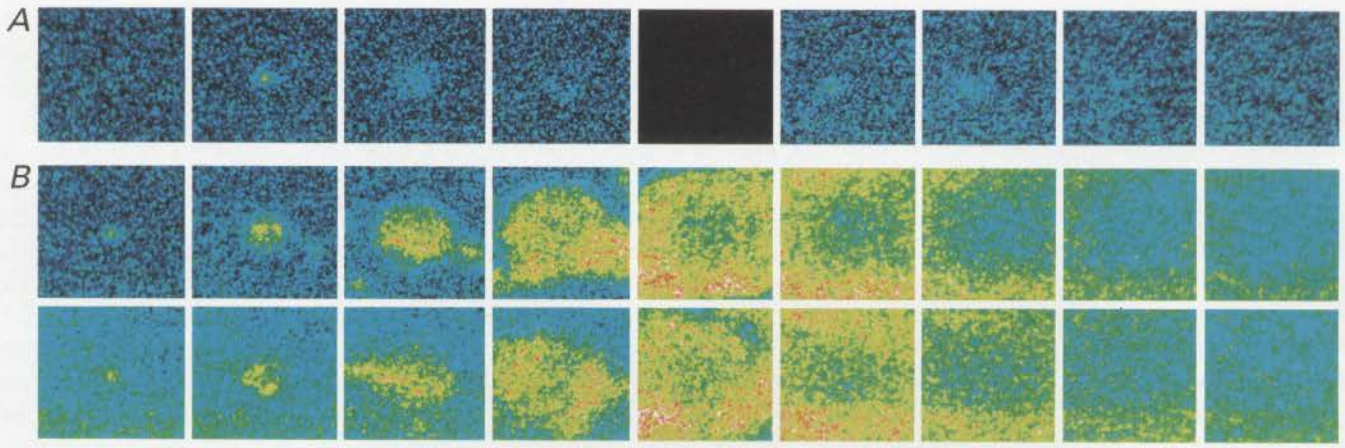

Figure 6. Effects of voltage on localized intracellular Ca^{2+} release
All images show Ca^{2+} distribution within a fixed area of the oocyte (80 × 80 μm), and sequential frames within each section of the figure were obtained at 1 s intervals. The resting fluorescence at a potential of +20 mV was subtracted from each image. Dark blue corresponds to no increase in Ca^{2+} and increasingly 'warm' colours to progressively higher Ca^{2+} levels. Each image shows a single video frame, low-pass filtered by a 3 × 3 pixel kernel. Data were collected by a real-time confocal microscope, as described in Methods. *A*, recording at a clamp potential of +20 mV, showing a transient, localized 'puff' of Ca^{2+} during frame 2, and a second puff at a different site 10 s later (blank square indicates a 6 s interruption in the recording sequence). *B*, repetitive Ca^{2+} waves were evoked from the first puff site in *A* after stepping to −60 mV. The two rows show a continuous sequence of images beginning immediately after stepping from +20 to −60 mV.

