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MEMBRANE CURRENTS ELICITED BY DIVALENT CATIONS IN *XENOPUS* OOCYTES

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SUMMARY

1. Membrane currents were recorded from voltage-clamped *Xenopus* oocytes in response to bath application of various divalent cations.

2. In oocytes from 93 of 160 frogs tested, Co^{2+} ions evoked slow, oscillatory membrane currents. Sensitivity to Co^{2+} varied greatly between oocytes from different frogs, but was relatively consistent for oocytes taken from the same ovary. Oocytes with high sensitivity had response thresholds of 5–10 μM, and gave currents > 1 μA to 1 mM-$CoCl_2$. In contrast, oocytes from some frogs gave no oscillatory response even to 10 mM-$CoCl_2$. With responsive oocytes, Cd^{2+}, Ni^{2+}, Zn^{2+}, Mn^{2+} and Cr^{2+} ions (5 μM to 1 mM) also elicited oscillations, whereas Sr^{2+}, Ba^{2+} and Ca^{2+} (0·1–10 mM) showed very little activity, and Mg^{2+} ions, none.

3. Responses to divalent cation were well preserved in defolliculated oocytes, indicating they were generated in the oocyte membrane itself, and were not dependent on the presence of enveloping follicular cells.

4. The oscillatory currents reversed around -20 mV (the chloride equilibrium potential) and rectified strongly at potentials more negative than about -60 mV. The oscillations were mimicked by intraoocyte injection of inositol 1,4,5-trisphosphate (IP_3), were largely preserved after removal of external Ca^{2+}, but were abolished following chelation of intracellular Ca^{2+} by EGTA. Intraoocyte injection of Co^{2+} ions failed to generate oscillatory currents.

5. Currents elicited by divalent cations resembled the oocyte's oscillatory responses to acetylcholine and a serum protein. However, the response to divalent cations was not blocked by atropine and furthermore, the relative sensitivities to these agonists varied independently between oocytes from different frogs.

6. We conclude that extracellular Cd^{2+}, Ni^{2+}, Zn^{2+}, Co^{2+}, Mn^{2+} and Cr^{2+} interact with the oocyte surface to raise cytosolic levels of inositol phosphates. This causes mobilization of intracellular Ca^{2+}, in turn activating Ca^{2+}-gated Cl^- channels in the oocyte membrane.

7. In addition to the large oscillatory currents, divalent cations generated small (5–50 nA), smooth, maintained currents associated with decreases in membrane conductance. The size and ionic basis of these currents varied between oocytes from different frogs.

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8. Zinc ions also elicited smooth currents, associated with an increase in membrane conductance, and carried predominantly by K^+. This response was specific to Zn^{2+} and occurred independently of oscillatory Cl^- currents. The K^+ current was abolished by defolliculation, was potentiated by the cyclic AMP phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine, and showed facilitation with K^+ currents generated by the adenylate cyclase activator forskolin. All this suggests that Zn^{2+} ions interact with a cyclic nucleotide-activated K^+ gating mechanism which appears to be located in follicular cells.

INTRODUCTION

Acetylcholine (ACh) elicits slow membrane currents in follicle-enclosed *Xenopus* oocytes (Kusano, Miledi & Stinnakre, 1977). These responses are mediated by muscarinic receptors and result from increases in conductance to Cl^- and K^+ ions (Kusano, Miledi & Stinnakre, 1982). One component of the Cl^- current exhibits slow oscillations/fluctuations in amplitude (Kusano *et al.* 1977, 1982). Large oscillatory Cl^- responses have also been found to a serum protein (G. Tigyi, C. M. Matute, D. L. Dyer & R. Miledi, unpublished results). These Cl^- currents are well preserved in defolliculated oocytes, indicating that they arise in the oocyte membrane itself, and are not associated with enveloping follicular cells (Kusano *et al.* 1982; Miledi & Woodward, 1989*a*).

The oscillatory Cl^- responses are generated through an intracellular messenger pathway involving hydrolysis of inositol phospholipids, mobilization of intracellular Ca^{2+} by inositol phosphates, and activation of Ca^{2+}-gated Cl^- channels in the oocyte membrane (Miledi & Parker, 1984; Oron, Dascal, Nadler & Lupu, 1985; Parker & Miledi, 1986; Parker & Miledi, 1987*b*). This same response mechanism is also activated by a variety of foreign receptors induced in the oocyte following injection of mammalian messenger RNAs (e.g. Gundersen, Miledi & Parker, 1983, 1984; Takahashi, Neher & Sakmann, 1987; Meyerhoff, Morely, Schwarz & Richter, 1988; see Dascal, 1987 for review), and has provided a sensitive electrophysiological assay for cloning and characterization of foreign receptors which couple to the oocyte's phosphoinositide messenger system (e.g. Fukuda, Kubo, Akiba, Maeda, Mishima & Numa, 1987; Masu, Nakayama, Tamaki, Harada, Kuno & Nakanishi, 1987; Jackson, Blair, Marshall, Goedert & Hanley, 1988; Julius, MacDermott, Axel & Jessell, 1988).

In addition to these responses, a Cl^- current can be evoked by entry of extracellular Ca^{2+}, through Ca^{2+} channels opened by depolarization (Miledi, 1982; Barish, 1983; Miledi & Parker, 1984; Leonard, Nargeot, Snutch, Davidson & Lester, 1987). Removing external Ca^{2+}, or bathing the oocyte in Ringer solution containing Co^{2+} or Mn^{2+} ions which block Ca^{2+} entry, abolishes the current (Miledi, 1982; Barish, 1983; Miledi & Parker, 1984; Leonard *et al.* 1987). When using divalent cations for this purpose we noticed that in some oocytes millimolar concentrations of $CoCl_2$ or $MnCl_2$ elicited oscillatory membrane currents, and moreover, that oocytes from some frogs were highly responsive to a variety of divalent cations. These observations were somewhat surprising, since they suggested that ions such as Cd^{2+}, Co^{2+} and Mn^{2+}, which are commonly used to block Ca^{2+} influxes, could themselves cause a rise in intracellular free Ca^{2+}. In this paper we characterize these and other effects of

divalent cations on the oocyte. A preliminary report has been presented to the Physiological Society (Miledi, Parker & Woodward, 1988).

METHODS

Experiments were started at University College London and completed at the University of California at Irvine. Laboratory-reared adult *Xenopus laevis* were purchased from Xenopus Ltd (Surrey, UK), Xenopus I (Ann Arbor, MI, USA), Nasco (Fort Atkinson, WI, USA) and wild-caught frogs from Scientific Animal Imports (Newark, NJ, USA). Frogs were killed by decerebration and pithing and oocytes at stages V and VI (Dumont, 1972) were dissected from the ovary and stored at 16 °C in Barth's medium (in mM): NaCl, 88; KCl, 1; $Ca(NO_3)_2$, 0·33; $CaCl_2$, 0·41; $MgSO_4$, 0·82; $NaHCO_3$, 2·4; HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulphonic acid), 5; at pH 7·4, usually with nystatin (50 U ml^{-1}) and gentamycin (0·1 mg ml^{-1}). Four different oocyte preparations were used during these experiments: (1) follicle-enclosed oocytes (ovarian follicles), the oocyte still surrounded by enveloping follicular, thecal and epithelial layers (Dumont & Brummett, 1978); (2) epithelium-removed (ER) oocytes, the oocyte dissected free of inner ovarian epithelium, to facilitate insertion of microelectrodes, but still surrounded by large numbers of follicular cells (follicle cells) (Kusano *et al.* 1982; Miledi & Woodward, 1989*a*); (3) oocytes treated with collagenase to remove surrounding cell layers (Kusano *et al.* 1982; Miledi & Parker, 1984); and (4) oocytes defolliculated manually, by dissection of epithelia, followed by rolling the oocyte along poly-L-lycine-treated slides to remove remaining follicular cells. For these latter oocytes, complete removal of follicular cells was in some cases confirmed by scanning electron microscopy (for details see Miledi & Woodward, 1989*a*).

