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ORIGINAL ARTICLE

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Capacitative calcium entry is colocalised with calcium release in *Xenopus* oocytes: evidence against a highly diffusible calcium influx factor

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Abstract Depletion of intracellular calcium stores activates the plasma membrane capacitative calcium entry pathway in many cell types. The nature of the signal that couples the depletion of the intracellular calcium stores to the activation of the plasma membrane calcium influx pathway is as yet unknown. It has recently been suggested that a highly diffusible calcium influx factor is involved in the activation of capacitative calcium entry, and that its action is potentiated by the protein phosphatase inhibitor okadaic acid. Depletion of intracellular calcium stores in a localised region of a *Xenopus* oocyte was found to evoke capacitative calcium entry exclusively colocalised across the stimulated area of the plasma membrane, arguing against the involvement of a highly diffusible calcium influx factor. Equally, no evidence could be found for the presence of a soluble calcium influx factor in the bulk cytosol of *Xenopus* oocytes. The potentiation of capacitative calcium entry by okadaic acid resembled that mediated by the activation of protein kinase C, thus suggesting that okadaic acid activity may not necessarily be related to the action of a putative calcium influx factor.

Key words Calcium · InsP_3 · Calcium entry · *Xenopus* oocytes

Introduction

Stimulation of many cell types with agonists linked to the phosphoinositide signalling pathway evokes biphasic rises in the cytosolic calcium concentration [1, 2]. Whereas the initial transient rise in cytosolic calcium concentration is due to InsP_3-evoked (i.e. inositol 1,4,5-trisphosphate) release of calcium from intracellular calcium stores [1], the secondary sustained phase of the calcium signal is due to calcium influx across the plasma membrane. This plasma membrane capacitative calcium influx pathway can be activated by a wide variety of protocols all involving the depletion of the intracellular calcium stores [3]. The capacitative calcium entry pathway thus appears to be controlled directly by the state of filling of the intracellular calcium stores [3], suggesting that there must be a retrograde signal from the stores to the plasma membrane controlling the depletion-activated calcium channels. One possibility for such a signal is that depleted intracellular calcium stores release a diffusible messenger into the cytosol, which would then activate the plasma membrane calcium channels [4–7]. Preliminary characterisations of such a putative calcium influx factor suggest that it may have a low molecular weight [5, 7] and that its action is potentiated by the protein phosphatase inhibitor okadaic acid [4, 6, 7].

Despite these efforts to characterise a putative calcium influx factor, there has been little attempt to obtain physiological evidence that capacitative calcium entry is controlled by a diffusible agent. Physiological evidence for a diffusible messenger involved in the activation of capacitative calcium entry comes from experiments involving the cramming of patches into *Xenopus* oocytes which have been treated in different ways [4]. The cramming of a patch into an oocyte with full intracellular calcium stores did not activate a calcium-permeable conductance, whereas cramming a patch into an agonist-stimulated oocyte activated calcium influx. However, in these experiments it is not clear whether the InsP_3 concentration had decreased at the time of cramming the patch into the oocyte. It is thus possible that the patch contains many organelles and that cramming it into an oocyte with high levels of InsP_3 simply releases calcium from the organelles attached to the patch membrane and thus activates calcium entry across the patch.

An alternative approach to investigating capacitative calcium entry in nasal epithelial cells revealed that calcium entry was exclusively localised to the region of the

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cells which had been stimulated by agonist application [8]. A highly diffusible calcium influx factor would be expected to diffuse from one end of the cell to the other in the many minutes between localised depletion of intracellular calcium stores and testing for calcium entry. Although the data of Paradiso et al. [8] would thus suggest that a highly diffusible calcium influx factor is not involved in the activation of capacitative calcium entry, it must also be noted that $InsP_3$ (which is known to be a diffusible messenger) was not observed to diffuse in this preparation, leaving it difficult to make any definitive interpretation of the data.

The data in this study show that capacitative calcium entry could be activated across a localised region of the *Xenopus* oocyte plasma membrane which had been stimulated by a short-lasting agonist application, whereas unstimulated areas of the same oocyte showed no calcium entry. This suggests that capacitative calcium entry is only activated near stores that have been depleted, which would fit the physiological role of the calcium entry pathway in refilling the depleted intracellular calcium stores. Furthermore these data argue against the involvement of a highly diffusible calcium influx factor in the activation of capacitative calcium entry. The injection of a large volume of cytoplasm from oocytes with depleted intracellular calcium stores into untreated oocytes failed to activate calcium entry, suggesting that there is no calcium influx factor present in the bulk cytosol of depleted oocytes. Further experiments suggested that the potentiation of capacitative calcium entry in *Xenopus* oocytes by okadaic acid is mediated by protein kinase C and thus not necessarily related to the activity of any calcium influx factor.