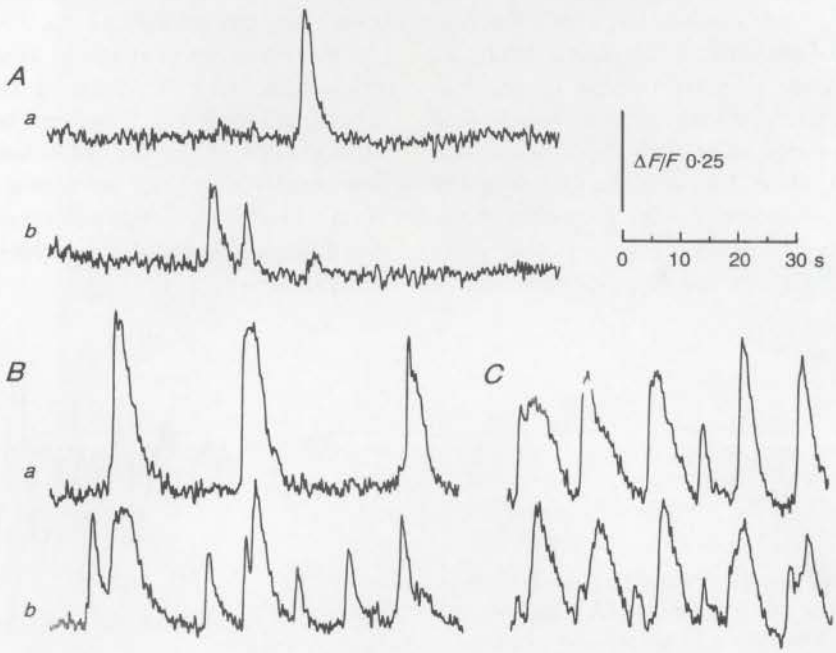

Figure 7. Polarization to more negative potentials increases the frequency of local Ca^{2+} release and promotes the propagation of Ca^{2+} waves
The two traces in each section are recordings of Ca^{2+}-dependent fluorescence made from two localized recording spots (*a* and *b*) within the recording field of Fig. 6. Ca^{2+} release occurring as localized 'puffs' at one of these sites appeared as a spike in the corresponding trace, while the other trace showed only a small signal due to diffusional spread of Ca^{2+}. Ca^{2+} waves, on the other hand, gave rise to nearly simultaneous spikes of large amplitude at both recording locations. *A*, during a 90 s recording period at +20 mV, site *a* showed a single localized 'puff' of Ca^{2+}, and site *b* showed two puffs. *B*, the clamp potential was stepped from +20 to −40 mV shortly before the beginning of the record, resulting in an increased frequency of Ca^{2+} puffs, some of which gave rise to propagating Ca^{2+} waves. Site *b* showed 5 localized puffs within 1 min, and 3 Ca^{2+} waves were recorded at both sites. *C*, clamping at −60 mV caused a further increase in Ca^{2+} spike frequency, which arose largely from repetitive Ca^{2+} waves.

