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Relation between intracellular Ca^{2+} signals and Ca^{2+}-activated Cl^- current in *Xenopus* oocytes

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Abstract — Activation of inositol 1,4,5-trisphosphate ($InsP_3$) signalling in *Xenopus* oocytes causes intracellular Ca^{2+} mobilization and thereby activates a Ca^{2+}-dependent Cl^- membrane conductance. Measurements of cytosolic Ca^{2+} levels using fluorescent indicators, however, revealed little correspondence with Cl^- currents. Intracellular photorelease of $InsP_3$ from a caged precursor evoked transient currents that peaked while the Ca^{2+}-fluorescence signal was rising, and subsequently declined within a few seconds, even though the Ca^{2+} signal remained elevated much longer. Also, Cl^- currents evoked by agonist activation showed transient spikes while a wave of Ca^{2+} liberation swept across the cell, but then decreased when the Ca^{2+} signal attained a maximal level. Thus, the Cl^- current corresponded better to the *rate of rise* of intracellular free Ca^{2+}, rather than to its steady state level. Experiments using paired flashes to photolyse caged $InsP_3$ and caged Ca^{2+} indicated that this relationship did not arise through desensitization or inactivation of the Cl^- conductance. Furthermore, fluorescence measurements made at different depths into the cell using a confocal microscope revealed no evidence that a rapid decline of local Ca^{2+} levels near the plasma membrane was responsible for the decay of Ca^{2+}-activated Cl^- current. Instead, Cl^- channels may show an adaptive or incremental response to Ca^{2+}, which is likely to be important for the encoding and transmission of information by Ca^{2+} spikes.

Oocytes of *Xenopus laevis* possess Ca^{2+}-activated Cl^- channels [1,2] which generate membrane current responses to various stimuli that elevate intracellular free Ca^{2+} levels. For example, transient Cl^- currents are activated when oocytes are depolarized to open voltage-gated Ca^{2+} channels [1] and activation of many different agonist receptors, either native to the oocyte or expressed from exogenous messenger RNA, leads to the generation of oscillatory Cl^- currents through stimulation of the inositol phospholipid messenger pathway [3–6]. As in many other cell types [7], inositol 1,4,5-trisphosphate ($InsP_3$) is formed in response to receptor activation and functions as a second messenger by liberating Ca^{2+} ions sequestered in intracellular stores as well as by allowing the influx of extracellular Ca^{2+} [5,8,9]. There is good evidence that all of these current responses arise because elevations of cytosolic free Ca^{2+} level cause Ca^{2+}-dependent Cl^- channels to open. In particular, the currents are abolished fol-

lowing intracellular injection of the Ca^{2+} chelating agent EGTA [2,4,10,11], whereas injections of Ca^{2+} directly evoke Cl^- currents [2,10,12]. Nevertheless, following the development of techniques to monitor intracellular Ca^{2+} by fluorescent probes [13,14], it has become increasingly apparent that the Cl^- currents do not simply mirror the corresponding intracellular free Ca^{2+} signals.

One difference is that membrane currents evoked by receptor activation or by elevations of intracellular $InsP_3$ display large fluctuations or oscillations, while Ca^{2+} signals monitored from the oocyte display a more sustained rise, superimposed on which fluctuations are sometimes, but not always, visible [5,15–17]. Another discrepancy is seen when light flashes are used to rapidly photorelease $InsP_3$ from a caged precursor loaded into the oocyte. The resulting Ca^{2+}-dependent currents are transient, lasting only one or two seconds, while the associated fluorescent Ca^{2+} signals persist for much longer [18,19].

An understanding of the relationship between cytosolic Ca^{2+} levels and activation of the Cl^- current is important in at least two respects. Firstly, Ca^{2+}-activated membrane current responses are an end result of the phosphoinositide/Ca^{2+} signal pathway in many diverse cell types, so it is important to know how information is transduced by this last step in the messenger pathway. A second, practical, consideration is that voltage clamp recordings of Cl^- current are widely used to assay the expression in *Xenopus* oocytes of exogenous phosphoinositide-linked receptors following injection of foreign mRNAs [20,21]. In the present paper we studied Ca^{2+} activation of Cl^- currents in intact oocytes, by using fluorescent indicators to monitor and image cytosolic free Ca^{2+} levels in the oocyte at the same time as recording membrane currents evoked by agonist applications or by photoreleased $InsP_3$. The results indicate that the apparent discrepancies between these two measures of intracellular Ca^{2+} arise because the Cl^- current magnitude is approximately proportional to the rate of rise of intracellular Ca^{2+} signal, rather than its absolute level. This ability of the membrane conductance to act as a high-pass filter may have important consequences for the way that signals are transduced by the $InsP_3$/Ca^{2+} messenger pathway.

An abstract describing some of this work has appeared [22].

Materials and methods

Experiments were done on oocytes from albino *Xenopus laevis*. Procedures for preparation of oocytes, voltage-clamp recording, photorelease of caged compounds and monitoring of intracellular free Ca^{2+} were as previously described [12,20,23,24]. Briefly, oocytes were loaded with 50–100 pmol of Fluo–3, calcium green-1 or calcium green-5N [14,25]. In experiments where Ca^{2+} was monitored from large areas of the oocyte, fluorescence was measured by a photomultiplier mounted on the microscope phototube, or by a photodiode positioned behind an image intensifier tube. Other experiments involved video imaging of Ca^{2+}-dependent fluorescence, utilizing an intensified CCD camera [26] or a 'real time' laser scanned confocal microscope (Odyssey; Noran Instruments, Middleton, WI, USA) and images were stored on S-VHS videotape for subsequent processing and analysis.