Materials and methods

Preparation of oocytes

Oocytes were obtained from *Xenopus laevis* and manually defolliculated. The oocytes were maintained at 17°C in an incubation medium containing (mM): 115 NaCl, 2 KCl, 1 $MgCl_2$, 2 $CaCl_2$, 50 μg/ml gentamicin, 5 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid (HEPES) (pH 7.4 adjusted by NaOH). All chemicals (except caged compounds) were obtained from Sigma.

Electrophysiological measurements

To record the calcium-activated chloride current, the oocyte was impaled with two microelectrodes (2 M KCl-filled, resistance 0.5–2 MΩ) and voltage-clamped to –60 mV using a Warner Instruments oocyte clamp amplifier (OC-725B). All experiments were carried out at room temperature 18–22°C. The perfusates contained (mM): 115 NaCl, 2 KCl, 1 $MgCl_2$, 5 HEPES (pH 7.4 adjusted by NaOH) with either 2 mM $CaCl_2$ added to give calcium-containing perfusate or 1 mM ethylenebis(oxonitrilo)tetraacetate (EGTA) added to give calcium-free perfusate.

Double perfusion system

A double perfusion system was constructed which enabled two different parts of a single oocyte to be perfused separately from each other. A small hole of approximately 200 μm diameter was made in the thin divide between two perfusion lanes, through which a small patch of oocyte could protrude and form a tight seal. The whole-cell-except-patch area of the oocyte and the protruding patch could then be perfused separately, while calcium release and entry were monitored by electrophysiological recording. The two perfusion lanes merged 20 mm downstream of the oocyte allowing both areas being perfused to be voltage-clamped at 0 mV by a virtual ground circuit.

Preparation of cell extracts

Cell extracts were prepared by homogenisation of thapsigargin-treated *Xenopus* oocytes in an ice-cold solution of 0.1 M HCl. The homogenate was centrifuged, the supernatant kept and the pH adjusted to 7.5 with KOH. The cell extract was concentrated using a lyophiliser to give a volume a tenth of the original volume of the oocytes. This concentrated cell extract was then introduced into oocytes using a Drummond Nanoject injector, while measuring the calcium-activated chloride current under two-electrode voltage-clamp.

Transfer of cytoplasm from oocyte to oocyte

Using the Drummond Nanoject apparatus fitted with a large diameter (50–100 μm) injection pipette, cytoplasm could be sucked out of an oocyte and injected into another oocyte within 30 s.

Flash photolysis of caged compounds

In some experiments, oocytes were injected using the Drumond Nanoject apparatus with 23 nl of 1 mM caged $InsP_3$ (Calbiochem, La Jolla, USA) corresponding to an intracellular concentration of approximately 20 μM. The oocytes were left to equilibrate for over 30 min before proceeding with the electrophysiological measurements. UV light flashes were produced by a xenon flash lamp system XF-10 (Hi-Tech Scientific, Salisbury, UK).

Results and discussion

Calcium release and capacitative calcium entry are colocalised

If a diffusible messenger is involved in the activation of capacitative calcium entry, then one would predict that depletion of intracellular calcium stores in a localised region would release the putative calcium influx factor into the cytosol. Since the characterisation of the putative calcium influx factor suggests that it is of a low molecular weight [5, 7], one would expect the putative calcium influx factor to diffuse away from the localised region of the depleted stores. The putative calcium influx factor could then activate calcium entry over a much larger area of the plasma membrane than the localised region of the depleted stores. In order to test this possibility, a perfusion system was constructed which allowed local applications of perfusate to two separate regions of a single *Xenopus* oocyte. The perfusion system had two lanes separated by a thin divide in which a 200-μm hole had been made. A small region of the oocyte (approximately 1% of the total plasma membrane area) protruded through this hole, allowing it to be perfused separately