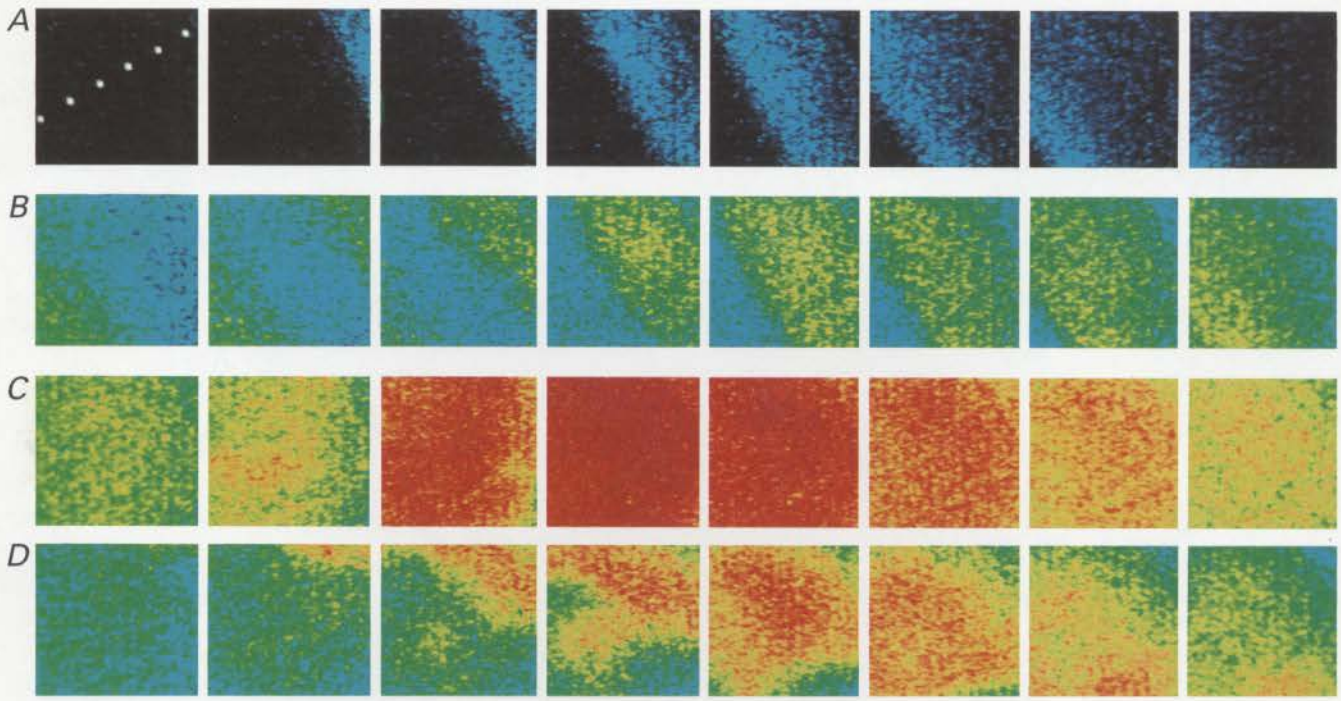

Figure 8. Effects of membrane potential changes on Ca^{2+} waves
Images were obtained by conventional epifluorescence microscopy (see Methods) in an oocyte where planar Ca^{2+} waves swept regularly across the recording field. Sequential images in *A* were obtained at 1·3 s intervals, whereas those in *B–D* were at 0·65 s intervals. The size of the image frame is 100 × 100 μm. Each image shows Ca^{2+}-dependent fluorescence after subtraction of resting fluorescence at a potential of +20 mV, and is an average of 4 sequential video frames. Increasingly 'warm' colours denote increasing Ca^{2+} levels. Spots marked in the first frame of *A* indicate the location of measuring sites used to derive Fig. 9. *A*, spontaneous Ca^{2+} wave recorded while the oocyte was clamped at +20 mV. *B*, the membrane potential was stepped to −80 mV a few seconds before the start of the image sequence, resulting in an overall increase of Ca^{2+} level and Ca^{2+} waves traversing the field at higher frequency and faster velocity. *C*, sequence of images beginning about 10 s after returning the potential to +20 mV, showing synchronized Ca^{2+} release across the recording field. *D*, Ca^{2+} release began from multiple foci about 35 s after returning to +20 mV.

Figure 9*C* shows measurements of Ca^{2+} wave velocity and frequency obtained while the oocyte was clamped at various potentials. An increase in frequency could be seen following a step from +20 to 0 mV, whereas an increase in propagation velocity was apparent only at the most negative potential examined (−80 mV). Voltage-dependent changes in propagation velocity of planar Ca^{2+} waves were seen in five out of six further oocytes examined, some of which showed a greater sensitivity than did the oocyte in Fig. 9. For example, negative-going steps of 30 or 40 mV (from a holding potential of +20 or 0 mV) caused a mean increase in velocity of 60 % in three oocytes.

DISCUSSION

Mechanism by which Ca^{2+} influx modulates intracellular Ca^{2+} signals

Our results demonstrate that changes in Ca^{2+} influx across the plasma membrane alter the frequency and amplitude of intracellular Ca^{2+} spikes as well as the spatial propagation of Ca^{2+} waves in *Xenopus* oocytes. After submission of this paper, Girard & Clapham (1993) also reported similar results showing increases in frequency and velocity of Ca^{2+} waves as a result of increased Ca^{2+} influx in *Xenopus* oocytes. It is generally accepted that Ca^{2+} spikes and waves are generated by liberation of Ca^{2+} from intracellular stores (Meyer & Stryer, 1991; Berridge, 1993) and, in agreement with this, Ca^{2+} oscillations persisted even when Ca^{2+} influx was virtually abolished by various means (Figs 1*B*, 2*B* and *C*, and 3*B*). Thus, it seems that cyclical changes in Ca^{2+} influx are not required to produce Ca^{2+} spikes but, instead, the incoming Ca^{2+} ions modulate the liberation of Ca^{2+} from intracellular stores. However, we cannot exclude the possibility that modulation of the Ca^{2+} influx pathway itself by cytosolic free Ca^{2+} may also contribute to the oscillations when influx is present. Such a mechanism was proposed by Loessberg, Zhao & Muallem, (1991) to account for oscillations in Ca^{2+} entry into AR42J cells at a frequency that matched that of intracellular Ca^{2+} spikes, but they also considered the possibility that

