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Ca^{2+} OSCILLATIONS AND Ca^{2+} INFLUX IN *XENOPUS* OOCYTES EXPRESSING A NOVEL 5-HYDROXYTRYPTAMINE RECEPTOR

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SUMMARY

1. We expressed a novel 5-hydroxytryptamine receptor (SRL) in *Xenopus* oocytes and monitored cytosolic Ca^{2+} through the endogenous Ca^{2+}-dependent Cl^- channel activity using the double electrode voltage-clamp technique.

2. 5-Hydroxytryptamine (5-HT; 200 nM) led to an initial rapid oscillatory current followed by a pronounced secondary one, which lasted long after 5-HT wash-out (20–40 min) and was not affected by the receptor antagonist yohimbine.

3. Both phases of the current were abolished by heparin demonstrating a key role for IP_3-induced Ca^{2+} release.

4. Caffeine (10 mM) alone did not evoke a current but reduced both phases of the current evoked by 5-HT. Ryanodine had no effect. No evidence for Ca^{2+}-induced Ca^{2+} release was found.

5. The secondary current activated by 5-HT was sensitive to changes in extracellular Ca^{2+}, suggesting it was evoked by Ca^{2+} influx. Reducing external Na^+ did not affect this current, demonstrating that it was rather specific for Ca^{2+}.

6. The Ca^{2+} influx pathway was much more sensitive to Cd^{2+} than other divalent ions (Co^{2+}, Mn^{2+}, Sr^{2+}, Ba^{2+}). It was insensitive to verapamil.

7. Injection of D-*myo*-inositol 1,4,5-trisphosphate,3-deoxy-3-fluoro (IP_3-F; an analogue not metabolized to D-*myo*-inositol 1,3,4,5-tetrakisphosphate (IP_4)), evoked either an oscillatory current or a rapid current followed by a sustained secondary one. The latter was sensitive to external Ca^{2+} and was blocked by Cd^{2+}. Heparin dramatically reduced the IP_3-F-evoked current.

8. Perfusion in Ca^{2+}-free solution, once a secondary current had been generated, significantly decreased the amount of intracellular Ca^{2+} mobilized by 5-HT, indicating that the Ca^{2+} influx pathway plays an important role in pool refilling.

9. Block of Ca^{2+} influx by Cd^{2+} in cells that were oscillating transiently increased the amplitude and then either abolished the oscillations or made them irregular. This effect was also elicited by increasing external Ca^{2+}.

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10. These results demonstrate that 5-HT, acting via IP_3, both releases Ca^{2+} from internal stores and evokes a pronounced Ca^{2+} influx. This last step is activated by pool depletion and is important for both refilling of the agonist-sensitive stores and modifying the oscillatory pattern.

INTRODUCTION

Activation of receptors linked to inositol 1,4,5-trisphosphate (IP_3) are capable of evoking oscillatory increases in cytosolic free Ca^{2+} in a variety of cell types (Berridge & Irvine, 1989). This form of signalling has several advantages over a simple elevation of free Ca^{2+} because information will be carried not only in the amplitude of each spike but also in its frequency. The mechanism of generating oscillations is therefore of widespread interest and several models have been put forward (reviewed by Tsien & Tsien, 1990). *Xenopus* oocytes represent a convenient system for studying oscillations and Ca^{2+} wave propagation (Berridge, 1988, 1991; Lechleiter & Clapham, 1992) because first they have numerous Ca^{2+}-dependent Cl^- channels in the plasma membrane (Barish, 1983; Takahashi, Neher & Sakmann, 1987) enabling rapid measurement of free Ca^{2+} and second, due to their large size, various components of the Ca^{2+} signalling system can be easily injected into oocytes (e.g. receptor RNA, drugs) without dialysing the cell and losing cytoplasmic components.

The mechanism of generating oscillations in *Xenopus* oocytes is unclear but IP_3 plays a central role since its injection is capable of evoking the oscillations (Oron, Dascal, Nadler & Lupu, 1985). Oscillations are present in Ca^{2+}-free solution (Parker & Miledi, 1986) suggesting intracellular Ca^{2+} is required for their generation. Fluctuations in the levels of IP_3 do not seem to be necessary because injection of non-metabolizable analogues of IP_3 evoke oscillations (Delisle, Krause, Denning, Potter & Walsh, 1990). Various intracellular models have been proposed. All require interaction between Ca^{2+} and IP_3, but differ in the nature of the interaction. One version suggests interaction through two intracellular Ca^{2+} pools via the process of Ca^{2+}-induced Ca^{2+} release (Berridge, 1988, 1991). Another postulates negative feedback by Ca^{2+} on the IP_3-induced Ca^{2+} release step (i.e. one store; Parker & Ivorra, 1990).

In this report, we have investigated the changes in cytosolic Ca^{2+} evoked by SRL, a novel 5-hydroxytryptamine receptor recently cloned from rat smooth muscle (Foguet *et al.* 1992). Our results suggest that IP_3 has two roles. First, it evokes Ca^{2+} oscillations which can be most easily accounted for in terms of a one pool model and second, IP_3-induced Ca^{2+} release activates a pharmacologically unusual Ca^{2+} influx pathway which refills the IP_3 pools, the signal for activation being the state of pool depletion. This Ca^{2+} influx modifies the oscillatory pattern.

METHODS

Preparation of complementary RNA

Complementary RNA (cRNA) was synthesized as described previously (Foguet *et al.* 1992).

Preparation of Xenopus oocytes

Oocytes were prepared using a protocol essentially as previously described (Methfessel, Witzemann, Takahashi, Mishina, Numa & Sakmann, 1986). Frogs were anaesthetized with tricaine and bundles of oocytes were removed after an abdominal incisure. The incision was then

surgically closed. Using such a procedure, frogs could be repeatedly used at intervals of 1 month or more.

Oocytes were incubated in nominally Ca^{2+}-free Barth's medium (composition below) with 2·8 mg ml^{-1} Worthingtons collagenase (type II) for around 3 h at room temperature (18–22 °C), in order to remove the follicular layer. Oocytes were then washed several times in normal Barth's medium and healthy ones selected under a microscope (sometimes parts of the follicular layer remained, and this was removed mechanically with fine forceps). The vitelline membrane was not removed.

Oocytes were injected manually with 20–30 ng cRNA using finely pulled microelectrodes. The oocytes were incubated in Barth's medium at 18 °C for 3–6 days. We have found that good expression occurs around this time following injection.