Because the long-wavelength indicators used in these experiments do not permit the use of ratio measurements to determine absolute free Ca^{2+} levels, fluorescence data are presented uncalibrated, or as fractional changes in fluorescence above the resting level ($\Delta F/F$). To estimate the absolute free Ca^{2+} concentrations in resting and stimulated oocytes, we obtained ratio measurements using Fura–2 [13]. Oocytes were loaded with 20–50 pmol Fura–2, which was excited by light from a 75 W xenon arc lamp passed through interference filters transmitting at 350 nm and 373 nm. The 373 nm filter was combined with a neutral density filter, so that the resting fluorescence emission in Fura–2-loaded oocytes was about the same at both wavelengths. After subtracting cell autofluorescence, ratio measurements were calibrated in terms of free Ca^{2+} concentration assuming a value of 200 nM for the apparent dissociation constant for Fura–2 [13], and measured values of minimal and maximal fluorescence ratios (350/370 nm) of 0.89 in Ca^{2+}-free solution and 15.0 in saturating Ca^{2+}. An alternative approach to estimate resting free Ca^{2+} concentrations involved

microinjecting oocytes loaded with Fluo–3 with buffers (mixture of 10 mM EGTA and 10 mM Ca-EGTA: Calcium Calibration Kit 1; Molecular Probes, Eugene, OR, USA) set to various free Ca^{2+} levels, and observing whether the fluorescence rose or fell from the resting level.

Caged $InsP_3$ (*myo*-inositol 1,4,5-trisphosphate, $P^{4(5)}$-1-(2-nitrophenyl)ethyl ester) [27] was injected as a 0.5 mM solution, to give a final intracellular loading of about 5 pmol per oocyte. DM-nitrophen (caged Ca^{2+}) [28] was prepared as a 30 mM solution, together with 1.42 mM Ca and 29.44 mM Mg [29], and oocytes were injected with about 30 nl to give a final intracellular concentration of about 1 mM. Photolysis of caged compounds was induced by applying UV light (350–400 nm) from xenon or mercury arc lamps, with the duration of exposure controlled by an electronic shutter and the intensity set by neutral density filters.

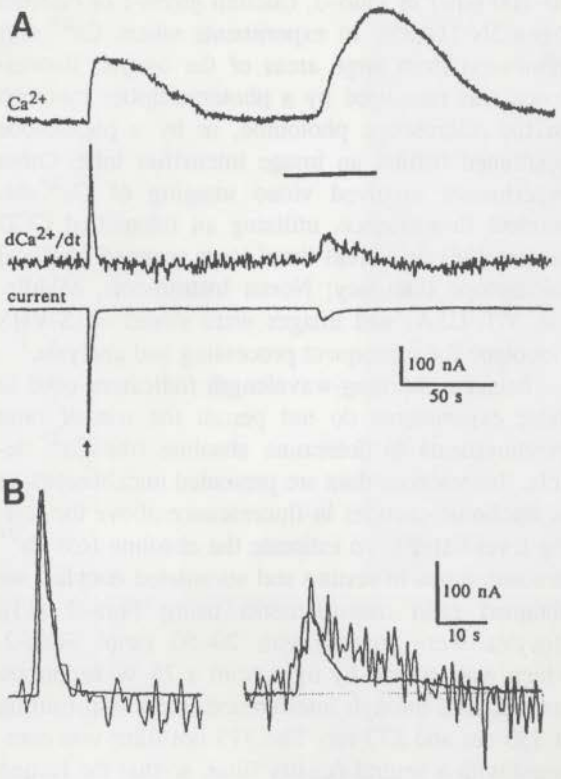

Fig. 1 Membrane currents evoked by photoreleased $InsP_3$ reflect the rate of rise of intracellular free Ca^{2+}. Records are from an oocyte loaded with 4 pmol caged $InsP_3$ and 40 pmol Fluo–3. (**A**) Traces show, from top to bottom, Ca^{2+}-dependent fluorescence, time derivative of the fluorescence signal, and size of Ca^{2+}-evoked membrane current. The oocyte was stimulated by a photolysis flash (50 ms duration) at full intensity when marked by the arrow, and by continuous exposure to photolysis light attenuated to 0.27% of maximal when indicated by the bar. The amplitudes of the Ca^{2+} trace and the differential trace are uncalibrated. (**B**) Superimposed traces, showing the time courses of the membrane current and Ca^{2+}-differential at a faster sweep speed.

During recording, oocytes were continually superfused with Ringer's solution (composition in mM; NaCl, 120; KCl, 2; $CaCl_2$, 1.8; HEPES, 5; at pH about 7.0) at room temperature. Ca^{2+}-free solution contained no added Ca^{2+}, and additionally 5 mM $MgCl_2$ and 1 mM EGTA. Membrane currents were recorded using a conventional two-electrode voltage clamp. Except for the experiment of Figure 7, the clamp potential was always set to –60 mV. At this voltage, Cl^- currents in the oocyte are inwardly directed, corresponding by convention to downward deflections of the current trace. However, some figures are presented with the current trace inverted, to facilitate comparison of increasing magnitudes of Cl^- current with increasing fluorescence Ca^{2+} signals.

Caged compounds were obtained from Calbiochem (La Jolla, CA, USA); Fura–2, Fluo–3, calcium green-1 and calcium green-5N were from Molecular Probes (Eugene, OR, USA); all other reagents were from Sigma Chemical Co. (St Louis, MO, USA).

Results

Membrane current signals rate of rise of intracellular Ca^{2+}

A complication in trying to correlate intracellular Ca^{2+} signals with membrane current is that $InsP_3$-mediated Ca^{2+} release occurs independently and asynchronously in different regions of the oocyte [7,17,23]. Membrane currents recorded with a two-electrode voltage clamp reflect a summation of Ca^{2+} activity over the whole membrane area of the cell, whereas fluorescence recordings of intracellular Ca^{2+} are restricted, at best, to one hemisphere. To simplify analysis, we therefore began by using experimental conditions where Ca^{2+} release was lo-

calized to the area from which fluorescence was monitored.

Figure 1 shows Ca^{2+} signals and membrane currents evoked by photorelease of $InsP_3$ from a caged precursor loaded into the oocyte. The photolysis light was arranged as a spot of 300 μm diameter, and measurements of Fluo–3 fluorescence were obtained from this same area of the cell. Stimulation by a brief (50 ms) photolysis flash at maximal intensity evoked a transient current and a rapid rise in Ca^{2+}-fluorescence. However, although the current decayed to the baseline within a few seconds, the Ca^{2+} signal remained nearly at its peak level at this time, and subsequently fell slowly over several tens of seconds. Further discrepancies were apparent when the oocyte was stimulated by prolonged (50 s) exposure to photolysis light attenuated by neutral density filters to 0.27% of the maximal intensity. This slow photorelease of $InsP_3$ evoked a slow rise in Ca^{2+}-fluorescence, that reached a peak higher than that evoked by the flash. In contrast, the associated current was much smaller than evoked by the flash, and occurred during the initial rise of the Ca^{2+} signal, so that almost no current remained when the Ca^{2+} signal was at its peak.