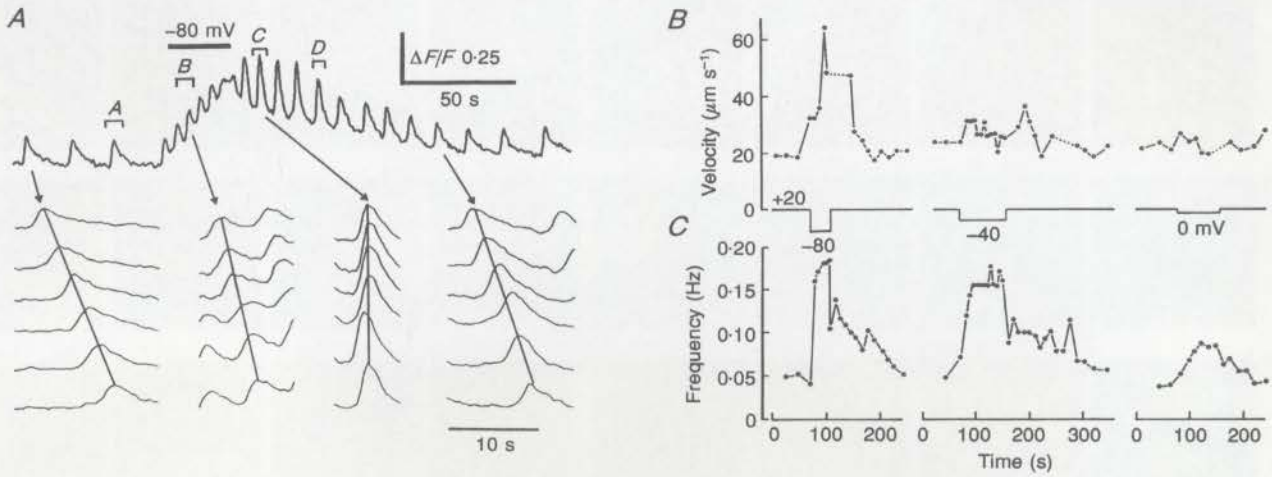

Figure 9. Changes in Ca^{2+} wave frequency and velocity with potential
A, trace shows Ca^{2+} spikes at a single recording location (third spot from right in Fig. 8*A*). The membrane potential was held at +20 mV and stepped to −80 mV for the time indicated by the bar. Measurements are from the same experiment as Fig. 8 and bars above the trace indicate the times corresponding to the image sequences in that figure. *B*, traces within each frame are simultaneous measurements of Ca^{2+} fluorescence recorded from spots at 21 μm increments along a line perpendicular to the Ca^{2+} wave-front (see first frame in Fig. 8*A*). The records illustrated correspond to the spikes indicated in *A*, and slopes of the lines joining peaks of Ca^{2+} spikes at successive locations give a measure of the velocity of propagation. Note that the line is vertical in frame 3, indicating that Ca^{2+} levels rose synchronously at all locations. *C*, changes in wave conduction velocity and spike frequency during polarization to the various potentials indicated from a holding potential of +20 mV. The measurements are part of a continuous recording, but were interrupted for a few minutes between trials at each potential. Dotted lines in the upper graph indicate periods when the velocity could not be determined because waves were not apparent or were not normal to the measuring line.

changes in membrane potential resulting from the opening of Ca^{2+}-sensitive ion channels might regulate Ca^{2+} entry. Our results are consistent with the latter explanation and emphasize the difficulty in interpreting Ca^{2+} spikes recorded from cells where the membrane potential is not monitored or, better, clamped to known values.

How, then, does Ca^{2+} influx modulate release of Ca^{2+} from intracellular stores? To begin, it is clear that this release requires the presence of $InsP_3$, and hence presumably occurs from $InsP_3$-sensitive stores. Thus, when $InsP_3$ is present in the oocyte, Ca^{2+} entry evokes Ca^{2+} oscillations, whereas elevations of cytosolic Ca^{2+} fail to evoke oscillations in the absence of $InsP_3$ (Miledi & Parker, 1984; Yao & Parker, 1992, 1993). Furthermore, there is little evidence in *Xenopus* oocytes for calcium-induced calcium release mediated by ryanodine- and caffeine-sensitive Ca^{2+} channels (Parker & Ivorra, 1991; DeLisle & Welsh, 1992).