Electrophysiology

Currents were recorded using the standard double electrode voltage-clamp technique, held at −80 mV unless otherwise indicated. Electrodes (resistance 1–3 MΩ and filled with 2 M KCl) were generally Sylgard-coated to a third of the distance down the shank to reduce electrode capacitance. Oocytes were perfused with normal frog Ringer (NFR) solution for at least 10 min before application of 5-HT. 5-HT was always applied for 1 min at 200 nM (since this concentration did not induce long-lasting desensitization). At least 15 min recovery time was allowed between 5-HT applications, depending on the degree of recovery of the holding current.

Solutions

The normal frog Ringer solution had the following composition (mM): NaCl, 115; KCl, 2·5; $CaCl_2$, 1·8; Hepes, 10 (pH 7·2 with NaOH at room temperature).

The Barth's medium, in which the cells were incubated, contained (mM): NaCl, 88; KCl, 1; $NaHCO_3$, 2·4; $MgSO_4$, 0·82; $Ca(NO_3)_2$, 0·3; $CaCl_2$, 0·41; Tris-HCl, 7·5; pH 7·4.

Injection of drugs into oocytes

In some experiments, drugs were injected directly into oocytes. This was achieved by removing the cell from the experimental chamber and injecting using a protocol identical to that for cRNA injection. At least 15 min was allowed before subsequent voltage clamping. For injections of Ca^{2+} or IP_3, a third electrode containing the solution was impaled whilst recordings were still being made from the cell. This method was used because the responses to these agents were rapid in onset.

Drugs used

5-Hydroxytryptamine (creatinine sulphate complex), yohimbine, caffeine, heparin (low molecular weight), niflumic acid, manganese chloride, guanosine 5′-*O*-(2-thiodiphosphate) (GDPβS), ethyleneglycol-bis-(β-aminoethylether)*N,N,N′,N′*-tetraacetic acid (EGTA), verapamil, barium and strontium chloride were all purchased from Sigma (Germany), cadmium and cobalt chloride from Merck (USA), and ryanodine and D-*myo*-inositol 1, 4, 5, trisphosphate, 3-deoxy-3-fluoro (IP_3-F) from Calbiochem (La Jolla, CA, USA). Thyrotrophin-releasing hormone receptor cRNA was a generous gift from Dr Barros, University of Oviedo, Spain.

RESULTS

Variability among oocytes

Those *Xenopus* oocytes that had been injected with SRL cRNA generally responded in one of two ways. The most common response (48 of 78 cells) consisted of a rapid inward current (range 40–900 nA) with a few oscillatory currents on the peak, a partial decline and then a smaller sustained secondary current (range 20–100 nA, duration 5–40 min, e.g. Fig. 1*A*). Other cells (8 of 78) exhibited a rapid initial current which then declined into a series of sustained oscillations (amplitude 20–400 nA). Such cells generally did not show a clear secondary current (Fig. 1*B*). A few cells (17 of 78) responded in a way intermediate between the above two extremes (Fig. 1*C*). Five cells responded with a transient non-oscillatory current and no secondary phase. There was no obvious correlation between the type of response

and membrane potential, cell size, morphology or the number of days after injection (3–6). Although the pattern of the current did not differ much during the time course of an experiment, the peak amplitudes tended to run down with time. For

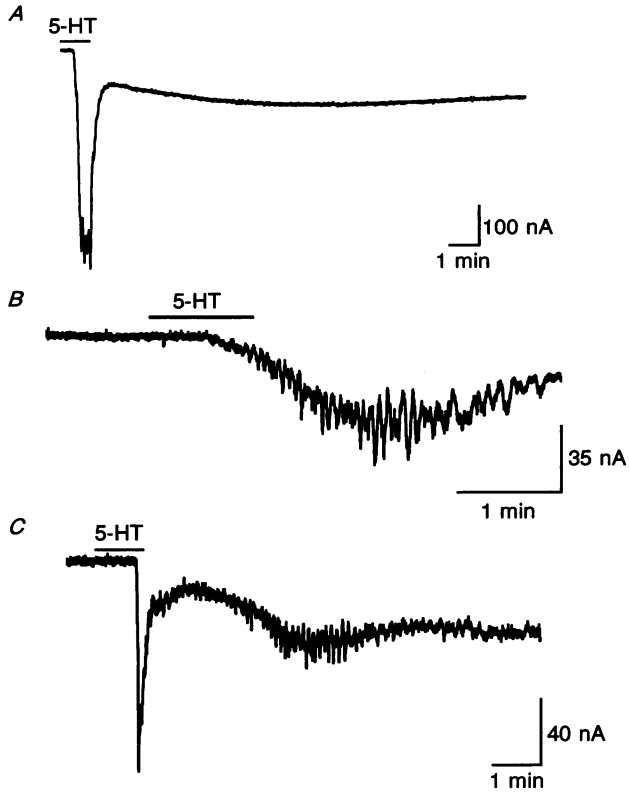

Fig. 1. Different patterns of current evoked by 200 nM 5-HT applied for 1 min as indicated. See text for details. *A*, *B* and *C* are from different cells. Inward current is depicted as downward.

example, the third application of 5-HT (at 30 min intervals) evoked currents which had peaks of 70–90 % of the first control. Run-down was more pronounced in those cells whose first response was large. Uninjected oocytes or those injected with distilled water consistently failed to respond to 5-HT ($n = 4$ and 8 respectively).

5-Hydroxytryptamine activates Ca^{2+}-dependent Cl^- channels

Activation of certain exogenous receptors can link to a transduction system endogenous to the oocyte resulting in an elevation of cytosolic free Ca^{2+} and subsequent activation of the Cl^- channels. SRL is also able to link into this endogenous transduction system for the following reasons. First, the currents reversed around −25 mV ($n = 3$). This corresponds to the chloride reversal potential (E_{Cl}) in oocytes under our conditions (Barish, 1983). Second, injection of the Ca^{2+}

chelator EGTA (final concentration *ca* 1 mM) abolished the currents. Third, the Ca_I^{2+}-dependent chloride channel blocker niflumic acid reversibly blocked the responses to 5-HT (250 μM, $n = 3$).

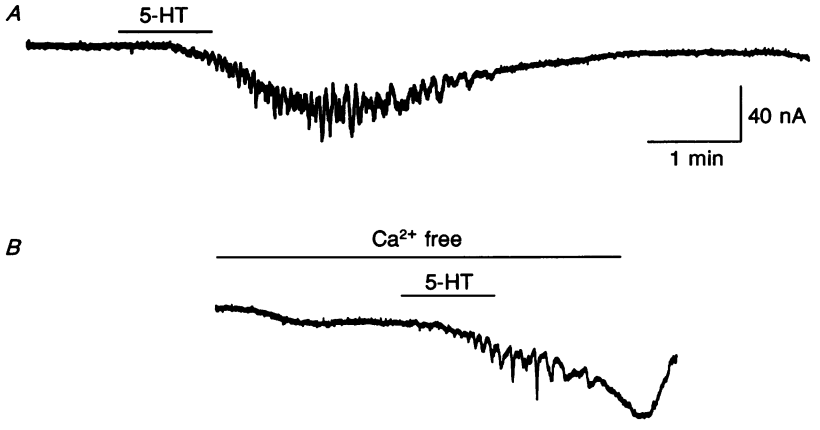

Fig. 2. Effects of Ca^{2+} removal on the 5-HT current. *A*, control response to 5-HT. *B*, after 2 min perfusion in Ca^{2+}-free 0·1 mM EGTA solution, 5-HT was applied in Ca^{2+}-free EGTA for 1 min followed by wash in the EGTA-containing solution.