From the records in Figure 1 it is clear that the current did not reflect the magnitude of the fluorescence Ca^{2+} signal, but instead was greatest at times when the fluorescence was rising rapidly. Further support for this idea is given by the middle trace in Figure 1A, which shows the first differential of the fluorescence record ($d[Ca^{2+}]/dt$). The time course and relative amplitude of the differentials matched well to the currents evoked by the light flash and sustained exposure (Fig. 1B), suggesting that activation of the Cl^- current corresponds closely to the rate of rise of the intracellular Ca^{2+} signal. Results similar to those illustrated were obtained in more than 20 oocytes loaded with Fluo–3; in all cases the duration of the Ca^{2+} signal outlasted that of the current.

Other Ca^{2+} indicator dyes

We were concerned that the slow time course of the Fluo–3 signal might arise through some artifactual property of the indicator; for example, because dissociation of Ca^{2+} from the dye was slow, or as a result of the exogenous Ca^{2+} buffering power introduced into the cell. Accordingly, we recorded Ca^{2+} fluorescence signals evoked by photorelease of $InsP_3$ using other long-wavelength indicator dyes including calcium green-1 and calcium green-5N. At least 10 oocytes were tested with each dye, and in all cases the decay of the fluorescence considerably outlasted the duration of the associated Cl^- current. The rate constant for Ca^{2+} dissociation is around 150 s^{-1} for calcium green-1 [25], thus ruling out the possibility that the decay of fluorescence signals over several seconds could be rate limited by dissociation of Ca^{2+} from this dye. Furthermore, the affinity of calcium is an order of magnitude lower than for the other indicators, so in this case buffering of intracellular Ca^{2+} would have been minimized.

Ca^{2+} images and currents evoked by agonist

To study Ca^{2+} signals and associated membrane currents evoked by receptor activation we used blood serum as an agonist, since virtually all oocytes possess endogenous receptors to a factor in serum that stimulates the $InsP_3/Ca^{2+}$ signalling pathway [30]. Figure 2 shows typical records of serum-evoked responses in an oocyte that had been loaded with Fluo–3 to allow imaging of intracellular Ca^{2+}, and was voltage-clamped to record Ca^{2+}-activated Cl^- currents. Images in the top row (Fig. 2A) show fluorescence images captured at various times (indicated in seconds) after beginning bath application of serum, and were derived after subtracting resting fluorescence. The trace at the bottom (Fig. 2C) shows Cl^- current magnitude (upward deflections = increasing inward current), and the arrows indicate the corresponding times at which the various images were captured.

Following addition of serum, fluorescence and current signals began after a latency of 21 s [30]. Ca^{2+} rose initially in a small region of the oocyte at 8 o'clock in the image, and this expanding Ca^{2+} signal was accompanied by a small current response that remained about the same amplitude for 5 s. A second region of the oocyte (3 o'clock) then showed a rise in Ca^{2+}, and the current showed an additional stepwise increase. Ca^{2+} signals from the initial two regions continued to expand until they fused

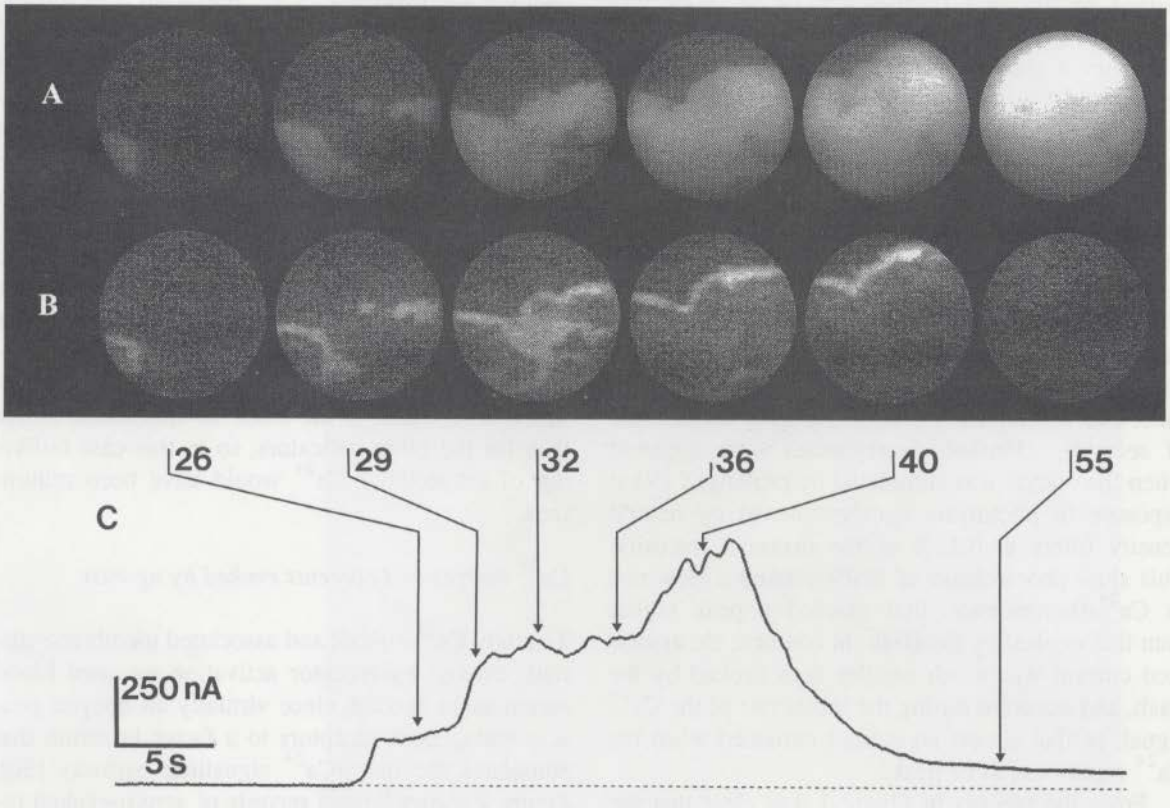

Fig. 2 Simultaneous records of membrane current and intracellular free Ca^{2+} images, obtained in an oocyte loaded with Fluo–3. The oocyte was bathed in Ca^{2+}-free solution, and serum at a dilution of 10^4 was added to the superfusate at time zero. (A) The upper row of images show fluorescence Ca^{2+} signals captured at different times (indicated in seconds) after adding serum. Resting background fluorescence was subtracted, so each image shows serum-evoked increases in Ca^{2+} on a scale from dark grey (no increase above background) to white (maximal increase). Absolute free Ca^{2+} concentrations were not calibrated. (B) Images showing regions of newly active Ca^{2+} release. These were formed by sequential subtraction of frames captured at 2 s intervals, and thus represent increases in Ca^{2+} occurring during 2 s intervals preceding each indicated time. (C) Trace shows membrane current, beginning 11 s after addition of serum, and lines indicate the times at which the images in A and B were obtained. Increasing inward currents are denoted as upward deflections, so as to facilitate comparison with intracellular Ca^{2+} levels.