One way in which Ca^{2+} influx might affect the frequency of Ca^{2+} spikes is via the rate of re-supply of Ca^{2+} ions to the $InsP_3$-sensitive stores (Kawanishi *et al.* 1989). A key observation in support of this idea was that after removing extracellular Ca^{2+} from hepatocytes only a few Ca^{2+} spikes were produced and these ceased after a short time (Kawanishi *et al.* 1989). In the much larger oocyte cell the situation is different, as Ca^{2+} spikes persist with undiminished amplitude long after removing external Ca^{2+} (Fig. 3*B*), which suggested that the slowing of spike frequency is not due to depletion of store contents. Furthermore, other experiments indicate that Ca^{2+} influx affects liberation from intracellular stores by a mechanism other than store replenishment. Firstly, hyperpolarizing pulses trigger Ca^{2+} release from $InsP_3$-sensitive stores with a latency as short as 100 ms (Yao & Parker, 1993). This appears insufficient time for the stores to fill to a point where they explosively release their contents, especially since very little pacemaker ramp of Ca^{2+} precedes the spike, as would be expected if Ca^{2+} ions passed through the cytosol in transit between the plasma membrane and the stores. Secondly, it is difficult to see how Ca^{2+} spikes could be initiated by *decreases* in Ca^{2+} influx (Fig. 8*C*), if the influx served only to replenish the stores.

A more attractive explanation arises from recent findings that cytosolic Ca^{2+} ions act on intracellular $InsP_3$-gated Ca^{2+} release channels, causing rapid facilitation at low concentrations and slower inhibition at higher concentrations (Iino, 1990; Parker & Ivorra, 1990*a*; Bezprozvanny *et al.* 1991; Finch *et al.* 1991; Yao & Parker, 1992). This dual positive and negative feedback has been proposed to underlie the generation of Ca^{2+} spikes and waves in the presence of steady levels of $InsP_3$ (Parker & Yao, 1991; DeLisle & Welsh, 1992; Lechleiter & Clapham, 1992; Yao & Parker, 1992), and it is probable that Ca^{2+}

liberation will be influenced by exogenous Ca^{2+} entering across the plasma membrane as well as by Ca^{2+} released into the cytoplasm from the stores. Thus, stimulation of intracellular Ca^{2+} liberation by Ca^{2+} influx is expected because the increase in cytosolic free Ca^{2+} will promote regenerative opening of Ca^{2+} release channels (Yao & Parker, 1992), and may account for the increase in number and frequency of wave initiation sites as well as the speeding of Ca^{2+} wave velocity. Furthermore, Ca^{2+} spikes may be triggered by decreases in Ca^{2+} influx, because the resulting fall in cytosolic free Ca^{2+} relieves the $InsP_3$-gated channel from Ca^{2+}-induced inhibition. Other proposed models for Ca^{2+} spiking involve Ca^{2+} stimulation of phospholipase C to increase $InsP_3$ formation (Harootunian, Kao, Paranjape & Tsien, 1991), or Ca^{2+}-induced release from $InsP_3$-insensitive stores (Goldbeter *et al.* 1990): but it is difficult to see how either scheme could explain stimulation of Ca^{2+} liberation by decreases in cytosolic free Ca^{2+}.

Synchronized Ca^{2+} liberation leads to large Ca^{2+}-activated membrane currents

The irregular Ca^{2+}-activated Cl^- currents evoked in oocytes by $InsP_3$ may be explained by the complex patterns of Ca^{2+} waves randomly activating different parts of the plasma membrane (Lechleiter *et al.* 1991; Berridge, 1993). Because the waves cover only a small proportion of the oocyte surface at a given time the whole-cell current is small. However, synchronous liberation of Ca^{2+} across wide areas of the cell will evoke larger currents. This mechanism appears to underlie the large current spikes evoked by synchronous Ca^{2+} liberation induced following positive- and negative-going voltage steps, and the subsequent damping of current oscillations may be explained by a loss of synchronization between different cellular areas (Fig. 5).