Electrical recordings were made using a conventional two-electrode voltage clamp (Miledi, 1982). During recording, the oocyte was continuously superfused with frog Ringer solution (in mM): NaCl, 115; KCl, 2; $CaCl_2$, 1·8; HEPES, 5; at pH 7·0 at room temperature (22–25 °C). Oocytes were exposed to divalent cations at concentrations from 1 μM to 10 mM, added to the Ringer solution. Agar bridges isolated bath electrodes from changes in ionic composition of the superfusate. Zero Ca^{2+} Ringer solution had the composition (in mM): NaCl, 115; KCl, 2; $MgCl_2$, 5; EGTA (ethyleneglycol-bis-(β-aminoethylether)-*N*,*N'*-tetraacetic acid), 1; HEPES, 5; pH 7·0. Intraoocyte injections of $CoCl_2$, inositol 1,4,5-trisphosphate (IP_3), EGTA and adenosine 3′,5′-cyclic monophosphate (cyclic AMP) were made by pneumatic pressure ejection from micropipettes (see Miledi & Parker, 1984). All injection solutions were made up in 10 mM-HEPES (pH 7·0) and filtered through 0·22 μm Millipore filter units. Micropipettes were filled with $CoCl_2$ and EGTA at 10–100 mM, cyclic AMP and IP_3 at 1 mM. Procedures for using aequorin to monitor intracellular Ca^{2+} were as previously described (Parker & Miledi, 1986).

Divalent cations were purchased as chloride salts from Mallinckrodt (Paris, KY, USA) or Sigma, and were made up as concentrated stocks at 0·01–1 M in distilled water. Upon storage at room temperature or 4 °C, stock solutions of some cations (e.g. $ZnCl_2$, $NiCl_2$, $CoCl_2$ and $CdCl_2$) developed light white precipitates, and were then replaced. Ringer solutions of divalent cations were made up daily, 1–10 mM cation solutions being checked, on first use, for changes in pH. In general, cations caused only a slight acidification (< 0·1 pH unit) of the Ringer solution. However, $CrCl_2$ at 1 mM reduced the pH to *ca* 6·0, and attempts to readjust this pH with NaOH caused precipitation. Ringer solution with 0·1 mM-$CrCl_2$ showed little shift in pH.

IP_3 and forskolin (*Coleus forskohlii*) were purchased from Calbiochem; all other drugs and reagents were from Sigma. Rabbit serum (T100) was purchased from Diagnostic Biochemicals (San Diego, CA, USA).

RESULTS

Oscillatory membrane currents elicited by divalent cations

Follicle-enclosed and defolliculated oocytes were routinely voltage clamped at −60 mV (Kusano *et al.* 1982), and responsiveness to divalent cations, for oocytes from different frogs, assessed by exposure to 1 mM-$CoCl_2$. This elicited predominantly

an inward, oscillatory membrane current, associated with an increase in membrane conductance. The responses to $CoCl_2$ closely resembled the oscillatory currents elicited by ACh and serum (see below), and grew in size with increasing concentration of Co^{2+} ions (Fig. 1). As with the responses to ACh (Kusano *et al.* 1982), sensitivity to Co^{2+} varied greatly when comparing oocytes taken from different frogs, but was relatively consistent in oocytes taken from the same ovary. Oocytes with highest sensitivity had response thresholds at 5–10 μM-$CoCl_2$, and produced oscillatory currents of > 1 μA when exposed to 1 mM-Co^{2+} ions. In contrast, oocytes from many frogs generated no detectable oscillatory current (< 2 nA) at concentrations up to 10 mM-$CoCl_2$. The distribution of response sizes evoked by 1 mM-$CoCl_2$, for > 500 oocytes obtained from 160 frogs, is shown in Fig. 2*A*. To simplify description we arbitrarily divided responsiveness into four groups: zero (< 2 nA), low (2–100 nA), medium (100–500 nA) and high (500–1500 nA) (Fig. 2*A*). A possible reason for the variability in sensitivity between oocytes from different frogs might be seasonal factors. However, as illustrated in Fig. 2*B*, there were no obvious seasonal variations, at least for these laboratory-reared animals, in the responsiveness of oocytes to $CoCl_2$. We also found that there were great variations in sensitivity even between frogs obtained at the same time from the same supplier. For example, freshly taken oocytes from two frogs (obtained from Xenopus I) were examined on the same day. Those from one frog responded to 1 mM-$CoCl_2$ with currents > 1 μA, whereas oocytes from the second failed to generate an oscillatory current (< 2 nA). All oocytes used in this study had diameters of 1·0–1·3 mm (stages V and VI, according to the classification of Dumont, 1972), and we did not record from oocytes at earlier stages of development.

Latencies and time courses of Co^{2+} responses were variable, and depended both on the sensitivity of oocytes and on the concentration of $CoCl_2$ applied (Fig. 1). Low-sensitivity oocytes exposed to 1 mM-Co^{2+} showed response latencies which varied between 0·2 and 6 min, while medium- and high-sensitivity oocytes typically showed latencies of 0·2–4 min with 100–10 μM-Co^{2+}, though at lower concentrations latencies could increase to as long as 12 min. When the response developed only very gradually, defining latency became problematic. The shortest latencies, ranging between about 10 and 30 s, were seen with high-sensitivity oocytes and high (1–10 mM) $CoCl_2$. These large responses to Co^{2+} often had a characteristic biphasic time course, also seen with ACh and serum responses, consisting of a 'spike' of oscillations with short latency which then subsided, to be followed by a more slowly developing peak of oscillations (Fig. 1). The relative size of these two phases varied greatly between different oocytes, and also between repeated responses in the same oocyte. In medium- and high-sensitivity oocytes oscillatory currents often persisted for several minutes after Co^{2+} had been washed from the recording chamber.

The reproducibility of responses, upon repeated exposures to $CoCl_2$, depended on concentrations applied and on sensitivities of the oocytes. Responses in low-sensitivity oocytes seldom repeated, and were often completely absent upon a second exposure to Co^{2+}. Oocytes with medium sensitivity gave more reproducible responses, but even so, application of 1 mM-Co^{2+}, given at intervals of 10–20 min, evoked progressively smaller currents (Fig. 3). Using low (50–100 μM) concentrations of $CoCl_2$ and high-sensitivity oocytes it was possible to obtain several reproducible

responses, but again there was a lot of variation between oocytes. In particular, when using concentrations close to threshold, response latencies tended to increase when repeated.