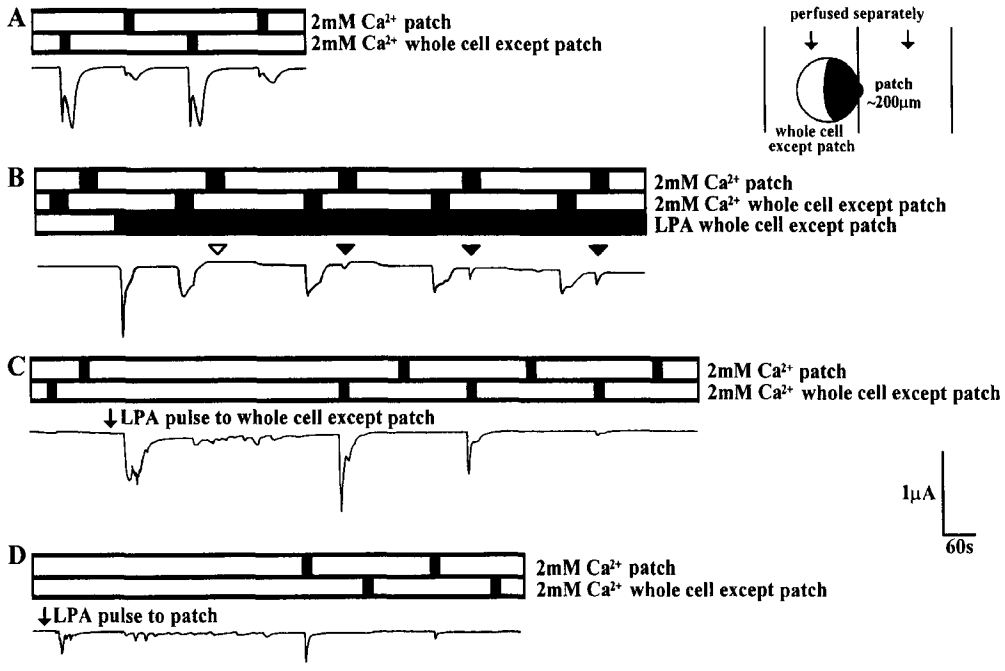

Fig. 1A–D Capacitative calcium entry is colocalised with calcium release in *Xenopus* oocytes. **A** Calcium pulses applied extracellularly to either the whole-cell-except-patch or the patch area of a thapsigargin-treated *Xenopus* oocyte evoked calcium-activated chloride currents monitored under voltage-clamp at –60 mV. This indicates that calcium entry can be detected independently across the two regions of the oocyte. **B** Long-lasting application of 5 μM lysophosphatidic acid (LPA) to the whole-cell-except-patch area of the oocyte evoked release of calcium from intracellular calcium stores as indicated by the initial transient response. Application of calcium pulses to the whole-cell-except-patch area showed that calcium entry was activated across this region by the agonist stimulation and remained activated for the remainder of the experiment. Calcium pulses to the patch area indicated that calcium entry was not initially activated (*open triangle*) but later calcium entry could be detected (*closed triangles*), suggesting that a cytosolic diffusible messenger (probably $InsP_3$) reached the patch area during prolonged agonist stimulation. **C** A 20-s application of 5 μM LPA to the whole-cell-except-patch area of the oocyte evoked calcium entry only across the stimulated region of the oocyte. Since no calcium entry was detected across the patch area, this experiment suggests that calcium entry is limited to regions of the plasma membrane beneath which the intracellular calcium stores have been depleted. **D** A 20-s application of 5 μM LPA to the patch area of the oocyte evoked calcium entry exclusively across the patch region of the oocyte, and remained active for many minutes

from the bulk of the oocyte. This small region of the plasma membrane will be referred to as the patch area, whereas the bulk of the oocyte membrane will be referred to as the whole-cell-except-patch area.

First it was essential to test whether calcium influx could be reliably detected across the patch area. In order to do this all the intracellular calcium stores were depleted by incubation of the *Xenopus* oocytes with 1 μM thapsigargin in calcium-free medium for over 3 h [9]. Calcium entry was monitored by applying 2 mM calcium pulses to the patch area and recording the calcium-entry-evoked inward chloride currents under voltage-clamp at –60 mV. *Xenopus* oocytes are polar cells showing distinct features in the animal and vegetal hemispheres, for example there are many more $InsP_3$ receptors in the animal than in the vegetal hemisphere [10]. Application of calcium pulses via the patch area of the perfusion system to the vegetal pole evoked currents which were 3%±1.5% (mean±SEM, n = 5) the magnitude of the calcium-entry-evoked currents at the animal pole (0.10 μA±0.02 μA, n = 5) of the same oocytes over the same area of plasma membrane. Thus orientation of the oocyte such that the animal pole protruded into the patch perfusion lane allowed capacitative calcium entry to be monitored reliably over a localised region of the oocyte. When calcium pulses were applied separately to either the patch or the whole-cell-except-patch area, calcium-entry-evoked currents could be recorded in a consistent manner (Fig. 1A, n = 5).

To test for any significant leak of perfusate from one perfusion lane to the other, calcium-entry-evoked currents were recorded from thapsigargin-treated oocytes by pulsed applications of 2 mM calcium to one region of the oocyte, whilst the other region of the oocyte was continually perfused with 2 mM calcium-containing medium. The currents evoked by these calcium pulses did not differ in magnitude or kinetics from those evoked in the absence of calcium on the other region of the cell. Thus it appears that the seal created around the patch area of the oocyte when placed in the double perfusion system is sufficiently tight to allow completely independent measurement of calcium entry at either region of the oocyte.

Xenopus oocytes express endogenous receptors for lysophosphatidic acid (LPA) which are linked to the phosphoinositide signalling pathway resulting in the activation of capacitative calcium entry [9]. Application of 5 μM LPA to the whole-cell-except-patch area of the cell in calcium-free medium caused release of calcium from intracellular stores as shown by the initial transient inward calcium-activated chloride current (Fig. 1B, n = 7). Continued application of LPA to the whole-cell-except-patch area of the cell caused a maximal activation of calcium entry within 2 min. At this stage there was no evi-

dence of calcium entry across the patch area (Fig. 1B, open triangle). With time, however, calcium entry was gradually activated on the patch membrane (Fig. 1B, closed triangles). This shows that a diffusible messenger was able to pass from the stimulated region to the resting area of the oocyte. This messenger is likely to be $InsP_3$, which is thought to be a highly diffusible messenger [11, 12]. During continuous stimulation of the whole-cell-except-patch area of the oocyte it is likely that a sufficiently high concentration of $InsP_3$ reached the patch area leading to store depletion and activation of calcium entry. Alternatively, a diffusible messenger generated by the depletion of the intracellular calcium stores [4–7] could have activated calcium entry in the patch area.