Mechanism of Ca^{2+} oscillations and waves

Repetitive Ca^{2+} spikes and waves are observed in many cell types (Berridge, 1993), and the mechanisms underlying their generation are a matter of much interest. Several models have been proposed for Ca^{2+} spiking, which can be divided into two classes depending on whether or not oscillations in level of $InsP_3$ accompany free Ca^{2+} oscillations (for discussion, see Harootunian *et al.* 1991; Yao & Parker, 1992). Similarly, there is controversy as to whether $InsP_3$ or Ca^{2+} is the diffusible messenger that propagates Ca^{2+} waves (Meyer, 1991; Lechleiter & Clapham, 1992). Our results support the idea that Ca^{2+} spikes and waves can occur in the presence of a stable, and spatially homogeneous level of $InsP_3$.

Firstly, Ca^{2+} spikes and waves could be recorded for a few hours in oocytes loaded with the poorly metabolized $InsP_3$ analogue, 3-F-$InsP_3$, similar to results obtained earlier with inositol 1,4,5-trisphosphorothioate in pancreatic acinar cells (Wakui, Potter & Petersen, 1989). Although Ca^{2+}-dependent activation of phospholipase C could impose fluctuating levels of endogenous $InsP_3$ on top of a stable level of exogenous 3-F-$InsP_3$, this seems unlikely since elevations of cytosolic free Ca^{2+} in the absence of $InsP_3$ fail to evoke cyclical Ca^{2+} liberation (Yao & Parker, 1992). Secondly, elevations of intracellular Ca^{2+} resulting from enhanced Ca^{2+} influx affected the propagation of Ca^{2+} signals in ways that appear consistent with a sensitization of the Ca^{2+} release mechanism by cytosolic Ca^{2+}. Specifically, elevations of Ca^{2+} speeded the propagation of Ca^{2+} waves, and transformed localized, decrementally spreading Ca^{2+} puffs into propagating Ca^{2+} waves.

Possible importance of modulation of Ca^{2+} liberation by Ca^{2+} influx

Because Ca^{2+} liberation from $InsP_3$-sensitive stores appears to be modulated by Ca^{2+} influx in many cell types (see Introduction), it is interesting to consider what functions may be served by this mechanism.

One aspect arises from the idea that Ca^{2+} signalling is frequency- or digitally encoded (Berridge, 1988; Meyer & Stryer, 1991), so that changes in an analog stimulus result in changes in frequency but not amplitude of Ca^{2+} spikes. Consistent with this, spike frequency varies with concentrations of agonists (Berridge, 1988), $InsP_3$ (Parker & Ivorra, 1993), and extracellular Ca^{2+} (Jacob *et al.* 1988; Kawanishi *et al.* 1989; Kurtz & Penner, 1989; Hajjar & Bonventre, 1991). Because Ca^{2+} influx varies with membrane potential, this imparts a voltage sensitivity to the biochemical $InsP_3$ signalling pathway. In *Xenopus* oocytes, as in other non-excitable cells (Pittet, Di Virgillio, Pozzan, Monod & Lew, 1990), Ca^{2+} influx and Ca^{2+} spike frequency reduce at more positive potentials, probably because of the decreased electrochemical gradient for Ca^{2+} entry. In contrast, cells expressing voltage-gated Ca^{2+} channels may show an opposite, or a biphasic, voltage dependence of Ca^{2+} spiking frequency.

A second aspect concerns the effects of Ca^{2+} influx on the spatial distribution of intracellular Ca^{2+} liberation. Ca^{2+} waves may serve as a means to transmit signals within cells and between coupled cells (Meyer, 1991). The Ca^{2+} wave velocity is speeded by increases in Ca^{2+} influx but, perhaps more importantly, rapid increases and decreases in Ca^{2+} influx can trigger spatially synchronized Ca^{2+} liberation. Thus, electrical changes may provide a means to 'leapfrog' slow chemical wave transmission and rapidly synchronize Ca^{2+} release within large individual cells and across populations of electrically coupled cells.

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