The responsiveness of oocytes to $CoCl_2$ generally declined over the first week of

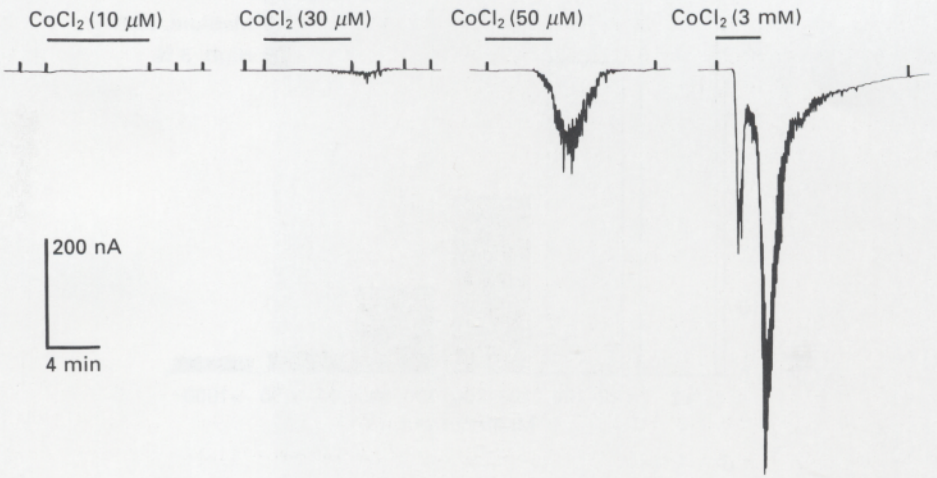

Fig. 1. Membrane currents elicited by $CoCl_2$ in a follicle-enclosed oocyte under voltage clamp. Holding potential -60 mV, periodically stepped to -50 mV to monitor changes in membrane conductance. The oocyte was continuously superfused with frog Ringer solution and exposed to different concentrations of $CoCl_2$. Exposures to $CoCl_2$ were separated by intervals of 6–15 min to minimize desensitization effects and there were no steps in command potential during exposures. Bars indicate the times of solution changes and in this case include a dead time of about 40 s. Inward current is denoted by downward deflection and capacitative transients on the voltage pulses have been touched out. Unless otherwise stated, these recording conditions were used in all following figures.

storage in Barth's medium. Low-sensitivity oocytes often lost their oscillatory response after only 1 day following isolation from the ovary, whereas high-sensitivity oocytes were sometimes responsive even after 7 days storage. Loss of responsiveness was still seen when antibiotics were omitted from the Barth's medium. Oocytes from frogs which initially showed no response did not develop sensitivity upon storage.

Oscillatory currents arise in the oocyte and not in follicular cells

Follicle-enclosed and ER oocytes are enveloped by a discontinuous monolayer of follicular cells (Dumont & Brummett, 1978; Miledi & Woodward, 1989*a*). These are coupled to the oocyte by gap junctions, which raises the possibility that membrane currents arising in follicular cells are monitored while voltage clamping the oocyte itself (Browne, Wiley & Dumont, 1979; Browne & Werner, 1984; van den Hoef, Dictus, Hage & Bluemink, 1984; authors' unpublished results). For example, cyclic nucleotide-activated K^+ conductances seen in follicle-enclosed and epithelium-removed oocytes are effectively abolished by defolliculation, suggesting that this current arises in follicular cells (Kusano *et al.* 1982; Smith, Brooker & Brooker, 1987; Miledi & Woodward, 1989*a*) (e.g. Fig. 4). In contrast, oocytes showing medium or high sensitivity to Co^{2+} ions still gave good oscillatory responses following treatment

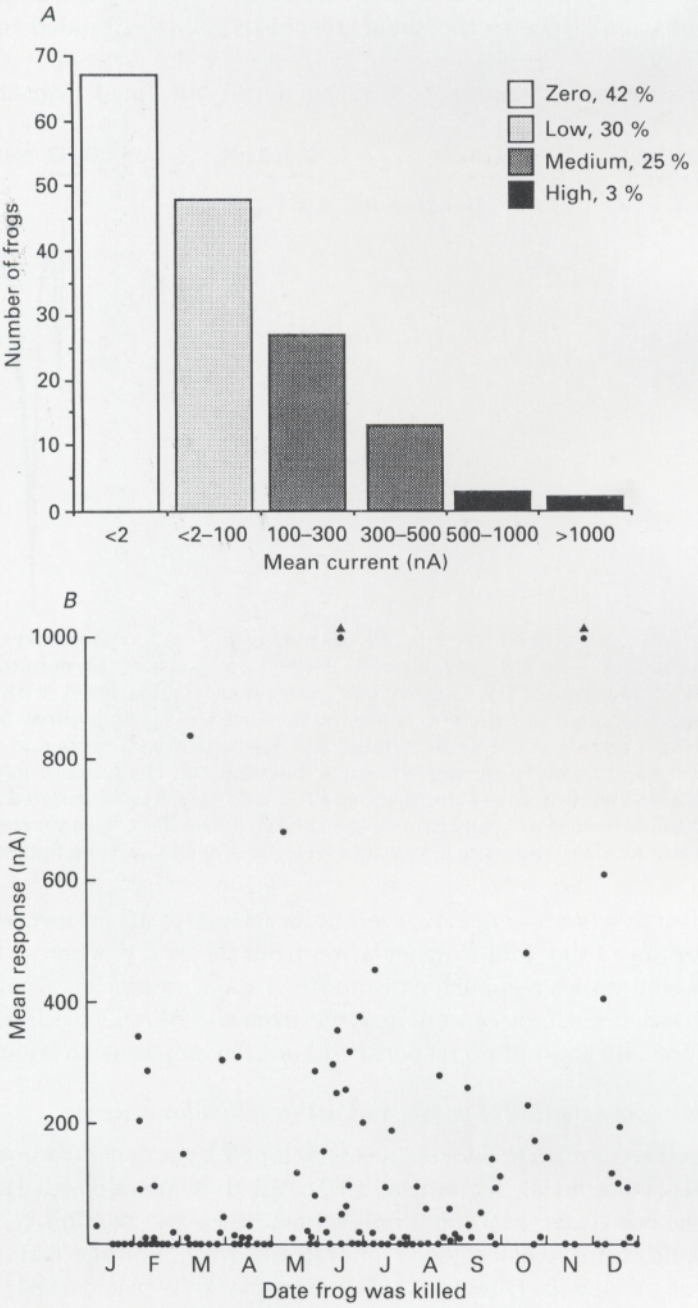

Fig. 2. *A*, distribution of responsiveness to $CoCl_2$ in oocytes from 160 frogs. For each frog two to six oocytes were exposed to 1 mM-Co^{2+} ions and the mean peak oscillatory membrane current determined. In the majority of cases, measurements were made within one day of killing the frog, using follicle-enclosed or ER oocytes. *B*, oocyte sensitivity as a function of season, in 121 laboratory-reared frogs obtained from two US suppliers (Xenopus I and Nasco). Mean responses from two to six oocytes per frog exposed to 1 mM-Co^{2+}; ▲, responses > 1 μA.

with collagenase or manual defolliculation by dissection and rolling over poly-L-lysine-treated slides (Fig. 4). For oocytes with low responsiveness to Co^{2+}, defolliculation often reduced and in some cases removed oscillatory currents.