In order to distinguish between these possibilities, short-lasting applications of LPA were made to the whole-cell-except-patch area of the cell. Under these conditions $InsP_3$ is generated transiently, thus limiting its ability to spread to the unstimulated region of the oocyte. Since the capacitative calcium entry pathway remains activated for as long as the stores remain depleted, one might expect a putative calcium influx factor to be able to diffuse to the patch area and activate calcium influx, but this was not the case. Application of 5 μM LPA for 20 s to the whole-cell-except-patch area evoked calcium release from intracellular stores as shown by the large initial inward chloride current (Fig. 1C, $n = 10$). Subsequent applications of calcium pulses to the whole-cell-except-patch area revealed that entry was activated across the region that had been stimulated by the agonist (Fig. 1C). However, calcium pulses to the patch showed that calcium entry had not been activated in this region up to 15 min later (Fig. 1C). Note that in the absence of a continuous application of LPA, the calcium entry responses gradually declined, presumably due to store refilling. In separate experiments where calcium pulsing was delayed to maximise the diffusion of a putative calcium influx factor, there was still no evidence of calcium entry across the patch (data not shown, $n = 5$). This suggests that the putative calcium influx factor was not able diffuse to the patch area, and that the diffusible agent observed to activate calcium entry under conditions of constant stimulation of the whole-cell-except-patch area was not the putative calcium influx factor, but probably $InsP_3$. Thus if a calcium influx factor is produced after depletion of intracellular calcium stores then it does not appear to be highly diffusible. Previous characterisations of the putative calcium influx factor suggest that it is of a low molecular weight [5, 7] and thus should diffuse rapidly in an inert medium.

If a low molecular weight soluble calcium influx factor is responsible for the activation of capacitative calcium entry in *Xenopus* oocytes, then the data of Fig. 1C suggest that the putative calcium influx factor is degraded very rapidly and is thus unable to diffuse any considerable distance, and in fact appears to be much less diffusible than $InsP_3$ according to the interpretation of the experiment shown in Fig. 1B. Another possibility is that a low molecular weight messenger is not involved in the activation of capacitative calcium entry.

If the putative calcium influx factor is very rapidly degraded then it seems unlikely that capacitative calcium entry could be activated locally across the region of the patch membrane, which faces a large bulk of unstimulated cytoplasm capable of diluting out and degrading a locally produced diffusible agent. Short-lasting (20 s) 5 μM LPA stimulation of the patch area alone showed that calcium entry was evoked only on the patch (Fig. 1D, $n = 7$) and not across the rest of the cell. The fact that calcium entry was still able to be detected across the patch area 10 min after agonist removal suggests that the putative calcium influx factor remains active in the localised region.

These experiments indicate that capacitative calcium entry is colocalised with calcium release evoked by short-lasting applications of agonist. This colocalisation suggests that the signal from the depleted intracellular calcium stores to the plasma membrane activating calcium influx is not highly diffusible. If a putative low molecular weight calcium influx factor is involved then the data indicate that the messenger must be both rapidly produced and rapidly degraded to allow for the observed tight spatial coupling of the signal. Alternatively, the signal involved in linking store depletion to calcium entry may not be a low molecular weight soluble messenger.

Injection of cell extracts or cytoplasm from thapsigargin-treated oocytes does not activate calcium entry when injected into control oocytes

Xenopus oocytes have previously been injected with cell extracts from thapsigargin-treated Jurkat cells to test for the presence of a putative calcium influx factor [7]. After injection of these Jurkat cell extracts, calcium influx could be detected as monitored by the activation of the calcium-activated chloride current in a manner dependent on extracellular calcium [7]. If *Xenopus* oocytes themselves produce such a calcium influx factor, then it should be possible to prepare cell extracts from *Xenopus* oocytes with depleted intracellular calcium stores and inject them back into other oocytes to activate calcium entry. To test this possibility, all the intracellular calcium stores of a group of approximately 100 *Xenopus* oocytes were depleted by thapsigargin treatment as described above. Cell extracts were then prepared following the method of Randriamampita and Tsien [5]. The thapsigargin-treated oocytes were homogenised under acidic conditions; concentrated in a lyophiliser (to approx. 10 times the normal cytoplasm concentration), neutralised and then injected into untreated *Xenopus* oocytes. Injection of up to 100 nl (10% oocyte volume) of this cell extract did not activate calcium entry as tested by applying pulses of 2 mM calcium and monitoring the calcium-activated chloride current as described above ($n = 14$).