Both intracellular and extracellular Ca^{2+} are required

The source of Ca^{2+} mobilized by 5-HT could be intracellular and/or extracellular. To discriminate between these possibilities, oocytes were perfused with Ca^{2+}-free 0·1 mM EGTA Ringer solution for 2 min. This resulted in a reversible increase in the holding current (20–400 nA) required to maintain the voltage clamp (see also Lupu-Meiri, Shapira & Oron, 1990) and may reflect a need for Ca^{2+} in maintaining membrane stability. Application of 5-HT in Ca^{2+}-free solution still evoked an oscillatory current (Fig. 2) but the amplitude was reduced by 20–45 % (33·5 $\pm$ 10·0 %, mean $\pm$ S.D., $n = 4$). This suggests that first, both intracellular and extracellular Ca^{2+} are required to maintain the size of the current and second, the basic mechanism for generating oscillations is intracellular in origin.

Role for IP_3: sensitivity to heparin

Since the diffusible second messenger IP_3 provides a link between receptor activation in the plasma membrane and Ca^{2+} release from internal stores (Berridge & Irvine, 1989), we injected heparin (an inhibitor of the IP_3 receptor; Ghosh, Eis, Mullaney, Ebert & Gill, 1988) to see whether IP_3 was involved in the currents to 5-HT. Prior injection of heparin (final concentration around 1 mg ml^{-1} which is supramaximal in many cell types) abolished the currents in four cells and reduced it by 90 % in a fifth (Fig. 3*A*). Oocytes injected with distilled water instead still responded to 5-HT (86 $\pm$ 24 % of control, $n = 4$). In three of these latter oocytes, subsequent injection of heparin abolished the currents in two cells and reduced it to 8 % in the third. Activation of the thyrotrophin-releasing hormone (TRH) receptor,

once exogeneously expressed in oocytes, evokes an oscillatory current and also increases IP_3 levels in oocytes (Oron, Gillo, Straub & Gershengorn, 1987). If heparin is working by blocking the IP_3 receptor, one predicts that the TRH current will also be blocked. Injection of the same stock solution of heparin that abolished the 5-

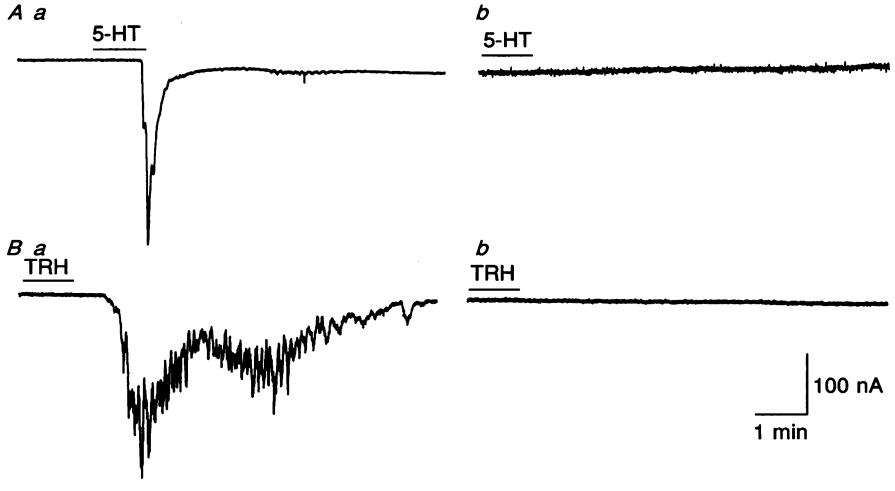

Fig. 3. The inhibitory effect of heparin on receptor-activated currents. *A a* and *b*, responses to 5-HT before and 30 min after injection of *ca* 1 mg ml^{-1} heparin, respectively. *Ba* and *b*, responses to 1 μM thyrotrophin-releasing hormone (TRH; following expression of the thyrotrophin-releasing hormone receptor) before and after injection of the same stock solution of heparin as in *A*).

HT currents also abolished the currents to TRH (3 of 3 cells, Fig. 3*B*). These results demonstrate that activation of the IP_3 receptor plays a pivotal role in all phases of the 5-HT currents.

Role for a G protein

Since receptor activation usually links to IP_3 production via a G protein, we reasoned that the SRL receptor also coupled to a G protein. Injection of the poorly hydrolysable GDP analogue, GDPβS (final concentration 500 μM), reduced the 5-HT currents to $11{\cdot}7 \pm 3{\cdot}0$ % ($n = 3$).

Caffeine sensitivity

Caffeine is capable of evoking intracellular Ca^{2+} release in a variety of cell types through the mechanism of Ca^{2+}-induced Ca^{2+} release (CICR) (Endo, 1977).

Cells were perfused with 10 mM caffeine for 4 min before application of 5-HT in the continuous presence of caffeine. In four cells the currents to 5-HT were abolished. If the cells were maintained in caffeine following 5-HT wash-out, the secondary current was also barely detectable. In four other cells, a small response was elicited by 5-HT (10–35 % of control) but the latency (after washing out 5-HT) was

increased from 40–63 s (measured from the onset of application of 5-HT) to 90–150 s. In these cells, the secondary current was also reduced (Fig. 4).