Specificity of oscillatory responses

Oocytes with medium or high sensitivity to $CoCl_2$ were assayed for their responsiveness to a variety of divalent cations. With respect to potency, the different

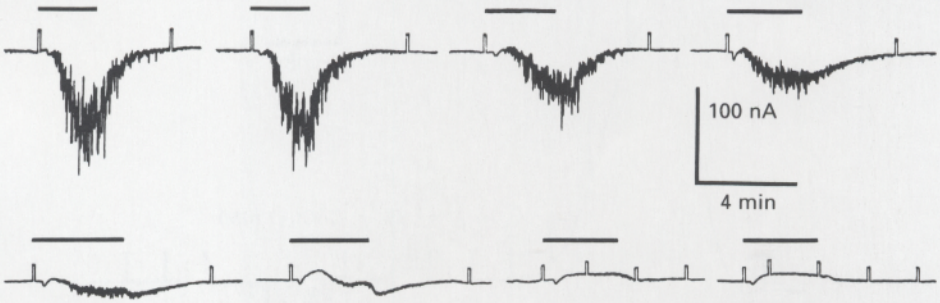

Fig. 3. Desensitization of an oscillatory response to Co^{2+} ions. An ER oocyte was repeatedly exposed to 1 mM-$CoCl_2$ at intervals of 10 min (bars). By the third exposure oscillations were markedly smaller and declined progressively over the following five exposures. When oscillations no longer dominated the Co^{2+} response (lower records), two other currents became clearly visible: (1) a small, apparently transient, inward current with short latency and (2) an underlying maintained outward current (see text for further description). In this and the following figures perfusion dead time was 10–20 s.

cations could be divided into three groups. The group with highest potency included Cd^{2+}, Ni^{2+}, Zn^{2+}, Mn^{2+}, Co^{2+} and Cr^{2+} ions, which were all active in eliciting oscillatory currents. The relative potencies of these cations were determined by comparing responses at low concentration (5–50 μM) and by defining response thresholds in the same oocyte. A problem in these experiments was the 'desensitization' described above, seen with repeated applications of different cations. To minimize this, oocytes were exposed first to the less active cations. Cd^{2+} and Ni^{2+} ions were generally more potent than Co^{+} and Mn^{2+}. Zn^{2+} ions were also active when applied at low concentrations, but at $\geqslant 1$ mM produced a curious effect, whereby the oscillatory currents became much larger shortly after Zn^{2+} was washed out. This probably arises because Zn^{2+} ions both activate the process generating oscillatory Cl^- currents and exert a direct blocking effect on the Cl^- channels themselves (R. Miledi, unpublished data). Thus upon washing, Zn^{2+} ions causing the direct block are quickly removed, while the activated oscillatory process persists for some time. Responses to Cr^{2+} were consistently smaller than to the other ions in this group, being about 10–20% of those to Co^{2+} or Mn^{2+} when compared at concentrations of 100 μM.

The second group of ions included Sr^{2+}, Ba^{2+} and Ca^{2+} (added to the 1·8 mM-Ca^{2+} already present in frog Ringer solution), which only very rarely ($< 2\%$ of frogs) elicited oscillations. These responses were always small (< 50 nA), were only seen at high concentrations of cations (5–10 mM) and were usually seen when superimposed upon a background of spontaneous oscillations. Finally, we categorized Mg^{2+} ions as

a third group, since at concentrations up to 10 mM oscillatory activity was never observed.

Though overall responsiveness to divalent cations varied greatly between oocytes taken from different frogs, sensitivity to the different cations invariably ran in

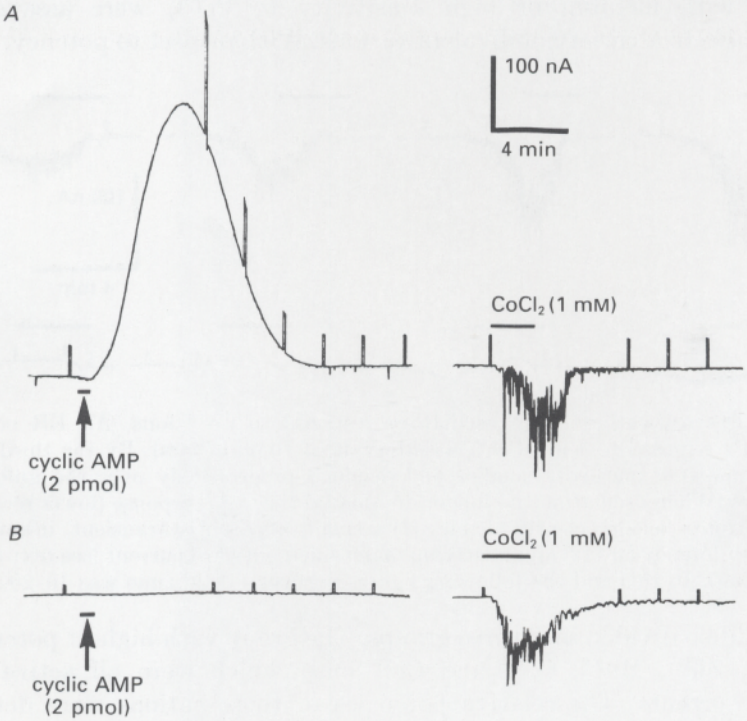

Fig. 4. Selective preservation of an oscillatory response to $CoCl_2$ through defolliculation. *A*, cyclic AMP (*ca* 2 pmol) injected (arrow) into a follicle-enclosed oocyte generated an outward K^+ current. Fifteen minutes later the follicle was exposed to 1 mM-$CoCl_2$. Electrodes and injection pipette were then removed, the oocyte's epithelial coverings dissected away and the oocyte rolled on a poly-L-lysine-treated slide to remove follicular cells. *B*, the oocyte was returned to the recording chamber after 2 h and the experiment repeated. K^+ current in response to intraoocyte cyclic AMP was abolished following defolliculation, but the oscillatory response to Co^{2+} ions was largely preserved.

parallel, with the same potency sequence. Furthermore, oscillatory responses elicited by all the different divalent cations had the same characteristics with respect to latency, time course, desensitization and preservation through defolliculation, as those described for Co^{2+} responses.

In experiments on a total of seventy-two oocytes from twenty-six frogs we determined the following potency sequence:

$$Cd^{2+} \geqslant Ni^{2+} \geqslant Zn^{2+} \geqslant Co^{2+} \geqslant Mn^{2+} > Cr^{2+} \gg Sr^{2+} \approx Ba^{2+} \approx Ca^{2+} : Mg^{2+}.$$

Given that Mg^{2+} could be > 1000 times less potent than active cations we tested whether it antagonized these responses. This was not the case, as Mg^{2+} at 10 mM had

no appreciable blocking effect on the oscillations produced by either Zn^{2+} or Cd^{2+} at 10 μM.

Ionic basis of the oscillatory currents elicited by divalent cations

The close similarity between oscillatory currents elicited by divalent cations and the oscillatory component of ACh responses strongly suggested that cation currents would likewise be carried predominantly by Cl^- ions. To confirm this, current–voltage relationships for cation responses were determined by briefly (10 s) stepping the clamp potential to different voltages during a prolonged, and reasonably stable, period of induced oscillations. Currents evoked by the same potential steps in the resting oocyte membrane were then subtracted to give the voltage dependence of divalent cation responses (Fig. 5*B*). Like spontaneous current fluctuations and ACh oscillatory responses, oscillations in response to divalent cations reversed *ca* −20 mV, the equilibrium potential for Cl^- in oocytes (e.g. Kusano *et al.* 1982; Barish, 1983), and the current rectified strongly at negative potentials. This voltage dependence is characteristic for Ca^{2+}-gated Cl^- channels in oocytes (Miledi & Parker, 1984) and suggests that responses to divalent cations involve the same chloride channels as those activated in responses to ACh and other agonists (Kusano *et al.* 1982; Takahashi *et al.* 1987).