To avoid the problems associated with the preparation of cell extracts, cytoplasm from thapsigargin-treated oocytes was directly injected into untreated oocytes in an attempt to activate capacitative calcium influx. The utilisat-

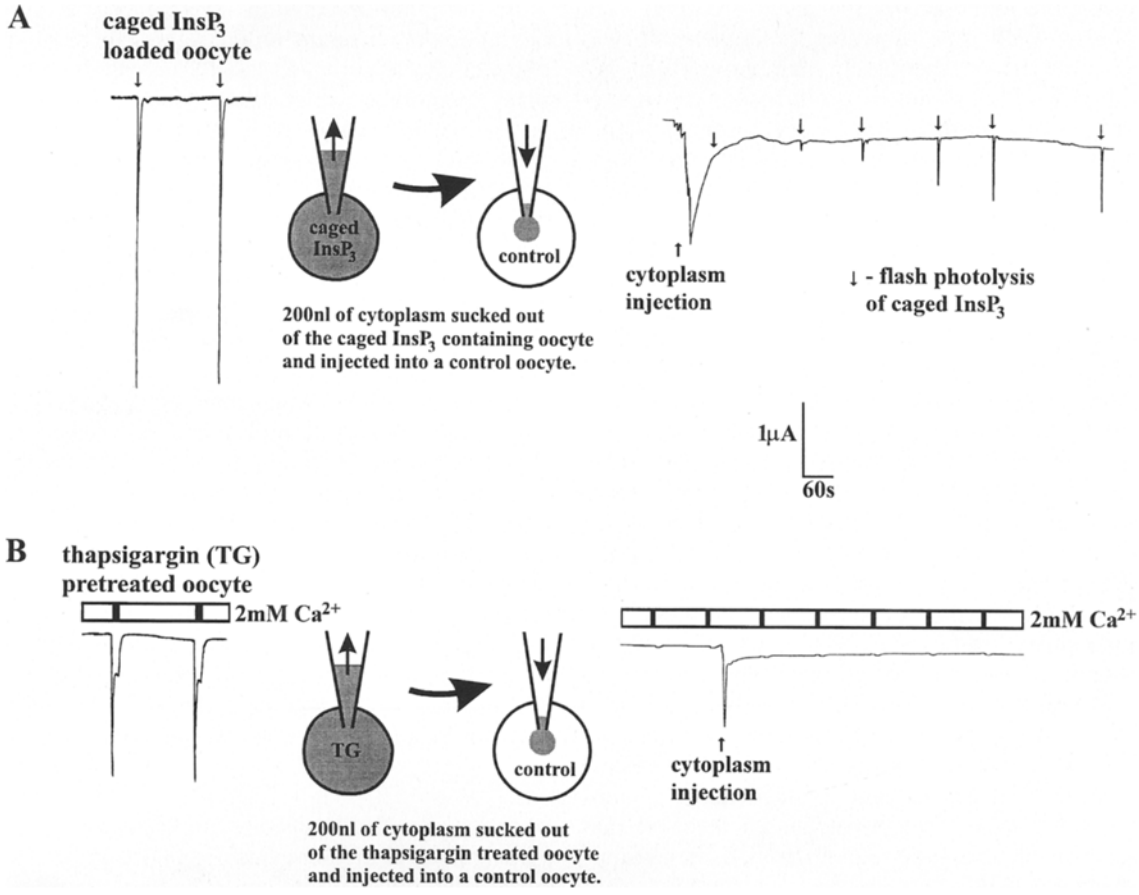

Fig. 2A,B Cytosol from *Xenopus* oocytes with depleted intracellular calcium stores does not appear to contain a putative calcium influx factor. **A** Pulses of UV light (*arrows*) to oocytes loaded with caged InsP_3 evoked calcium release detected as an inward calcium-activated chloride current under voltage-clamp. Cytoplasm (200 nl) was sucked out from the oocyte and injected into a control oocyte within 30 s. Subsequent UV flashes to the injected oocyte again evoked calcium-activated chloride currents, suggesting that the injection of 200 nl of cytoplasm does not destroy the calcium signalling apparatus of the oocyte. **B** Calcium pulses to a thapsigargin-treated oocyte evoked calcium-activated chloride currents, showing that capacitative calcium entry was activated. Cytoplasm (200 nl) from this oocyte was removed and injected into a control oocyte within 30 s. Subsequent calcium pulses to the injected oocyte show that calcium entry was not activated, suggesting that the bulk cytoplasm of an oocyte with depleted intracellular calcium stores does not contain a putative calcium influx factor

ion of cytoplasm has several advantages over cell extracts. Firstly, a single thapsigargin-treated oocyte can be used in which the capacitative calcium influx pathway is known to be activated. Secondly, any factors present in the cytoplasm will remain undisturbed in their native environment until injected into the control oocyte, preventing any degradation until the injection stage. If a diffusible calcium influx factor is present in the bulk cytosol of store-depleted oocytes then an injection of 200 nl (approx. 20% of the volume of a *Xenopus* oocyte) of cytosol from a thapsigargin-treated oocyte would be expected to reveal an easily observable calcium influx in the control oocyte. Another advantage of injecting bulk cytoplasm is that the recipient oocyte will receive calcium-depleted endoplasmic reticulum, which should continue to generate the putative calcium influx factor, since the inhibition of calcium pumps by thapsigargin is essentially irreversible.