We altered the protocol such that caffeine was applied 4–8 min after 5-HT wash-out in ten cells expressing either a sustained secondary current or an oscillatory

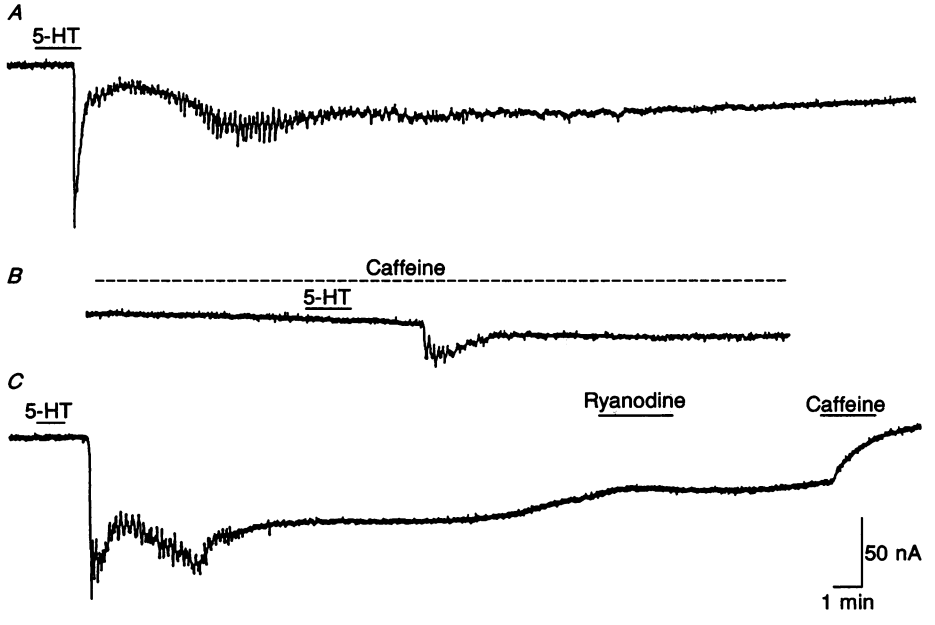

Fig. 4. Effects of caffeine on currents evoked by 5-HT. *A*, control current. This returned to the prestimulation value after 26 min. *B*, after 4 min pretreatment with 10 mM caffeine. Note the lack of effect of caffeine itself. *A* and *B* are from the same cell. *C*, comparison of ryanodine (100 μM) and caffeine (10 mM) on the same cell.

response. Caffeine (10 mM) rapidly attenuated the current (abolished in 3 cells and reduced by 50–85 % in the others) although the rate at which this occurred varied (between 1 and 4 min). This effect was reversible. The caffeine block is therefore not not a process which has brief kinetics following receptor stimulation.

In all cells (18), we failed to observe a current in response to caffeine alone. Particularly in those cells where caffeine abolished the responses, one would have anticipated a large current if the drug was acting through CICR. Previous experiments on the actions of caffeine in *Xenopus* oocytes have led to rather different conclusions, although actions on the Cl^- channel and through cAMP phosphodiesterase inhibition have been ruled out. Berridge (1991) suggested that caffeine triggered CICR from an IP_3-insensitive store whereas Parker & Ivorra (1991) proposed that the drug was a low-affinity antagonist of the IP_3 receptor. Although we also failed to observe a current to caffeine, one could argue that this inability by caffeine to evoke a current itself is not due to the absence of CICR but reflects low cytosolic free Ca^{2+}. This possibility arises because caffeine increases the sensitivity of the release mechanism to Ca^{2+}. Hence in low cytosolic Ca^{2+}, the rate of Ca^{2+} efflux

may be such that pool emptying occurs at a rate slow enough for the released Ca^{2+} to be adequately buffered by the cell. The following experiments were designed to see if CICR was occurring in our oocytes.

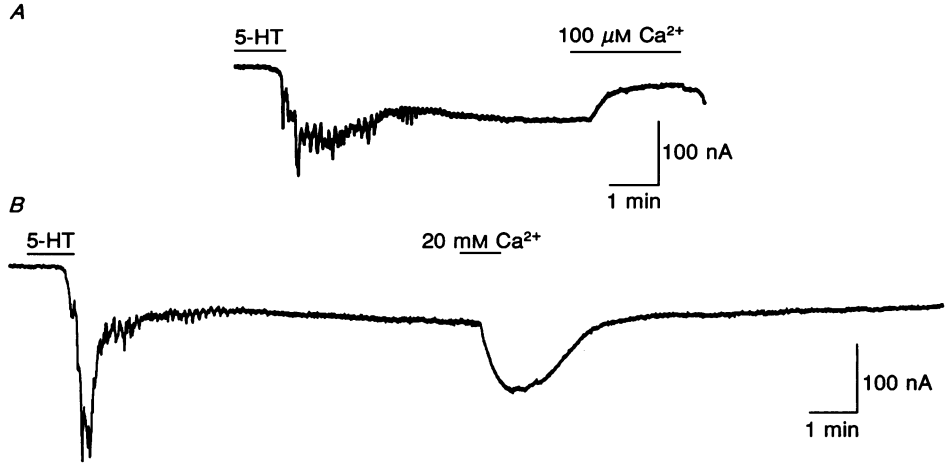

Fig. 5. The dependence of the secondary current on the external Ca^{2+} concentration. *A*, 100 μM Ca^{2+} reduced the current; *B*, 20 mM Ca^{2+} increased the current. This latter effect was the largest we observed. Note that the effects of Ca^{2+} were present more than 4 min after 5-HT wash-out. *A* and *B* are from different cells.

First, we perfused cells with high Ca^{2+}-containing Ringer solution (20 mM) in an attempt to increase cytosolic Ca^{2+}. In five of fifteen cells, this manoeuvre reversibly increased the Ca^{2+}-activated Cl^- current by 20–50 nA. In all cells, even those where the Cl^- current was activated, 10 mM caffeine was without effect.

Second, we applied ryanodine, an agent that locks the CICR channel in an open state of lowered conductance, thereby depleting the store (Rousseau, Smith & Meissner, 1987). When applied during the secondary current, after 5-HT wash-out, ryanodine had only a weak effect yet, once again, 10 mM caffeine rapidly reduced the currents in the same cells (Fig. 4*C*). These results indicate that the site of action of caffeine is different to the ryanodine receptor, which is widely taken as the CICR channel.

Properties of the secondary current

An increase in membrane Ca^{2+} permeability

Reducing external Ca^{2+} (to 100 μM) reduced the secondary current (by 30–70 %; $n = 4$, Fig. 5*A*). Perfusion with Ca^{2+}-free 0·1 mM EGTA solution (containing 5 mM Mg^{2+} to stabilize the membrane) reduced the current by around 80–90 % ($n = 3$). The remaining current presumably reflects declining intracellular Ca^{2+} release. Increasing the external Ca^{2+} (20 mM) generally increased the current (10–46 %) (6 of 9 cells, Fig. 5*B*). In three cells, 20 mM Ca^{2+} alone did not evoke a current whereas it

did after 5-HT treatment. This suggests that 5-HT is increasing membrane permeability to Ca^{2+}. This effect was time dependent in that 20 mM Ca^{2+} increased inward current shortly after 5-HT exposure (2–20 min), but was without effect a