Mechanism generating oscillatory currents

In zero Ca^{2+} Ringer solution currents evoked by divalent cations were sometimes reduced by 50–90%, though in other oocytes responses were almost unchanged. External Ca^{2+} might therefore modulate responses to divalent cations but, as with the responses to ACh and serum, it is not requisite. In contrast, chelation of intracellular Ca^{2+} by intraoocyte injection of EGTA (20–200 pmol) consistently abolished the oscillatory currents to divalent cations (eight oocytes), indicating that intracellular Ca^{2+} is necessary for the response (Fig. 6*A*) (cf. Parker, Gundersen & Miledi, 1985*a*). A role for intracellular Ca^{2+} was further suggested by experiments using aequorin as a calcium monitor (Parker & Miledi, 1986). These showed that a rise in intracellular free Ca^{2+} accompanied the membrane current response to Mn^{2+}. All this lends further support to the idea that divalent cations in some way interact with the signalling system which involves mobilization of intraoocyte Ca^{2+} by inositol phosphates (Miledi & Parker, 1984; Oron *et al.* 1985; Parker & Miledi, 1987*b*).

Divalent cation responses showed many other similarities with responses evoked through the phosphoinositide signalling system. Cation responses were to a large extent mimicked in the same oocyte by injections of IP_3 (Fig. 5*A*). Furthermore, there was clearly facilitation between responses to divalent cations and to ACh (Fig. 7) and serum, similar to the facilitation previously demonstrated between pairs of agonists (e.g. ACh and serotonin) both of which activate the phosphoinositide signalling pathway (Parker, Miledi & Sumikawa, 1987). Facilitation was not seen in oocytes which were sensitive only to ACh, and had zero responsiveness to cations (see below).

As well as causing liberation of Ca^{2+} from intracellular stores, activation of the inositol cascade in oocytes leads to an entry of extracellular Ca^{2+} through IP_3-modulated Ca^{2+} channels in the surface membrane (Parker & Miledi, 1987*a*: Snyder,

Krause & Welsh, 1988). To see whether this was also the case for divalent cation responses, we applied brief hyperpolarizing pulses during administration of Co^{2+} (Fig. 6*B* and *C*), since hyperpolarization enhances the calcium influx and gives rise to a transient inward (T_{in}) Cl^- current (Parker, Gundersen & Miledi, 1985*b*). While

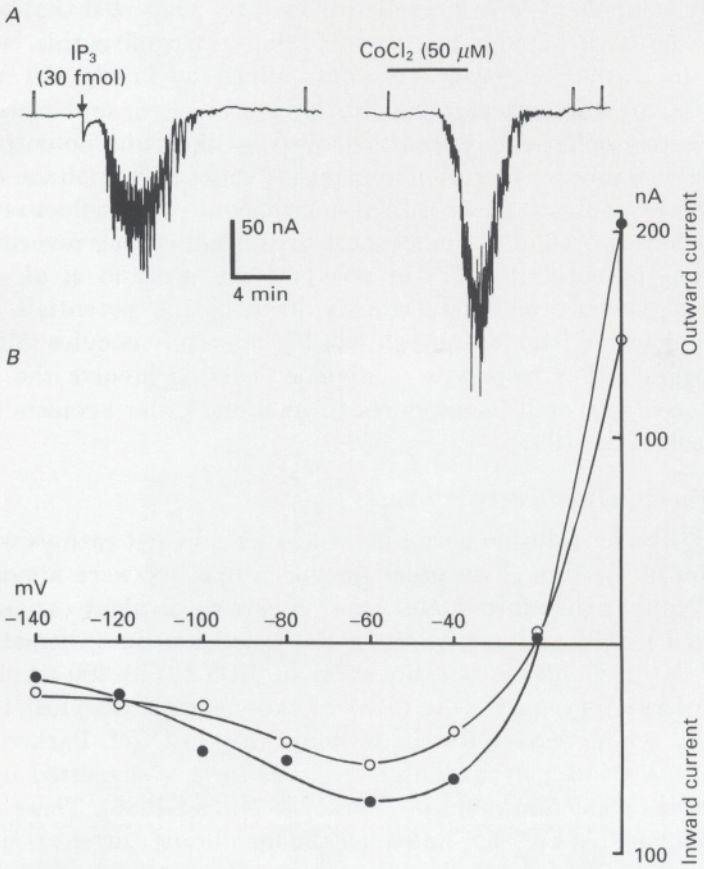

Fig. 5. Similarities between membrane currents elicited by intracellular injection of IP_3 and by bath application of $CoCl_2$. *A*, currents generated by intraoocyte injection (arrow) of IP_3 into a collagenase-treated oocyte and, after an interval of 20 min, a response to bath application of 50 μM-Co^{2+}. *B*, current–voltage relationships of oscillatory currents induced by IP_3 and Co^{2+}. Measurements were made during responses like those in (*A*), by stepping the potential to different levels during the oscillatory current. Data points show the induced currents at each potential, after subtraction of passive currents evoked by same potential steps in the resting oocyte membrane. Different oocyte to (*A*) but taken from the same frog. (○) *ca* 100 fmol IP_3, (●) 50 μM-$CoCl_2$.

Co^{2+} (1 mM) was present in the bathing solution no T_{in} current was apparent, even though a small oscillatory response was evoked. However, large currents developed shortly after washing out Co^{2+}, and subsequently declined over a few minutes. The probable explanation for this is that Co^{2+} activated the inositol signalling system, hence opening membrane Ca^{2+} channels, but at the same time Co^{2+} ions blocked entry of Ca^{2+} through these channels so that no T_{in} current was seen while the divalent ions were present (cf. Parker *et al.* 1985*b*). On washing out Co^{2+}, a T_{in}