together about 32 s after adding serum, after which Ca^{2+} release spread to engulf the entire visible hemisphere of the oocyte. The maximal fluorescence response was attained about 55 s after adding serum, at which time the Ca^{2+}-fluorescence was highest near the animal pole, situated at the top of the image. Although the initial increases in current corresponded to the appearance of Ca^{2+} signals at discrete regions of the oocyte, there was little correlation between the fluorescence images and the current at later times. Most notably, the overall fluorescence increased progressively to reach a maximum about 55 s after adding serum, whereas the current amplitude peaked after about 41 s, and had declined close to the baseline when the fluorescence was greatest.

A clue as to the relation between the fluorescence and current signals arose from the observation that larger currents occurred at times when Ca^{2+} rose at new regions of the oocyte, or spread rapidly to invade new areas. To further explore this idea, we processed images by pair-wise subtraction of se-

quential frames captured at 2 s intervals, so as to display only the advancing wavefronts of active Ca^{2+} release where the free Ca^{2+} level had risen during each preceding 2 s interval. As shown in Figure 2B, these images displaying areas of new Ca^{2+} release correspond much better to the current record than do the images of 'raw' Ca^{2+} (Fig. 2A). In particular, the maximal current response occurred when Ca^{2+} release was spreading rapidly towards the animal pole (image at 40 s), whereas after 55 s, when the current had largely decayed, there was almost no further increase in Ca^{2+}, even though the Ca^{2+} level was maximal.

Membrane current correlates with rate of spread of intracellular Ca^{2+}

Figure 3A shows measurements derived from the experiment of Figure 2. The upper trace represents the overall brightness of the Ca^{2+}-fluorescence signal, integrated over the oocyte. Although the Ca^{2+} signal began to rise at the same time as the current, there was otherwise little correlation between the two records, and the Ca^{2+} was maximal at the end of the trace when the current (lower trace) had fallen to a low level. The middle trace shows the rate of spread of Ca^{2+} across the oocyte ('spatial derivative'), obtained by measuring the fractional area of the oocyte image over which the fluorescence signal increased by more than a threshold amount (6 intensity units; just above the noise level of the recordings) during sequential 1 s intervals. This derivative of the Ca^{2+} signal clearly corresponds better to the time course of the Ca^{2+}-activated Cl^- current than does the overall Ca^{2+} signal.

The records in Figure 3B are from the same oocyte as Figure 3A, but show responses evoked by a 10-fold higher dilution of serum (10^4 dilution rather than 10^3). The peak current was about 5-times smaller than that evoked by the lower dilution, and the current and Ca^{2+} signals began nearly simultaneously following a longer latency (about 37 s). However, the overall Ca^{2+} fluorescence again showed little correlation with the current. It increased progressively throughout the recording period, whereas the current was transient and, despite the much smaller current amplitude, the maximal fluorescence was almost as great as that with the lower dilution of serum. Different to this, the spatial derivative of the Ca^{2+} corresponded more closely to the current record. Its peak amplitude was about 10-times smaller than with the lower dilution of serum, and the time course showed an initial transient peak followed by a slow rise resembling the time course of the current.

The analysis of the spatial derivative of intracellular Ca^{2+} changes described above is subject to several errors, and can only be approximate. We

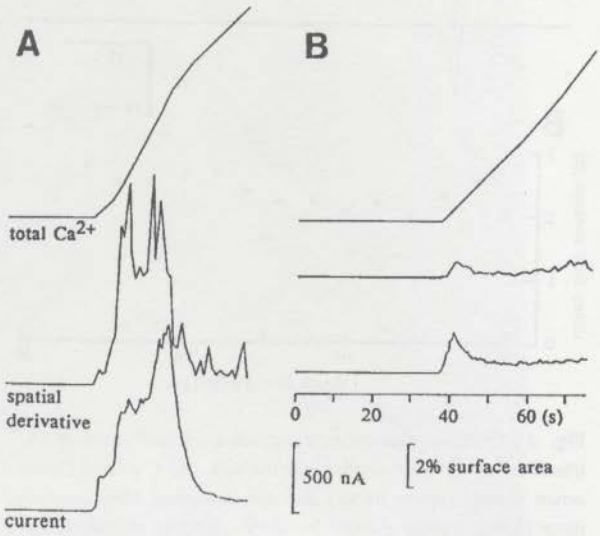

Fig. 3 The amplitude of the agonist-evoked membrane current correlates better with the spatial derivative of intracellular free Ca^{2+} than with the overall Ca^{2+} level. The upper trace in each section shows the total Ca^{2+}-dependent fluorescence recorded from the entire visible area of the oocyte. The amplitude is not calibrated in terms of absolute free Ca^{2+} concentration, but the scale is the same in A and B. Middle traces show the spatial derivative of Ca^{2+} (i.e. the relative area of the oocyte over which Ca^{2+} increased during successive 1 s intervals). Measurements were made by sequentially subtracting fluorescence images captured at 1 s intervals, and measuring the area of the cell where the Ca^{2+} signal had increased by more than 6 intensity units. Calibration bar corresponds to an area 2% of the visible surface of the oocyte. Lower traces show current amplitudes (upward deflections = increasing magnitude of inward current). (A) Responses evoked by addition of serum at a dilution of 10^3, beginning at the start of the traces. The oocyte was bathed in Ca^{2+}-free Ringer, and the data are from the same experiment as Figure 2. (B) Responses in the same oocyte following addition of serum at a dilution of 10^4 at the beginning of the traces.