To check whether the calcium signalling machinery remains intact after a cytoplasm injection, cytoplasm was transferred from an oocyte injected with caged InsP_3 into a control oocyte. The donor oocytes injected with caged InsP_3 gave typical responses to UV light flashes of 3.3 μA±0.4 μA (n = 4) (Fig. 2A, as previously described by Petersen and Berridge [9]). Injection of 200 nl of cytoplasm from these caged InsP_3-containing oocytes initially caused a transient injection artefact caused by the large diameter pipettes required for cytoplasm injections. After injection, responses to UV light flashes gradually increased to give responses of approximately 1 μA (n = 4) (Fig. 2A). An oocyte injected with 200 nl of cytoplasm is thus able to function normally with respect to InsP_3-evoked release and activation of chloride current. In the next experiment, cytoplasm was obtained from oocytes treated with thapsigargin to activate calcium influx. Application of 2 mM calcium pulses to these thapsigargin-pretreated donor oocytes evoked typical responses of 1.4 μA±0.2 μA (n = 13) (Fig. 2B). Transfer of 200 nl of cytoplasm from such thapsigargin-treated oocytes into control oocytes did not activate calcium entry (n = 13) (Fig. 2B).

Thus the bulk cytoplasm of the *Xenopus* oocytes with depleted intracellular calcium stores did not appear to contain a highly diffusible calcium influx factor. If a pu-