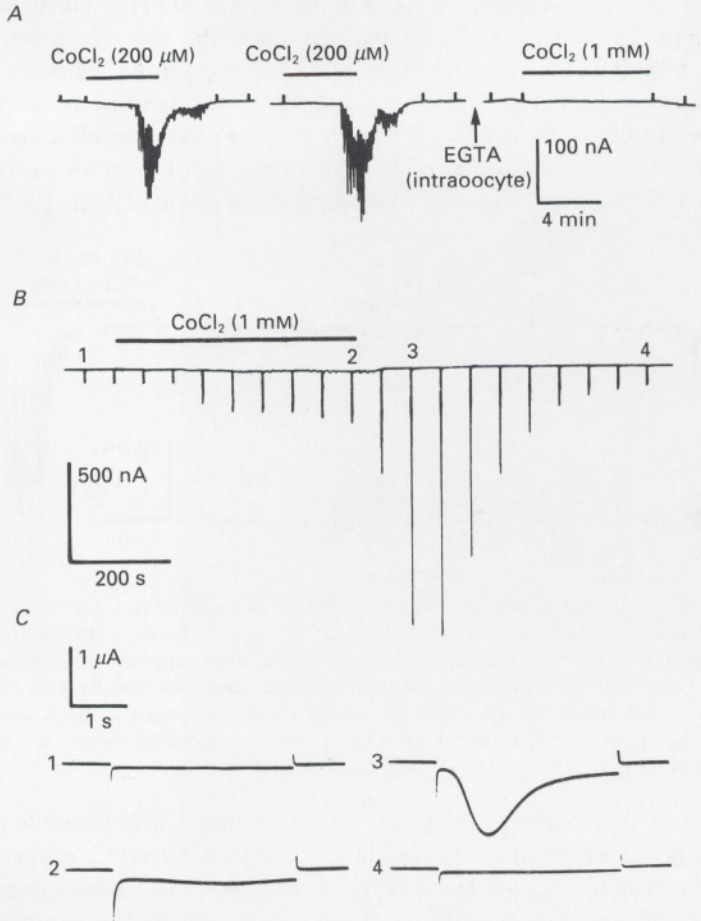

Fig. 6. *A*, dependence of the $CoCl_2$ response upon intracellular Ca^{2+}. A manually defolliculated oocyte exposed twice to 200 μM-$CoCl_2$ at an interval of 10 min gave consistent responses. A micropipette containing 50 mM-EGTA was inserted intracellularly and the oocyte loaded with *ca* 90 pmol EGTA. Ten minutes following EGTA injection the oocyte was exposed to 1 mM-$CoCl_2$, but even this higher concentration of Co^{2+} now failed to elicit any oscillatory current. *B* and *C*, activation of T_{in} current by Co^{2+}. *B*, a collagenase-treated oocyte was briefly (3 s) stepped from a holding potential of −40 to −130 mV at intervals of 1 min. Co^{2+} (1 mM) was applied for the time indicated by bar. Traces in (*C*) show selected responses to the hyperpolarizing pulses recorded at faster sweep speed.

current was then seen because the IP_3 signalling system remains activated for some time, while extracellular blocking effects of Co^{2+} are quickly removed.

Site of action of divalent cations

Intracellular injection of Co^{2+} was used to test whether responses to bath-applied divalent cations occur because they act on an external site on the oocyte membrane, or whether they cross the membrane to act intracellularly. Using defolliculated oocytes with high sensitivity, membrane current oscillations could be elicited by

pressure pulses of Co^{2+} applied from micropipettes placed close to the oocyte (vitelline) surface. To check for blockage of pipettes, the size of ejected droplets was measured in air both before and after intraoocyte injections. Though extracellular pressure pulses of Co^{2+} consistently elicited oscillatory currents, in the same oocytes intracellular injections either gave no responses, or very small currents (Fig. 8), which may have arisen because some Co^{2+} leaked out of oocytes and acted on the external surface of the membrane (cf. Parker, Gundersen & Miledi, 1985*c*).

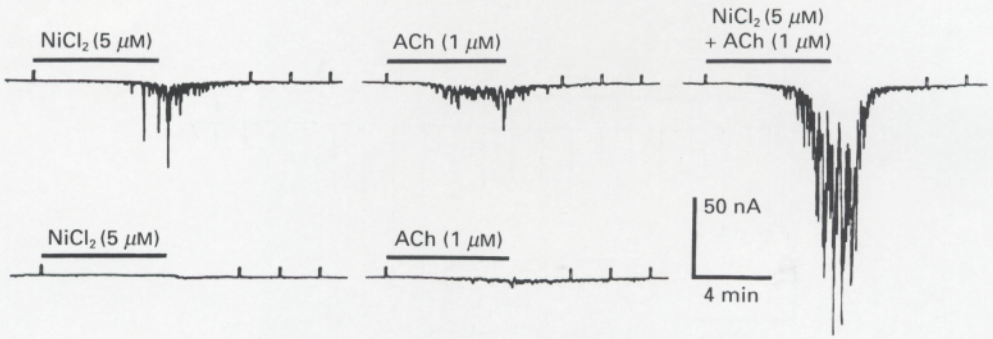

Fig. 7. Facilitation between oscillatory responses to $NiCl_2$ and ACh. Upper records: oocyte was exposed separately to 5 μM-Ni^{2+} and 1 μM-ACh, concentrations selected to be close to response thresholds in this oocyte. When agonists were applied simultaneously the response was considerably larger than the sum of currents elicited by each drug alone. Lower records: following the simultaneous application, responses to both Ni^{2+} and ACh showed desensitization. Intervals of 16 min between exposures, using an ER oocyte. Perfusion dead time *ca* 25 s.

Divalent cations therefore appear to act extracellularly, and possible sites of action include the receptors which mediate oscillatory responses to ACh and serum. Several observations allow us to exclude these. Firstly, responses to cations were not blocked by atropine, applied at concentrations (1–10 μM) which in the same cell abolished responses to ACh. Moreover, when comparing oocytes taken from different frogs, the responsiveness to ACh was independent of that to cations. For example, oocytes from some ovaries consistently responded to ACh but gave no response to $CoCl_2$ (Fig. 9*B*). In oocytes from other frogs there were responses to $CoCl_2$ but little or no current evoked by ACh (Fig. 9*C*), yet others responded to both agonists, and some to neither (Fig. 9*A* and *D*). Similarly, when comparing oocytes from different frogs for relative sensitivities to cations and serum, $CoCl_2$ responses varied between 0 and 500% of responses to a 1:25000 dilution of rabbit serum (Fig. 9*E* and *F*). Finally, there also appeared to be no consistent correlation between the presence of spontaneous oscillatory activity in an oocyte (Kusano *et al.* 1977, 1982) and its responsiveness to divalent cations. Oocytes which showed spontaneous Cl^- current fluctuations usually responded to divalent cations, but again this was not consistent, and some oocytes with spontaneous activity showed only a marginal intensification of oscillations in response to 1 mM-$CoCl_2$.

Divalent cations block 'leakage' currents

In oocytes with good sensitivity, oscillatory currents dominated the response to divalent cations. However, in oocytes which gave small or no oscillatory currents,

and in oocytes where the oscillatory currents desensitized on repeated exposure (e.g. Fig. 3), it was clear that divalent cations also elicited small underlying maintained currents. Occasionally, in follicle-enclosed and ER oocytes, the maintained currents were preceded by small transient inward currents (Fig. 3) which remain to be

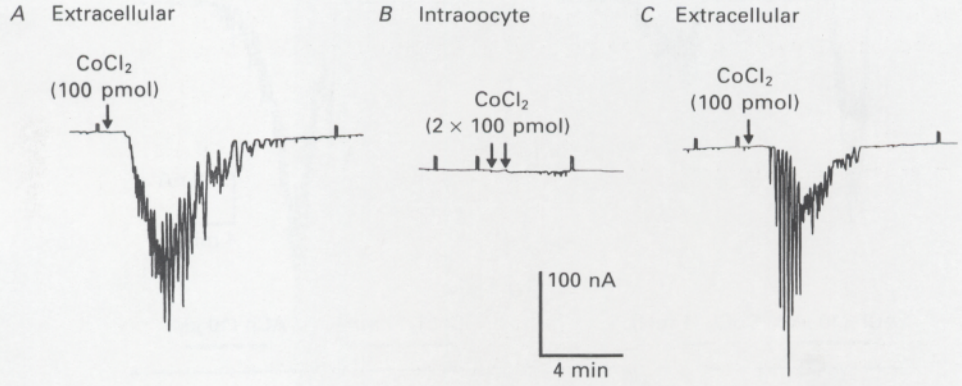

Fig. 8. Intracellular injection of $CoCl_2$ fails to elicit appreciable responses. *A*, a pipette containing 50 mM-$CoCl_2$ was brought close to the surface of an ER oocyte and a pressure pulse applied, ejecting *ca* 100 pmol of $CoCl_2$, which elicited an oscillatory response. *B*, the pipette was then inserted into the oocyte, causing some loss of input resistance. Two intracellular injections, each *ca* 100 pmol, produced little oscillatory current over the following 10 min. The pipette was withdrawn from the oocyte and 15 min allowed for resealing of membranes. The pipette tip was again placed close to the oocyte surface where the same pressure pulse again elicited an oscillatory response. Diameters of injection droplets were measured in air both before and after intraoocyte injections and showed no blockage of pipette. Perfusion of the recording chamber was stopped during this experiment.