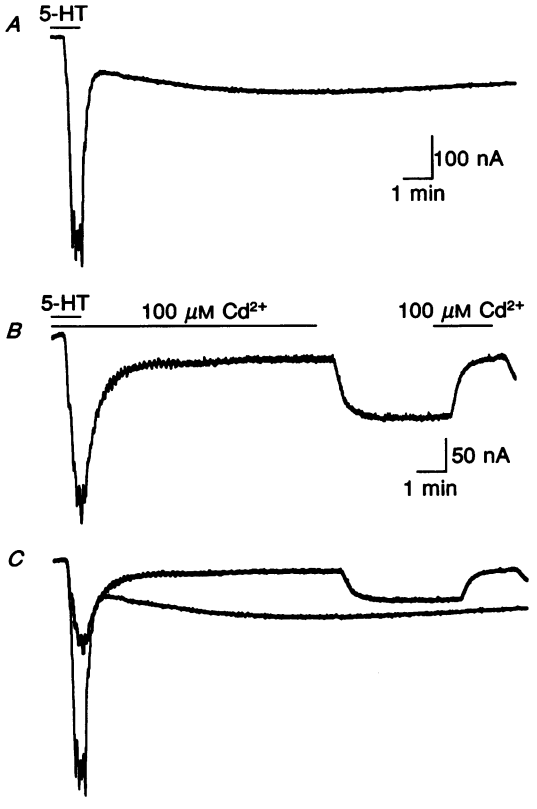

Fig. 6. Inhibitory effects of Cd^{2+} on the secondary current. *A*, control response to 5-HT. *B*, response in the same cell after simultaneous exposure to 5-HT and Cd^{2+}. The cell was maintained in Cd^{2+} for the time shown. *C*, superposition of the currents from *A* and *B*. Upper record is *B* and lower is *A*.

long time after 5-HT wash-out (*ca* 30 min). The secondary current therefore reflects Ca^{2+} influx which is maintained long after 5-HT wash-out.

Cd^{2+} block

Cadmium is widely used as a blocker of Ca^{2+} influx (Hille, 1992) and in *Xenopus* oocytes it appears to be a potent blocker of endogenous Ca^{2+} channels (Lory, Rassendren, Richard, Tiaho & Nargeot, 1990). In nine of eleven cells, 100 μM Cd^{2+} reversibly reduced the secondary current (abolished in 5 cells and reduced by 52·8 ± 16 % in the other 4; Fig. 6). In four cells where we applied Cd^{2+} simultaneously with 5-HT, the early peak current was reduced by 20–45 % (Fig.

6 *C*). The subsequent controls ran down less than this (5–20 %), suggesting the Cd^{2+}-sensitive step is activated soon after receptor stimulation.

These divalents are not blocking the Cl^- channel because they do not affect the ability of Ca^{2+} injections to evoke a Cl^- current (Mn^{2+}, Miledi & Parker, 1984; Cd^{2+}, data not shown, this study).

Ion selectivity

In some non-excitable cells, Ca^{2+} influx following IP_3-induced Ca^{2+} release permeates a non-selective cation channel (von Tscharner, Prod'hom, Baggiolini & Reuter, 1986). If such a channel was activated by SRL, then one would expect a significant decrease in the current if external Na^+ were to be reduced. Lowering Na^+ (from 115 to 10 mM, replaced by TrisCl), once the secondary current had been evoked, produced a small but reversible decrease in the current (5–20 %, $n = 4$). If KCl replaced TrisCl as the salt substituting for NaCl, then the decrease was more or less prevented, indicating that K^+ could replace Na^+. The current we are measuring, however, has two components; that activated by 5-HT and the background leak current. To see whether the small changes obtained on reducing Na^+ could be attributed to changes in the leak current, we lowered Na^+ in cells that had not been exposed to 5-HT. In five out of five cells, lowering Na^+ (115 to 10 mM, replaced by TrisCl), reduced the holding current by around 10–30 nA, which was similar to the reduction achieved on lowering Na^+ for the 5-HT-activated current. If K^+ replaced Tris as the substituting cation, then the current not only recovered but slightly increased further (4 of 5 cells). Lowering Ca^{2+} did not discernibly change the leak current. These results suggest that both a Na^+ and K^+ permeability contribute to the background current and it is this that causes the small reduction in the 5-HT current when Na^+ is reduced. Hence the influx pathway activated by 5-HT is rather selective for Ca^{2+}.

Pharmacology of the Ca^{2+} influx

Since we were voltage clamping at −80 mV, a contribution of voltage-gated Ca^{2+} channels to the Ca^{2+} influx was unlikely. Consistent with this was that verapamil, an organic blocker of voltage-gated Ca^{2+} channels, did not affect the secondary current ($n = 3$). Because Cd^{2+} blocked the Ca^{2+} influx (see above), we compared the actions of a series of divalent cations to gain some understanding of the Ca^{2+} entry pathway. Each divalent was compared to Cd^{2+} (to correct for differing sensitivities). The potency series was (all at 200 μM, and each tested in 3 cells): $Cd^{2+} \gg Co^{2+} = Mn^{2+} > Ba^{2+} = Sr^{2+}$.

It should be stressed that these currents were rather small (30–70 nA) and therefore small differences between divalents would not be picked up. What is clear is the much larger block by Cd^{2+} compared to both Mn^{2+} and Co^{2+}, which are widely used as Ca^{2+} channel blockers (Hille, 1992).

Yohimbine does not block the secondary current

Yohimbine is a potent receptor blocker of SRL (Foguet *et al.* 1992). To see whether the secondary current was independent of receptor stimulation and not due to slow

wash-out of agonist (arising from the diffusion barriers of vitelline and plasma membranes), we applied 1 μM yohimbine 2–3 min after washing out 5-HT. The currents were unaltered ($n = 3$). This suggests that, once initiated, the currents become receptor independent.

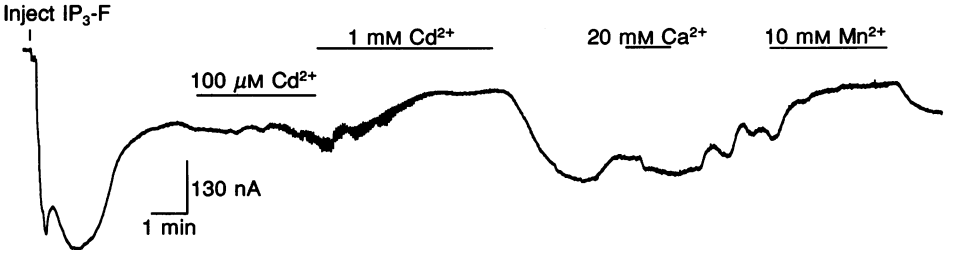

Fig. 7. Currents arising from the injection of IP_3-F and the sensitivity to divalent cations. 20 nl of 360 μM IP_3-F was injected at the bar. The initial small deflection (*ca* 20 nA) is the injection artifact. In this cell, the injection electrode was not withdrawn after injection.