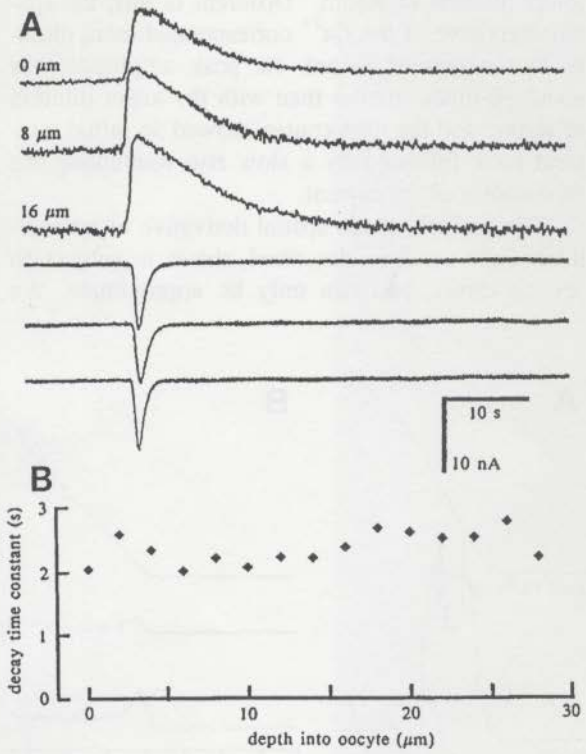

Fig. 4 Confocal fluorescence recording of $InsP_3$-evoked Ca^{2+} transients at different depths into the cell. (A) Confocal fluorescence signals (upper traces) and corresponding membrane currents (lower traces) evoked by three identical photolysis light flashes (10 ms duration) with the microscope focussed at different depths, indicated in μm, inward from the cell surface. (B) Time constant of decay of the fluorescence signal plotted as a function of depth of the confocal slice into the cell. Measurements were obtained from traces like those in A, by fitting exponential curves to the fluorescence decay beginning after the fluorescence had declined about 20% from the peak.

were able to visualize only one half of the oocyte at any time, and thus had no knowledge of Ca^{2+} changes occurring on the hidden side. To minimize this problem, the oocyte was placed in a metal cup which shielded the underside from the flow of solution. Also, measurements of areas of Ca^{2+} release would be distorted by geometric foreshortening around the periphery of the cell, and we took no account of the differing sensitivity of the Cl^- current to intracellular Ca^{2+} between the hemispheres [2]. Nevertheless the main conclusion – that the current reflects the spread of Ca^{2+} release across the oocyte rather than the overall Ca^{2+} level – seems well founded.

Free Ca^{2+} concentrations at rest and during stimulation

The long wavelength indicator dyes used for these experiments do not permit ratiometric calibration of fluorescence signals in terms of free Ca^{2+} concentrations. Accordingly, we used other approaches to estimate the resting free Ca^{2+} level in the oocyte and the peak level attained during $InsP_3$-mediated signalling.

One method was to use Fura–2 to make ratio measurements [13] but, because we do not have a rapid filter changer, this was possible only in resting oocytes, and during the sustained phase of Ca^{2+} release induced by bath application of serum (10^3 dilution). The mean fluorescence ratio (350/373 nm) in 8 resting oocytes was 1.15 ± 0.03, corresponding to an estimated free Ca^{2+} concentration of about 33 nM (*see* Materials and methods). During stimulation by serum the ratio measured near the animal pole rose to 2.14 ± 0.12 (5 oocytes), corresponding to 180 nM free Ca^{2+}. Another approach to estimate the resting free Ca^{2+} level involved injecting Fluo–3-loaded oocytes with Ca^{2+}/EGTA buffers set to different free Ca^{2+} levels, and determining whether these caused an increase or decrease in fluorescence. Two oocytes showed a null point between 37 and 64 nM, and in a further two oocytes this lay between 64 and 100 nM.

Confocal Ca^{2+} signals at different depths in the cytoplasm

One explanation for the discrepancy in time course between the membrane current and fluorescence Ca^{2+} signals could be that the Cl^- current accurately reflects a transient increase in free Ca^{2+} close to the inner surface of the plasma membrane, whereas the fluorescent Ca^{2+} monitor senses a more prolonged increase in Ca^{2+} deeper into the cytoplasm. We had previously tested this idea by using a 'homemade' confocal microscope to monitor Ca^{2+} signals near

the membrane and deeper into the cell, but detected no differences in decay time of the Ca^{2+} transient at these locations [31]. However, as we now have available a commercial 'real time' laser scanned confocal microscope (Noran Odyssey) with better resolution, it seemed worthwhile to repeat this experiment.

Figure 4 shows an experiment in which $InsP_3$ was photoreleased by a pinhole of UV light focussed to a spot of about 2 μm diameter on the oocyte. The oocyte was loaded with calcium green-5N, and fluorescence was monitored from a spot about 5 μm across, concentric with the photolysis spot. The microscope was equipped with a 40 X oil immersion objective (numerical aperture 1.3; Nikon) and a 50 μm confocal detector slit was used, providing an optical section of 1.5–2 μm thickness at half-maximal intensity, as measured by reflectance from a mirrored slide (Noran Odyssey users manual). The upper traces in Figure 4A show fluorescence signals evoked by photolysis flashes (60 ms duration), recorded with the microscope focussed at the cell surface and at depths of 8 and 16 μm into the oocyte. The Ca^{2+} signals decayed over several seconds, with little difference in time course at different depths of the confocal section.. This decay was more rapid than in the experiment of Figure 1A, probably because diffusion of $InsP_3$ and Ca^{2+} away from the near point stimulation contributed to the fall in Ca^{2+} fluorescence, in contrast to photorelease of $InsP_3$ over a wide area in Figure 1. Nevertheless, Ca^{2+} levels remained elevated for several times longer than the duration of the membrane current responses (lower traces, Fig. 4A).

Measurements of decay time constant of the Ca^{2+} signal are plotted in Figure 4B as a function of depth of the confocal slice within the oocyte. The decay rate remained about constant from the oocyte surface to a depth of at least 25 μm. In particular, we found no evidence for a more transient Ca^{2+} signal near the surface membrane in this, and in 5 similar experiments.