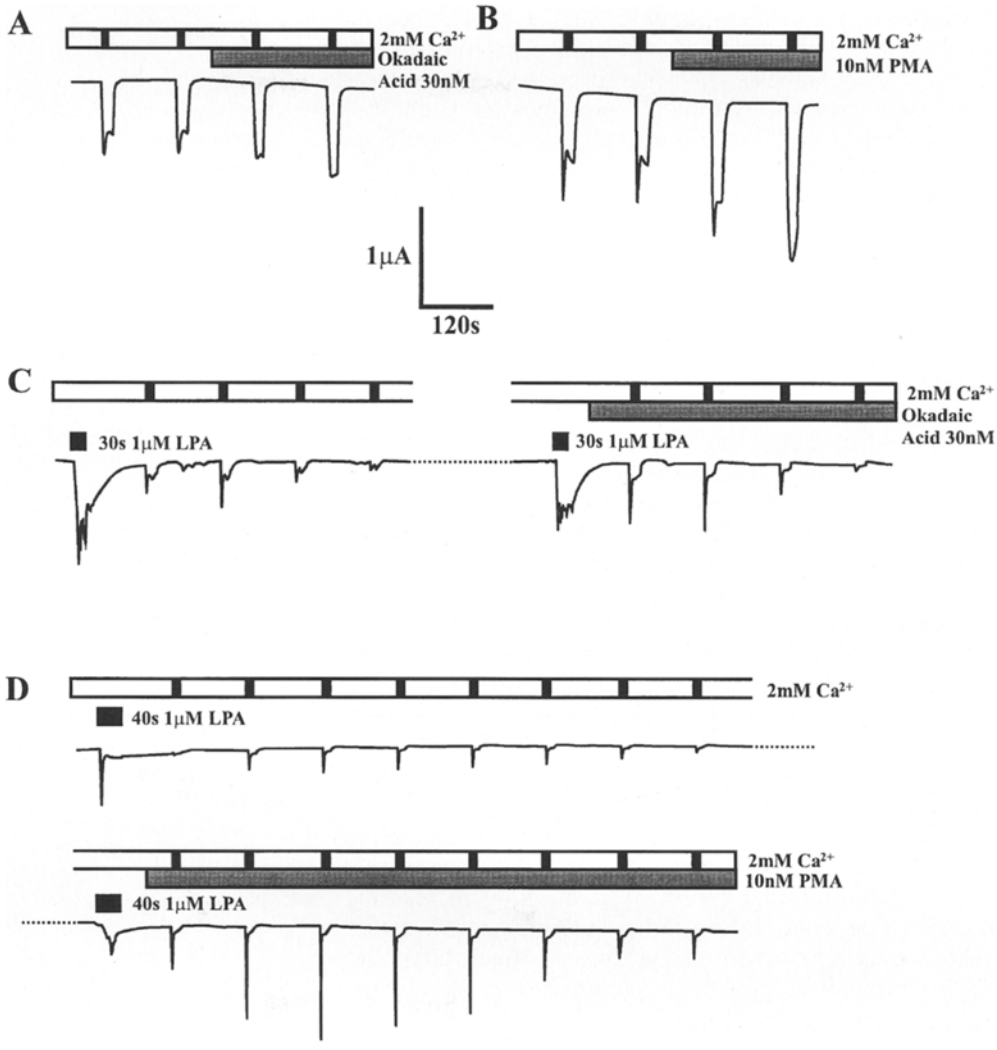

Fig. 3A–D Potentiation of capacitative calcium influx by okadaic acid may be mediated by increased protein kinase C phosphorylation. **A** Calcium-entry-evoked currents recorded from thapsigargin-treated oocytes were potentiated by application of 30 nM of the protein phosphatase inhibitor okadaic acid. Additionally the early inactivation kinetics of the calcium-entry-evoked currents disappeared after application of okadaic acid. **B** Stimulation of protein kinase C by application of 10 nM phorbol myristate acetate (PMA) potentiated calcium-entry-evoked currents in thapsigargin-treated oocytes. Again the early inactivation phase of the currents is blocked by protein kinase C stimulation. **C** Okadaic acid (30 nM) increased the calcium entry evoked by identical 30-s applications of 1 μM LPA. **D** Calcium entry evoked by 40-s applications of 1 μM LPA was potentiated after stimulation of protein kinase C by 10 nM PMA

tative low molecular weight calcium influx factor exists then it may be localised to the region beneath the plasma membrane by both rapid generation and rapid degradation (as discussed above) and thus is not present in the bulk cytoplasm. Alternatively, the signal coupling store depletion to calcium entry may not be a low molecular weight soluble messenger.

Okadaic acid potentiates calcium entry via protein kinase C

Previous reports agree that the protein phosphatase inhibitor okadaic acid potentiates calcium influx stimulated by the putative calcium influx factor [4, 6, 7]. Randriamampita and Tsien [6] suggested that okadaic acid inhibited the degradation of the calcium influx factor. Zweifach and Lewis [13] have also found that calcium entry is potentiated by okadaic acid, although in a manner apparently independent of the protein phosphatase activity of okadaic acid. It thus appears useful to discern in further detail the molecular mechanism by which okadaic acid potentiates calcium influx in *Xenopus* oocytes.

As shown in Fig. 1A, capacitative calcium entry can be reliably monitored by applications of 2 mM calcium to thapsigargin-treated oocytes. Application of 30 nM okadaic acid potentiated the calcium influx-evoked currents by 42%±10% (n = 10), but did not stimulate calcium entry in untreated control oocytes (n = 5) as previously reported [4, 7]. The calcium-entry-evoked currents before application of okadaic acid display complex kinetics during the calcium pulse (Fig. 3A) presumably due to calcium-dependent feedback processes observed in many cell types [9, 14, 15]. After okadaic acid application, the early rapid inactivation phase of the evoked currents disappeared and this may account for the potentiation of calcium entry (Fig. 3A). These effects are very similar to those observed upon stimulation of protein kinase C [9, 16] following application of 10 nM of the phorbol ester PMA (potentiation 32%±7%, n = 8) (Fig. 3B). Calcium influx evoked by LPA stimulation can also be potentiated by 30 nM okadaic acid (n = 9, Fig. 3C). Again a very similar potentiation of calcium influx is attained by application of 10 nM PMA (n = 5, Fig. 3D). These results suggest that okadaic acid may mediate its effects by inhibiting the phosphatase involved

in removing the protein-kinase-C-mediated phosphorylation events responsible for potentiating calcium influx. To test this hypothesis, thapsigargin-treated oocytes were incubated with 100 nM of the protein kinase inhibitor staurosporine for 1 h. Staurosporine has no significant effect on the calcium-entry-evoked currents in thapsigargin-treated oocytes, but does completely abolish the effects on calcium entry evoked by 10 nM PMA (n = 6). Application of okadaic acid to these staurosporine-treated oocytes did not potentiate thapsigargin-evoked calcium influx (inhibition of 6%±4%, n = 6), suggesting that the okadaic acid effects do not occur in the absence of protein kinase activity. These experiments confirm that okadaic acid can modulate calcium entry. While previous authors have interpreted such an effect in terms of enhancing the action of a putative calcium influx factor [4, 6, 7], an alternative explanation is that okadaic acid may act by augmenting protein-kinase-C-mediated phosphorylation, which can enhance calcium entry in *Xenopus* oocytes.

Capacitative calcium entry in *Xenopus* oocytes appears to be highly localised to the region of stimulation. These results seem to indicate that capacitative calcium entry might be localised to the membrane regions lying in close juxtaposition to empty stores. This colocalisation of capacitative calcium entry with calcium release suggests that a highly diffusible calcium influx factor is not involved in the activation of capacitative calcium entry. These data would also fit the physiological role of this calcium entry pathway functioning in refilling only the depleted intracellular calcium stores, rather than activating calcium entry across a much larger region including areas where calcium stores are already full.