characterized. The size and polarity of maintained currents varied considerably between oocytes from different frogs. With cation concentrations of 1–10 mM the maintained current was usually between 5 and 30 nA and was outwardly directed at −60 mV. On the other hand, oocytes from some frogs showed small (5–15 nA), smooth currents which were apparently inward at −60 mV. In both cases the currents were associated with a *decrease* in membrane conductance, indicating that they arose because of a reduction in resting currents which were, respectively, inwardly and outwardly directed.

These currents were seen at divalent cation concentrations as low as 0·1 mM and increased progressively with doses up to at least 10 mM (Fig. 10*A*). Ni^{2+}, Co^{2+} and Mn^{2+} ions were generally more potent than Mg^{2+}. Current–voltage relationships were determined during the reduction in conductance, by stepping clamp potential to different levels, and were subtracted from currents obtained in the absence of added cation, thus giving the voltage dependence of currents actually being blocked (Fig. 10*B*). At potentials between about −100 and 0 mV these currents varied roughly linearly with voltage, but with polarization beyond this range, steep increases in Co^{2+}-sensitive currents were often observed. The non-linear current at strongly negative potentials occurred because Co^{2+} reduces a calcium-independent chloride current activated by hyperpolarization (cf. Parker & Miledi, 1988). Similarly, at positive potentials Co^{2+} ions reduce a slowly developing voltage-gated outward

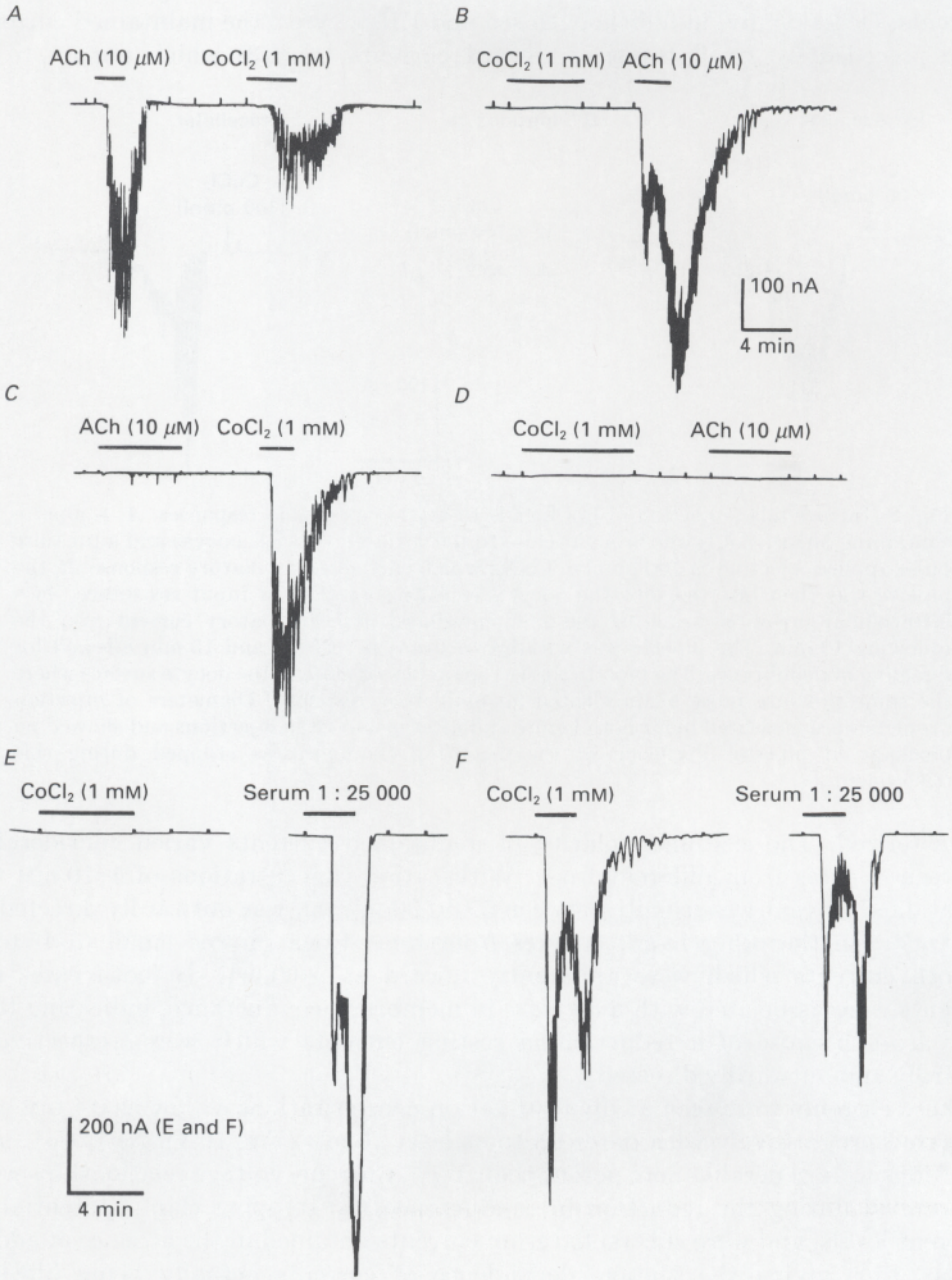

Fig. 9. *A–D*, comparison of relative sensitivities to 1 mM-$CoCl_2$ and 100 μM-ACh in four oocytes taken from four different frogs. Examples: oocyte which responded to both agonists (*A*), to ACh but not to Co^{2+} (*B*), to Co^{2+} but little to ACh (*C*), and to neither agonist (*D*). *E* and *F*, comparison of relative sensitivities to 1 mM-$CoCl_2$ and serum in two ER oocytes taken from different frogs. The oocyte in *E* gave no current in response to Co^{2+} ions but responded well to serum, whereas that in *F* responded to both agonists.