Decline of Cl^- current does not arise through desensitization

Another explanation for the rapid decay of the membrane current following photorelease of $InsP_3$ may be that the Ca^{2+}-activated Cl^- channels inactivate or desensitize, so that they no longer open even if free Ca^{2+} levels remain high in the vicinity of the membrane. Figure 5 illustrates two experiments which indicate this is not the case, but that instead the current appears to show an incremental or adaptive response characteristic.

In Figure 5A an oocyte that was loaded with caged $InsP_3$ and calcium green-1 was stimulated by

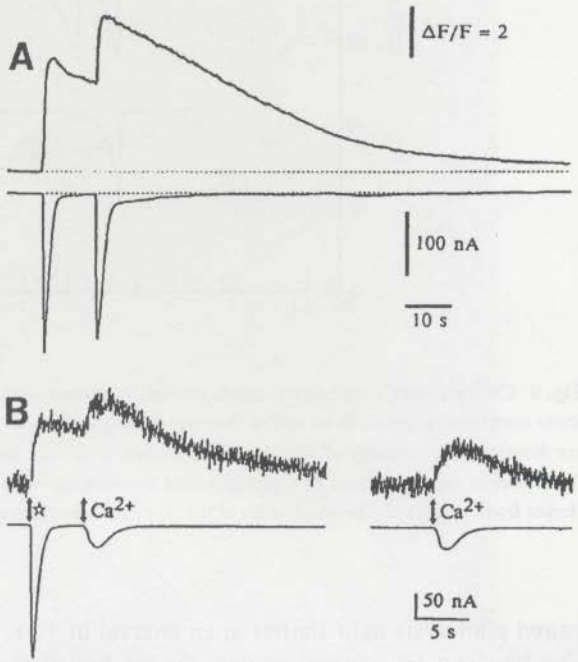

Fig. 5 Decline of Cl^- current during sustained elevations of intracellular Ca^{2+} does not arise from desensitization. (A) Traces show calcium green-1 fluorescence (upper) and membrane current (lower). Paired photolysis flashes were delivered at an interval of 10 s to cause intracellular photorelease of $InsP_3$. The flash duration was increased from 30 ms to 40 ms for the second stimulus, to compensate for depression of $InsP_3$-evoked Ca^{2+} release seen at short inter-stimulus intervals. Results illustrated are representative of 10 trials. (B) Upper trace in each frame shows Fluo–3 fluorescence and lower trace shows membrane current. In the left frame $InsP_3$ was photoreleased by a flash of 75 ms duration delivered when indicated by the star, and 75 fmol Ca^{2+} (15 pl of 5 mM $CaCl_2$) was then injected into the oocyte after an interval of 5 s, through a micropipette centered in the photolysis and fluorescence excitation light spots. The right frame shows responses evoked by a similar injection of Ca^{2+} after allowing the oocyte to rest for 90 s.

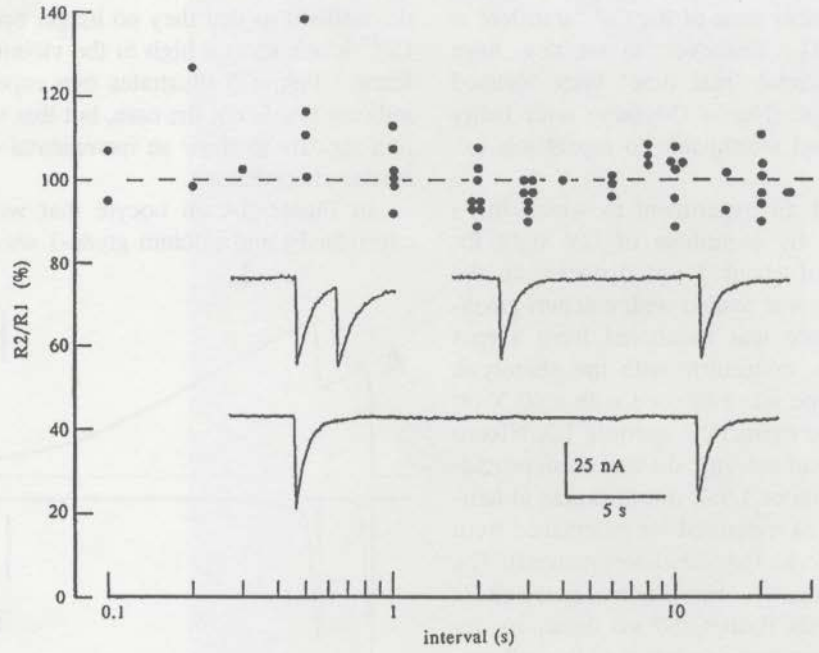

Fig. 6 Ca^{2+}-activated membrane currents evoked by photorelease of Ca^{2+} from a caged precursor show little inactivation. Inset traces show membrane currents in an oocyte that was loaded with about 700 pmol DM-nitrophen, and stimulated by identical light flashes (60 ms duration, full intensity of illuminator) delivered at various inter-flash intervals. Graph shows the sizes of responses to the second flash in each pair, expressed as a percentage of the response to the first flash. Each point is a single measurement, and pooled data are shown from 7 oocytes. The amplitudes of the membrane currents varied in different oocytes between 15 and 270 nA.

paired photolysis light flashes at an interval of 10 s. The transient Cl^- current evoked by the first flash had decayed almost to the baseline when the second flash was given, even though the Ca^{2+} fluorescence remained high. The cessation of the current in the presence of a sustained elevation of Ca^{2+} cannot readily be explained by desensitization, since a further increment in Ca^{2+} evoked by the second flash resulted in a Cl^- current only slightly smaller than the first. A similar result is shown in Figure 5B, except that here the sensitivity of the Cl^- current to further increases in Ca^{2+} following photorelease of $InsP_3$ was tested by intracellular injection of Ca^{2+} through a micropipette. In the left-hand record, photorelease of $InsP_3$ evoked a transient current response, together with a more slowly decaying Fluo–3 Ca^{2+} signal. The current had decayed almost completely to the baseline 5 s after the light flash, yet injection of Ca^{2+} at this time evoked a current almost identical to the control response without preceding photorelease of $InsP_3$ (right). Because the Ca^{2+} pipette was centered in the photolysis light spot, the current evoked by Ca^{2+} injection presumably arose through activation of channels in the same region of membrane as that giving rise to the $InsP_3$ response, indicating that the decline in current following the light flash did not result from a reduced sensitivity to Ca^{2+}.