If a putative low molecular weight calcium influx factor is the signal coupling the depletion of intracellular calcium stores to the activation of calcium entry, then its diffusibility appears to be limited by rapid degradation, whilst its continued activity in a localised region requires that it also be rapidly generated. Alternatively the signal for the activation of capacitative calcium entry may not involve a low molecular weight soluble messenger. One possibility is that small GTP-binding proteins are involved in the activation of capacitative calcium entry [17, 18], although in *Xenopus* oocytes there is no evidence for this mechanism [16]. Another possibility is that the capacitative calcium entry channel protein is involved in a direct interaction with a regulatory protein, such as the InsP_3 receptor, on the intracellular calcium stores (recently reviewed by Berridge [15]). Homologues of *Drosophila trp* have recently been implicated as capacitative calcium entry channels [19–22] and interestingly have been found to have ankyrin-like repeats which could be involved in protein interactions, in particular with the recently identified ankyrin binding region of the InsP_3 receptor [23].

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References

1. Berridge MJ (1993) Inositol trisphosphate and calcium signalling. Nature 361:315–325
2. Clapham DE (1995) Calcium signaling. Cell 80:259–268
3. Putney JW Jr (1990) Capacitative calcium entry revisited. Cell Calcium 11:611–624
4. Parekh AB, Terlau H, Stühmer W (1993) Depletion of $InsP_3$ stores activates a Ca^{2+} and K^+ current by means of a phosphatase and a diffusible messenger. Nature 364:814–818
5. Randriamampita C, Tsien RY (1993) Emptying of intracellular Ca^{2+} stores releases a novel small messenger that stimulates Ca^{2+} influx. Nature 364:809–814
6. Randriamampita C, Tsien RY (1995) Degradation of a calcium influx factor (CIF) can be blocked by phosphatase inhibitors or chelation of Ca^{2+}. J Biol Chem 270:29–32
7. Thomas D, Hanley MR (1995) Evaluation of calcium influx factors from stimulated Jurkat T-lymphocytes by microinjection into *Xenopus* oocytes. J Biol Chem 270:6429–6432
8. Paradiso AM, Mason SJ, Lazarowski ER, Boucher RC (1995) Membrane-restricted regulation of Ca^{2+} release and influx in polarized epithelia. Nature 377:643–646
9. Petersen CCH, Berridge MJ (1994) Regulation of capacitative calcium entry in *Xenopus* oocytes by calcium and PKC. J Biol Chem 269:32246–32253
10. Kume S, Muto A, Aruga J, Nakagawa J, Michikawa T, Furuichi T, Nakade S, Okano H, Mikoshiba K (1993) The *Xenopus* IP_3 receptor: structure, function, and localization in oocytes and eggs. Cell 73:555–570
11. Allbritton NL, Meyer T, Stryer L (1992) Range of messenger action of calcium ion and inositol 1,4,5-trisphosphate. Science 258:1812–1815
12. Kasai H, Petersen OH (1994) Spatial dynamics of 2nd messengers – IP_3 and cAMP as long-range and associative messengers. Trends Neurosci 17:95–101
13. Zweifach A, Lewis RS (1995) Slow calcium-dependent inactivation of depletion-activated calcium current. J Biol Chem 270:14445–14451
14. Hoth M, Penner R (1993) Calcium release-activated calcium current in rat mast cells. J Physiol (Lond) 465:359–386
15. Berridge MJ (1995) Capacitative calcium entry. Biochem J 312:1–11
16. Petersen CCH, Berridge MJ (1995) G-protein regulation of capacitative calcium entry in *Xenopus* oocytes may be mediated by protein kinases A and C. Biochem J 307:663–668
17. Bird G St J, Putney JW Jr (1993) Inhibition of thapsigargin-induced calcium entry by mircoinjected guanine nucleotide analogues. J Biol Chem 268:21486–21488
18. Fasolato C, Hoth M, Penner R (1993) A GTP-dependent step in the activation mechanism of capacitative calcium influx. J Biol Chem 268:20737–20740
19. Hardie RC, Minke B (1993) Novel Ca^{2+} channels underlying transduction in *Drosophila* photoreceptors: implications for phosphoinositide-mediated Ca^{2+} mobilization. Trends Neurosci 16:371–376
20. Vaca L, Sinkins WG, Hu Y, Kunze DL, Schilling WP (1994) Activation of recombinant *trp* by thapsigargin in Sf9 insect cells. Am J Physiol 267:C1501–C1505
21. Petersen CCH, Berridge MJ, Borgese MF, Bennett DL (1995) Putative capacitative calcium entry channels – expression of *Drosophila trp* and evidence for the existence of vertebrate homologs. Biochem J 311:41–44
22. Wes PD, Chevesich J, Jeromin A, Rosenberg C, Stetten G, Montell C (1995) TRPC1, a human homolog of a *Drosophila* store-operated channel. Proc Natl Acad Sci USA 92:9652–9656
23. Bourguignon LYW, Jin H (1995) Identification of the ankyrin-binding domain of the mouse T-lymphoma cell inositol 1,4,5-trisphosphate (IP_3) receptor and its role in the regulation of IP_3-mediated internal Ca^{2+} release. J Biol Chem 270:7257–7260