Injection of IP3-F

Since heparin abolished both the oscillatory currents (which are due to intracellular Ca^{2+} release) and the sustained secondary current (due to Ca^{2+} influx), it seems reasonable to suppose that IP_3 binding to its intracellular receptor is activating both these processes. We are not the first to inject IP_3 into oocytes. Other groups have done this and observed a pronounced Ca^{2+} influx (Snyder, Krause & Welsh, 1988; Lupu-Meiri *et al.* 1990). Although not investigated, these results have all been interpreted in terms of IP_3 directly gating a Ca^{2+} influx pathway. If our interpretation is true, then it makes the testable prediction that IP_3-induced Ca^{2+} release, independent of receptor activation, should activate a Cd^{2+}-sensitive Ca^{2+} influx pathway and this should be reduced by heparin. We tested this by injection of IP_3-F, a new analogue that is as potent as IP_3 in evoking intracellular Ca^{2+} release, but is not a substrate for the 3-kinase that produces IP_4 (Kozikowski, Faruq, Aksoy, Seewald & Powis, 1990). Hence an effect would be due to IP_3 and not IP_4 or IP_3–IP_4 synergy. Injection of a large concentration of IP_3-F (final concentration *ca* 12 μM) evoked one of two types of current. One type, resembling the common 5-HT current, consisted of a rapid transient current (range 80–350 nA, mean 193 $\pm$ 64 nA) followed by a pronounced, sustained secondary phase (range 60–250 nA, mean 140 $\pm$ 21 nA) (Fig. 7). The other type of response consisted of a series of oscillations with no clear sustained phase. The type of response seemed to be determined by the donor frog, in that oscillations were generally predominant in certain donors whereas the sustained current was typical of others. For the latter type of current, the secondary phase was sensitive to the external Ca^{2+} concentration in that 100 μM Ca^{2+} perfusion reduced the current (by 30–60 %) and elevating Ca^{2+} (20 mM) increased it (by 10–40 %). The currents were sensitive to Cd^{2+}, although less so than the receptor-activated current (Fig. 7). Cd^{2+} at a concentration of 100 μM had variable effects in that it slightly decreased the current in three cells but did not have much of an

effect in two others. In four of these cells, 100 μM Cd^{2+} induced a series of small oscillations. This may reflect Ca^{2+} inhibition of Ca^{2+} release (see below). Cd^{2+} (1 mM) clearly reduced the current (abolished in 2 cells, reduced by almost 70 % in a third). Consistent with the properties of the receptor-activated current, much higher

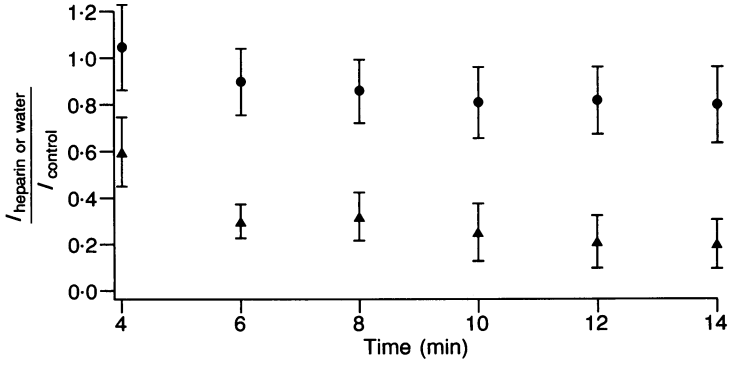

Fig. 8. Plot of the ratio of current obtained after IP_3-F injection in the absence and then presence of heparin (final concentration *ca* 1 mg ml^{-1}) or water (control). ●, water; ▲, heparin.

concentrations of Mn^{2+} were required to achieve the same block. Analysis of the oscillatory current is described later.

We took two approaches to study the effects of heparin on the IP_3-F currents. First, we compared responses of control cells with those injected with heparin taken from the same donor. Eight out of eight control cells responded to IP_3-F injection (5 cells gave oscillatory responses whereas 3 gave long-lasting secondary currents). Three of the eight cells injected with heparin failed to respond and in the other five, only oscillatory currents were observed. These results suggest that heparin reduces the probability that IP_3-F evokes a current. This sort of protocol where one compares responses between different cells has, however, the drawback that one does not know what the control current to IP_3-F was in the heparin-treated cells. This makes it difficult to accurately quantify the effect. We therefore compared the effects of IP_3-F in the absence then presence of heparin in the same cell. In these experiments, we first obtained a current to IP_3-F. Then the cell was removed and heparin (or water, in which the heparin had been dissolved) injected. Around 1 h later, the cell was reimpaled and IP_3-F injected again. Figure 8 is a plot of the ratio of the current in heparin (or water) to the control current as a function of time (data from 4 cells in each). It is evident that the current in heparin is dramatically reduced compared to those cells where water has been injected and this is more pronounced at longer times. Since heparin is a competitive antagonist of the IP_3 receptor, the relatively larger currents at short time intervals may reflect a high local IP_3-F concentration which displaces heparin before IP_3-F diffuses away and is diluted to a level unable to significantly displace the heparin from other stores.

Role of Ca^{2+} influx

Refilling the intracellular stores

The obvious question is the role of this pronounced Ca^{2+} influx. One simple possibility is that it refills the IP_3-sensitive stores, which have been emptied by

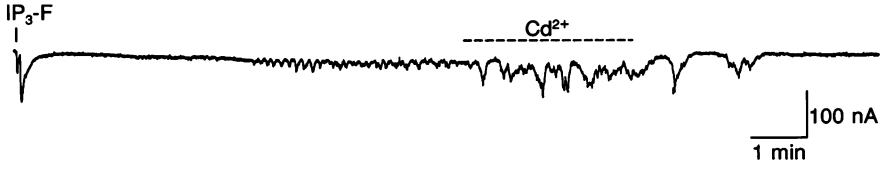

Fig. 9. Effect of 200 μM Cd^{2+} on the oscillatory currents evoked by IP_3-F. Note the increase in size as well as the irregular nature after Cd^{2+} application. This cell gave the biggest effect we observed.

agonist. To test this, we exposed cells to Ca^{2+}-free EGTA solution once a secondary current had been evoked. Cells were maintained in Ca^{2+}-free solution for around 30 min (recovery time between applications of 5-HT) before 5-HT was applied in Ca^{2+}-free solution. After exposure to Ca^{2+}-free solution, the time for onset of the current was increased (from 40 ± 3 to 83 ± 10 s, measured from time of 5-HT application) and the current (measured after 2 min) was reduced (from 85 ± 8 to 41 ± 10 %). For this analysis, we compared the currents (in the presence of Ca^{2+}) before and after exposure to Ca^{2+}-free solution with those in Ca^{2+}-free solution to correct for run-down. These results demonstrate a role for the Ca^{2+} influx pathway in pool refilling. After 5-HT wash-out, no clear current was seen at times of 4 min or longer in Ca^{2+}-free solution (i.e. no sustained Ca^{2+} influx), as expected if Ca^{2+} was the major permeating species. Readmission of Ca^{2+} around 10 min after 5-HT wash-out evoked a current with a similar time course to the secondary current (in Ca^{2+}) which would have been obtained at that time. This lends additional support to the idea that 5-HT increases membrane permeability to Ca^{2+}.