Currents evoked by photoreleased Ca^{2+} show little desensitization

To examine further whether Ca^{2+}-activated Cl^- currents show desensitization, we used caged Ca^{2+} (DM-nitrophen) to generate controlled increases in intracellular free Ca^{2+} [12]. Figure 6 illustrates cur-

rents evoked by paired light flashes delivered at various intervals. Responses to the second stimulus in each pair were of similar size to the first, and measurements in 7 oocytes (Fig. 6) indicated that the second response differed little from the control size at any interval between 0.1 and 20 s.

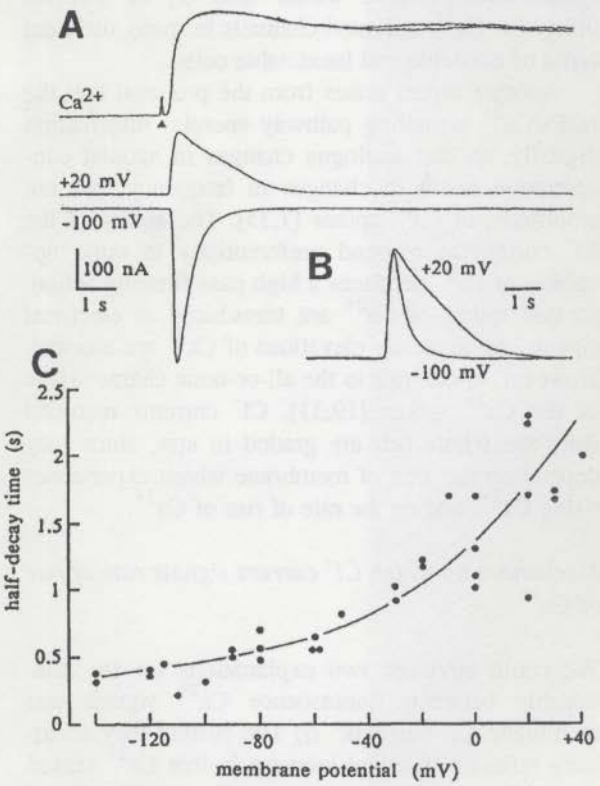

Fig. 7 Voltage dependence of Cl^- current decay. (A) Calcium green-1 fluorescence signals and membrane currents evoked by photorelease of $InsP_3$ while clamping the oocyte to potentials of +20 and –100 mV. The upper trace shows superimposed fluorescence records obtained at each potential, and the lower traces show corresponding membrane currents. Photolysis flashes were delivered at the arrowhead, and produced a small artifact in the fluorescence trace. The flash duration was lengthened slightly (from 40 to 45 ms) for the second flash, so as to evoke a peak Ca^{2+} signal of similar amplitude to the first response. Fluorescence was monitored using a confocal microscope focussed just under the oocyte surface from a 5 μm diameter spot, centered within the 50 μm diameter photolysis light spot. (B) Superimposed current traces from A, scaled to the same peak amplitude and direction so as to facilitate comparison of their kinetics. (C) Measurements of time for Cl^- current to decay to one-half of the peak, plotted as a function of membrane potential. Each point is a single measurement from traces like those in A. Data are from 4 oocytes, and the curve is drawn by eye.

Voltage sensitivity of Cl^- current decay

Figure 7A,B shows membrane currents and calcium green-1 fluorescence signals evoked by photorelease of $InsP_3$ while the oocyte was clamped to potentials of +20 mV and –100 mV. Although virtually identical fluorescence signals were observed at each voltage, the decay of the Cl^- current was about 3-times slower at +20 mV than –100 mV, though still rapid as compared to the fall in Ca^{2+}.

Measurements of Cl^- current decay in 4 oocytes are plotted in Figure 7C as a function of membrane potential. Because the current decay often did not follow single exponential kinetics at more positive voltages (e.g. Fig. 7A), we simply measured the time to fall to one-half of the peak. This shortened from about 2 s at +20 mV to about 0.4 s at potentials negative to –100 mV.

Discussion

Relation between time courses of Cl^- current and intracellular free Ca^{2+}

This work was prompted by a discrepancy between Ca^{2+}-activated Cl^- currents and intracellular Ca^{2+} signals monitored by fluorescent probes in *Xenopus* oocytes in response to $InsP_3$-evoked Ca^{2+} liberation. Large currents were observed when the fluorescence signal was rising rapidly, but then decayed within a few seconds even though the fluorescence signal persisted much longer. Thus, the current corresponded better with the **rate** of increase of fluorescence than with its absolute level. This relationship has several consequences for the patterns of electrical responses evoked by Ca^{2+}-mobilizing stimuli.

Currents evoked in the oocyte by Ca^{2+}-mobilizing agonists and intracellular injections of $InsP_3$ comprise several components; an initial large, transient spike, followed by a slowly developing sustained component on which oscillations are usually superimposed [5,32]. A part of the sustained com-

ponent arises as a consequence of Ca^{2+} influx into the cell, whereas the spike and oscillatory components involve intracellular Ca^{2+} liberation [33]. However, the mechanisms giving rise to the spike and oscillatory current components have been unclear, especially since free Ca^{2+} signals monitored from the whole oocyte peak well after the initial current spike, and show little evidence of oscillations in Ca^{2+} corresponding to the current oscillations [5,15,16]. The present results, taken together with observations of the spatiotemporal aspects of Ca^{2+} signalling revealed by Ca^{2+} imaging [17], suggest an explanation for the components of the Cl^- current response. Specifically, the initial current spike arises because Ca^{2+} levels increase rapidly across wide areas of the cell, but is transient because the Cl^- current declines even though intracellular Ca^{2+} levels remain high (Fig. 2). Patterns of intracellular Ca^{2+} often then become more complex, consisting of a chaotic sequence of repetitive Ca^{2+} waves propagating across the cell [17]. These are accompanied by irregular oscillations in membrane current, which presumably arise at the leading edge of Ca^{2+} waves where Cl^- channels are exposed to a rapid rise in free Ca^{2+}. Although such localized Ca^{2+} spikes are revealed by Ca^{2+} imaging [17,23] and by confocal recording from minute spots [19,31], they would have been obscured in measures of Ca^{2+} integrated over the cell, since the overall Ca^{2+} level remains roughly constant even though its spatial distribution is constantly changing.

One important aspect of the Cl^- conductance is that it allows transient currents to be generated in response to small increments of intracellular free Ca^{2+}, even though little or no current is generated at the resting free Ca^{2+} level. The peak amplitudes of transient Cl^- currents evoked by Ca^{2+} injections and photorelease of Ca^{2+} from a caged precursor increase linearly with amount of Ca^{2+} [12], but Ca^{2+} levels during $InsP_3$ signalling are only about 5-fold above the resting level. Thus, an appreciable standing current would be expected at rest if the current amplitude were linearly proportional to the steady-state concentration of intracellular Ca^{2+}, whereas the membrane permeability of the resting oocyte to Cl^- is actually small or negligible [21]. In the oocyte the functional importance of this characteristic may be to allow generation of a fertilization potential as a wave of Ca^{2+} spreads over the cell from the point of sperm entry [34], with little standing current at the resting Ca^{2+} level. A similar incremental response characteristic would also be of obvious utility for Ca^{2+}-activated channels in many different types of excitable and inexcitable cells.

Another aspect arises from the proposal that the $InsP_3/Ca^{2+}$ signalling pathway encodes information digitally, so that analogue changes in agonist concentration result in changes in frequency, but not amplitude, of Ca^{2+} spikes [7,35]. The ability of the Cl^- current to respond preferentially to rapid upstrokes of Ca^{2+} produces a high pass filtering action, so that spikes of Ca^{2+} are transduced as electrical signals, while steady elevations of Ca^{2+} are blocked. However, in contrast to the all-or-none characteristic of the Ca^{2+} spikes [19,31], Cl^- currents recorded from the whole cell are graded in size, since they depend on the area of membrane which experiences rising Ca^{2+}, and on the rate of rise of Ca^{2+}.

Mechanism by which Cl^- current signals rate of rise of Ca^{2+}

We could envisage two explanations for the relationship between fluorescence Ca^{2+} signals and membrane Cl^- currents. (i) The current may accurately reflect a transient increase in free Ca^{2+} sensed by Cl^- channels at the inner surface of the plasma membrane, which was not apparent in the fluorescent dye signals. (ii) Conversely, the fluorescence signals may give a good indication of free Ca^{2+} in the vicinity of the channels, while Cl^- current through the channels is proportional to the rate of rise of Ca^{2+} rather than its steady-state level. As discussed below, we favour the latter possibility, but kinetic studies on Cl^- channels in inside-out membrane patches are needed to confirm this interpretation and further elucidate the mechanism by which the Cl^- current preferentially signals increments in cytosolic Ca^{2+} level.

Firstly, the fluorescence signal should accurately track bulk changes in cytosolic free Ca^{2+}, since the rate constant for Ca^{2+} dissociation introduces a lag of only several milliseconds [25]. Although high levels of dye loading may artifactually slow intracellular Ca^{2+} transients because of the introduction of exogenous Ca^{2+}-buffering power, the time course of

the fluorescence signal is still expected to correspond to that of free Ca^{2+} because of the rapid association and dissociation of Ca^{2+} with the dyes. More directly, fluorescence signals remained slower than the Cl^- current even when interference with normal Ca^{2+} homeostasis was minimized by using an indicator with low affinity for Ca^{2+} (calcium green-5N; K_d = 10–12 μM).

Secondly, it seems that the differences in decay rates of fluorescence and membrane current Ca^{2+} signals do not arise because of a spatial gradient of cytosolic free Ca^{2+} near the plasma membrane. Conventional epifluorescence techniques provide a weighted measure of Ca^{2+} levels to a depth of 20–30 μm into the oocyte, limited by turbidity of the cytoplasm [31]. However, the Ca^{2+} signal near the membrane might be more transient than this bulk measure of Ca^{2+} if, for example, $InsP_3$-gated release sites are located close to the plasma membrane or if Ca^{2+} ions are rapidly extruded across the membrane. Experiments using confocal microscopy to monitor Ca^{2+} signals at different depths into the cell failed to show any differences in kinetics near the membrane but, because the axial resolution was limited to 1 or 2 μm, a very steep gradient could have escaped detection. However, it seems improbable that a steep gradient could be maintained over sub-μm distances for long enough (1 or 2 s) to account for the decay time of the Cl^- current. Furthermore, the observation that Cl^- currents were slowed at positive membrane potentials is consistent with the idea that the decline of the Cl^- current is an intrinsic property of the Ca^{2+}-activated Cl^- channels themselves. Because of their location in the plasma membrane, the Cl^- channels will sense the membrane potential of the cell, whereas Ca^{2+} release and re-sequestration into intracellular stores were neither expected nor observed to be inherently sensitive to membrane potential (Fig. 7). Although Ca^{2+} extrusion across the plasma membrane (via electrogenic pumping and Na^+/Ca^{2+} exchange) will, presumably, be affected by membrane potential, the expected result is the opposite of that seen. Depolarization reduces the electrochemical gradient against which extrusion must occur and hence should accelerate the fall in Ca^{2+} levels near the inner membrane surface, whereas decay of the Ca^{2+}-activated Cl^- current was actually slowed.

A third possibility is that the Cl^- channels desensitize, so that the current declines in the continued presence of Ca^{2+}. This seems unlikely, since we found no evidence for desensitization in recordings of currents evoked by paired photorelease of intracellular Ca^{2+}. Furthermore, when the current had decayed almost to zero during a prolonged elevation of Ca^{2+}, the sensitivity to additional elevations of Ca^{2+} was little reduced. Thus, the kinetics of Ca^{2+}-dependent activation of Cl^- current appear to show an 'adapting' behaviour, rather than desensitization, such that successive transient currents are evoked by successive increments of free Ca^{2+}. The molecular basis of this behaviour remains mysterious, but may be analogous to the adaptive response characteristic recently described for cardiac ryanodine receptor/channels [36].

Although a large fraction of the Cl^- current is transient, smaller maintained currents persist during prolonged elevations of intracellular free Ca^{2+} [37]. Boton et al. [37] suggested that these components may arise because of multiple types of Ca^{2+}-activated Cl^- channel in the oocyte membrane, but it is unclear at present whether the sustained current could instead arise through incomplete adaptation of a single type of channel. Patch clamp studies of excised membrane patches are needed to resolve this question, and to explore the basis of the adaptive behaviour.

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