Modulation of Ca^{2+} oscillations

Since Cd^{2+} is a blocker of Ca^{2+} influx, we studied its effects on the pattern of oscillations following injection of IP_3-F in eight cells. Cd^{2+} (200 μM) produced a transient increase in amplitude of the oscillations ($1{\cdot}83 \pm 0{\cdot}30$-fold increase, we measured 5 spikes before and around 30 s after onset of the Cd^{2+} effect; Fig. 9). Cd^{2+} alone prior to IP_3-F injection did not have this effect. In three cells, the continuous presence of Cd^{2+} subsequently resulted in rapid loss of the oscillations whereas in the five others the oscillations tended to become more irregular (Fig. 9). Elevating Ca^{2+} to 20 mM, once oscillations had been evoked, resulted in a large inward current and this either abolished the oscillations ($n = 2$) or converted them into a series of irregular fluctuations ($n = 2$). These results demonstrate that IP_3-induced Ca^{2+} influx through the Cd^{2+}-sensitive pathway can modify the pattern of the Ca^{2+} oscillations.

DISCUSSION

Our main findings are that (i) 5-HT, acting through IP_3, induces oscillations in cytosolic Ca^{2+} that can be adequately explained in terms of one internal Ca^{2+} pool; (ii) the increase in IP_3 leads to a prolonged Ca^{2+} influx which is activated by IP_3-induced Ca^{2+} release; (iii) this Ca^{2+} influx pathway is rather selective for Ca^{2+} and has an unusual pharmacology; and (iv) the Ca^{2+} influx makes a significant contribution to store refilling and also modifies the pattern of oscillations.

Limitations of our approach

We have used the Ca^{2+}-dependent Cl^- channel endogenous to the *Xenopus* oocyte to monitor cytosolic free Ca^{2+}. This is an indirect method and only measures Ca^{2+} below the surface membrane. Changes in Ca^{2+} deep in the oocyte will be sensed less accurately, if at all.

We also observed much variability in the response pattern between cells (either injected or uninjected). Several sequential steps link receptor activation to the Cl^- channel. If these steps are expressed to various degrees in different cells, then such variability will be inevitable.

Mechanism for generating oscillations

Of the models describing oscillations in cytosolic free Ca^{2+}, we have been unable to demonstrate CICR in oocytes, at least in the conventional sense (activation by Ca^{2+}, ryanodine-sensitive, store depletion by caffeine with a resulting inward current). Nakai, Imagawa, Hakamata, Shigekawa, Takeshima & Numa (1990) only observed currents to caffeine following injection of RNA coding for the cardiac ryanodine receptor, suggesting little endogenous release mechanisms. The key question is how caffeine interferes with Ca^{2+} signalling. A direct action by caffeine on the IP_3 channel has been demonstrated in rat cerebellum (Brown, Sayers, Kirk, Michell & Michelangeli, 1992). Although this can explain why the currents to 5-HT were more or less abolished by caffeine pretreatment in our study, it cannot convincingly explain all our observations. Caffeine reduced the currents even 8 min after agonist wash-out. Some cells were still oscillating 8 min after removal of 5-HT. Under these conditions, one would expect IP_3 levels to have returned to prestimulation levels, since the second messenger has a half-life of the order of tens of seconds (e.g. PC12 cells, Fasolato, Pandiella, Meldolesi & Pozzan, 1988) and in oocytes IP_3 returns to basal within 5 min (Nomura, Kaneko, Kato, Yamagishi & Sugiyama, 1987). In the absence of IP_3, it is difficult to see how the IP_3 channel is still conducting Ca^{2+}. These difficulties may be reconciled by considering a model recently proposed by Lechleiter & Clapham (1992). A consequence of the bell-shaped dependence of IP_3-induced Ca^{2+} release on Ca^{2+} could be that, despite lowered levels of IP_3, the sensitivity to IP_3 is enhanced such that release continues (Bezprovanny, Watras & Ehrlich, 1991; Finch, Turner & Goldin, 1991). This could therefore confer the prolonged caffeine sensitivity. It is interesting that an increase in cytosolic Ca^{2+} through Ca^{2+} injection (Miledi & Parker, 1984), elevating extracellular Ca^{2+}, or through the Cd^{2+}-sensitive Ca^{2+} influx pathway (this study) did not evoke

oscillations despite the current being of a similar size to that of an oscillation (suggesting that this level of Ca^{2+} was not inhibiting IP_3-induced Ca^{2+} release). A simple interpretation is that an increase in cytosolic free Ca^{2+} alone is not sufficient to trigger IP_3-induced Ca^{2+} release (i.e. it cannot shift the sensitivity of IP_3-induced release to such an extent that release occurs at resting levels of IP_3). Instead it may require some elevation (or recent prior increase) in IP_3.

Although extracellular Ca^{2+} was not necessary for generating oscillations, it modified them. Reduction of Ca^{2+} influx with Cd^{2+} transiently increased the size of oscillations. This can be explained by Ca^{2+} influx contributing to Ca^{2+} feedback inhibition on IP_3-induced Ca^{2+} release. Removal of this block by Cd^{2+} enables more Ca^{2+} to be released. In a few cells, the oscillations were rapidly abolished by Cd^{2+} whereas in others they became more irregular. This would suggest that the Ca^{2+} influx pathway not only modifies the pattern of the oscillations but also contributes to rapid refilling of the stores so that oscillations can be sustained. We observed the converse of this when Ca^{2+} was increased, namely increased Ca^{2+} influx reduced the oscillations. This result is consistent with that of Delisle *et al.* (1990), who used a different approach in which, on readmitting external Ca^{2+} to a cell oscillating in response to IP_3, the oscillations were lost. IP_3-mediated Ca^{2+} influx may therefore play an important role in regulating the spatio-temporal pattern of Ca^{2+} signalling.

Link between the IP_3-sensitive store and Ca^{2+} influx

We found that 5-HT activated a Ca^{2+} influx pathway that functioned for a considerable time after agonist removal. It therefore cannot be regulated directly by a receptor (i.e. not a ligand-gated channel). Possibilities whereby this influx could be activated include the following. First, interaction between IP_3 and IP_4 to promote Ca^{2+} entry (Morris, Gallacher, Irvine & Petersen, 1987). Second, IP_3 itself directly gates a Ca^{2+} channel (Kuno & Gardner, 1987). Third, IP_3-induced Ca^{2+} release directly activates a Ca^{2+}-permeable non-selective cation channel (von Tscharner *et al.* 1986). Fourth, some factor links the extent of pool depletion to a Ca^{2+} influx mechanism (Putney, 1986). Let us consider each in turn.

Since IP_3-F (which is not metabolized to IP_4) was capable of activating the Ca^{2+} influx pathway, IP_4 is not necessary for this process. This does not rule out a contribution of this latter metabolite in the response evoked by receptor stimulation, however. It has been suggested that Ca^{2+} influx enhances metabolism of IP_3 to IP_4 (Delisle, Pittet, Potter, Lew & Welsh, 1992). This mechanism may be involved during the early stages of the 5-HT-activated Ca^{2+} influx but it is unlikely to account for the long sustained influx because IP_3 would have returned to resting values with the consequences of first, there would be no substrate for IP_4, and second, in the study of Delisle *et al.* IP_4 alone was ineffective and required the presence of IP_3.

It has been suggested that IP_3 directly gates the Ca^{2+} influx mechanism (Snyder *et al.* 1988; Lupu-Meiri *et al.* 1990). Although this may contribute to the early phase of the current, we find it unlikely that this is the only action of IP_3. Even 20–40 min after brief receptor stimulation, the current was present. The metabolism of IP_3 is incompatible with such a direct action. Moreover, the pronounced Ca^{2+} influx

evoked by injection of IP_3-F could be dramatically reduced by preinjection of heparin. Interpretation is not so simple because heparin can inhibit IP_3-gated Ca^{2+} channels in neuronal plasma membranes (Fadool & Ache, 1992). If the latter mechanism is operating in the oocyte, it means that Ca^{2+} influx through plasma membrane Ca^{2+} channels evokes the secondary current. In its simplest form this postulates Ca^{2+}-induced Ca^{2+} influx.

Ca^{2+}-induced Ca^{2+} influx has been observed in a variety of cells (e.g. von Tscharner *et al.* 1986; Lückhoff & Clapham, 1992). However, it is unlikely to have a major role since Ca^{2+} injections into oocytes evoke transient currents and not the characteristic long-lasting secondary ones described here (Miledi & Parker, 1984; our unpublished observations).

In his capacitance model, Putney proposed that depletion of the IP_3-sensitive store will trigger secondary Ca^{2+} influx (Putney, 1986). This has been observed in several cell types but not all (e.g. Jacob, 1990; Bird, Rossier, Hughes, Shears, Armstrong & Putney, 1991; but see Shuttleworth, 1990). Our results demonstrate that a similar mechanism is operating in oocytes. One obvious question is the nature of the regulatory system that relates the Ca^{2+} state of the store to the plasma membrane. Although the regulatory system is unknown, it is unlikely to be an inositol polyphosphate, judging from the kinetics (Takemura, Hughes, Thastrup & Putney, 1989; this study). A fascinating model has been suggested by Irvine (1990), who proposes that the IP_3 receptor links to an IP_4 receptor (or a Ca^{2+} pathway) in the plasma membrane, thereby linking the internal store to the membrane. This is analagous to the coupling in skeletal muscle of the ryanodine-sensitive Ca^{2+} release channel in the sarcoplasmic reticulum with the dihydropyridine-sensitive Ca^{2+} channel in the T-tubules. It is interesting that IP_3-sensitive stores have been observed close to the membrane in oocytes (Parker & Yao, 1992). It is not clear what turns off the Ca^{2+} influx. A straightforward mechanism would be refilling of the store. Although one would expect subplasmalemmal pool refilling to occur faster than the 20–40 min duration of the Ca^{2+} influx, especially since Ca^{2+} can be of the order of several tens of micromolar here, after an increase in Ca^{2+} conductance (Augustine & Neher, 1992), pools more distal to the membrane will also be activated (through interaction of the diffusing IP_3 and released Ca^{2+}) and will refill more slowly, as the cytosolic Ca^{2+} will be more effectively buffered. The duration of the Ca^{2+} influx may therefore reflect a summation of signals from depleted pools dispersed throughout the cytoplasm.

Nature of the Ca^{2+} influx pathway

Since the currents evoked by 5-HT reversed at E_{Cl} and were markedly reduced by Cl^- channel block, it is clear that the Ca^{2+} influx must be rather small. Calculations show that a whole-cell Ca^{2+}-selective current of only 2 pA can increase cytosolic Ca^{2+} at a rate of 100 nM s^{-1} in a typical cell of 10 μm diameter (Neher, 1992). Assuming the same Ca^{2+} current density and buffering capacity, this would correspond to a whole-cell current of around 200 nA in the oocyte. This is well below the pure Ca^{2+} current we detected (<10 nA). Indeed, Ca^{2+} currents in oocytes are only revealed following replacement of Ca^{2+} with high concentrations of Ba^{2+},

an unphysiological situation (Lory *et al.* 1990). We therefore used indirect approaches to probe the nature of the Ca^{2+} influx pathway. In contrast to lowering external Ca^{2+}, a reduction in Na^+ led to only a minor decrease in the current (this was in the opposite direction to what one would expect if a Na^+–Ca^{2+} exchanger were compromised) and this decrease was predominantly on the background leak current. This suggests that the influx pathway is rather selective for Ca^{2+} and is not a non-selective cation channel. This Ca^{2+} influx pathway is rather different from conventional voltage-gated Ca^{2+} channels in that lowering Ca^{2+} did not render the pathway non-selective (i.e. increased permeability to Na^+; Hess, 1990). Consistent with this was the insensitivity of the Ca^{2+} influx to organic Ca^{2+} channel blockers. Reduction of the current by divalents showed that Cd^{2+} was much better than other divalents. A Ca^{2+} current has been found in mast cells following IP_3 pool depletion (Hoth & Penner, 1992*a*) which has a divalent sensitivity similar to that observed for the Ca^{2+} influx in the oocyte (Hoth & Penner, 1992*b*). An endogenous voltage-dependent Ca^{2+} channel has been characterized in oocytes (Lory *et al.* 1990), and it has an almost identical pharmacology to the Ca^{2+} entry following pool depletion found here. If this is indeed the same channel, then pool depletion is not only activating a voltage-gated Ca^{2+} channel but also dramatically shifting its gating such that it conducts at −80 mV.

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