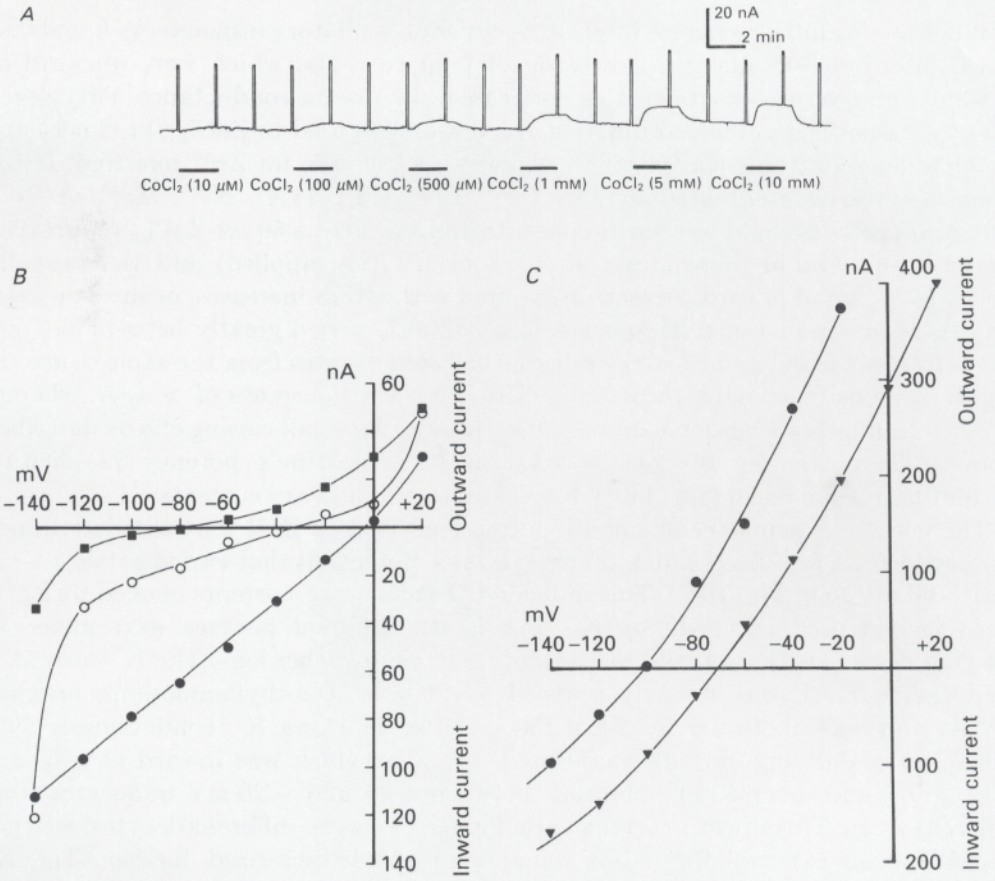

Fig. 10. *A*, dose dependence of the apparent outward current elicited by Co^{2+} ions. Holding potential was −80 mV, with brief pulses to −60 mV. Note that the conductance, monitored by the test pulses, declined during Co^{2+} applications. *B*, voltage dependence of the currents blocked by 10 mM-$CoCl_2$ in three oocytes from different frogs, none of which gave detectable oscillatory currents. (○), a collagenase-treated oocyte from the same frog as (*A*). In this case the Co^{2+}-sensitive currents varied linearly with voltage between −100 and 0 mV and reversed at −35 mV. (■), an ER oocyte in which blocked currents reversed at −80 mV. (●), collagenase-treated oocyte where blocked currents reversed at −5 mV. The direction of currents refer to the pre-existing current which was reduced by Co^{2+}. *C*, voltage dependence of currents elicited by 100 μM-$ZnCl_2$ (▼) and 5 μM-forskolin (●) in an ER oocyte.

current (authors' unpublished data). Reversal potentials of currents blocked by Co^{2+} varied between about 0 mV and −80 mV in oocytes from different frogs (Fig. 10*B*). The blocking of resting currents was clearly seen in defolliculated oocytes, but whether defolliculation affected reversal potential was not examined. Channel-closing effects were still present in zero Ca^{2+} Ringer solution and in oocytes injected with EGTA (200–900 pmol).

K^+ currents elicited by Zn^{2+} ions

When testing follicle-enclosed and ER oocytes for oscillatory responses we found that $ZnCl_2$ often elicited smooth, slowly developing currents, which were outward at -60 mV, and were associated with an increase in membrane conductance. Fifty-seven oocytes, taken from nineteen different frogs, were exposed to $ZnCl_2$ (0·1–2 mM) and *ca* 90% generated this current. The response was specific for Zn^{2+} ions and, in the same oocytes, was not activated by Cd^{2+}, Co^{2+} (Fig. 11*A*), Ni^{2+}, Mn^{2+} or Cr^{2+}. Outward currents could be seen at concentrations as low as 50 μM-$ZnCl_2$, began after latencies of 1–7 min (depending on the concentration applied) and were usually preceded by small inward currents associated with either increases, or in some cases decreases, in conductance. Responsiveness to $ZnCl_2$ varied greatly between oocytes from different frogs, and to a lesser degree between oocytes from the same ovary. In highly responsive oocytes 1 mM-Zn^{2+} elicited outward currents of $> 1\mu$A, whereas oocytes from other frogs gave no response, or only the small closing effects described above. There was no obvious correlation between zinc's potency in eliciting oscillatory responses and its ability to evoke smooth outward currents.

The voltage dependence of smooth currents elicited by Zn^{2+} ions was determined in oocytes from four frogs which showed reversal potentials that varied between -70 and -90 mV (e.g. Fig. 10*C*). This indicated that outward currents evoked by $ZnCl_2$ were carried predominantly by K^+, but in the different oocytes were mixed to varying degrees with a current, or currents, carried by other ions. The K^+ current in response to $ZnCl_2$ was strongly reduced by 20 mM-tetraethylammonium bromide (TEA) and was abolished by 2 mM-$BaCl_2$. After blocking K^+ conductances with $BaCl_2$, an underlying current was usually revealed which was inward at -60 mV (Fig. 11*B*), and reversed at potentials between -45 and -20 mV in oocytes from different frogs. This inward current varied in size between different oocytes, was not dependent on external Ca^{2+}, but remains to be characterized further. The K^+ responses to Zn^{2+} were also seen in zero Ca^{2+} Ringer solution and, in contrast to the oscillatory responses, were still present following intraoocyte injections of 800–1000 pmol EGTA (two oocytes).

Zinc ions only elicited K^+ currents in follicle-enclosed or ER oocytes and this type of response was abolished by defolliculation. For example, in six ER oocytes from one frog the K^+ current in response to 1 mM-$ZnCl^2$ was 448 ± 95 nA (mean $\pm$ S.D., using an exposure of 4 min). In contrast, four manually defolliculated oocytes from the same frog (with follicular cells removed by rolling on poly-L-lysine-treated slides; Miledi & Woodward, 1989*a*) either produced no outward current to 1 mM-$ZnCl_2$, or, in three cases, small residual inward currents (5–20 nA).

Follicle-enclosed and ER oocytes can generate K^+ currents through a variety of receptors coupled to adenylate cyclase (e.g. Kusano *et al.* 1982; Lotan, Dascal, Oron, Cohen & Lass, 1985; Van Renterghem, Penit-Soria & Stinnakre, 1985; Woodward & Miledi, 1987; Miledi & Woodward, 1989*b*). As previously described, these responses are essentially abolished by defolliculation (e.g. Smith *et al.* 1987; Woodward & Miledi, 1987; Miledi & Woodward, 1989*a*). Removal of Zn^{2+} responses by defolliculation, and similar sensitivities to K^+ channel blockers, suggested involvement of the same K^+ gating mechanism. We therefore checked to see if K^+ currents elicited by $ZnCl_2$ were potentiated by the phosphodiesterase inhibitor

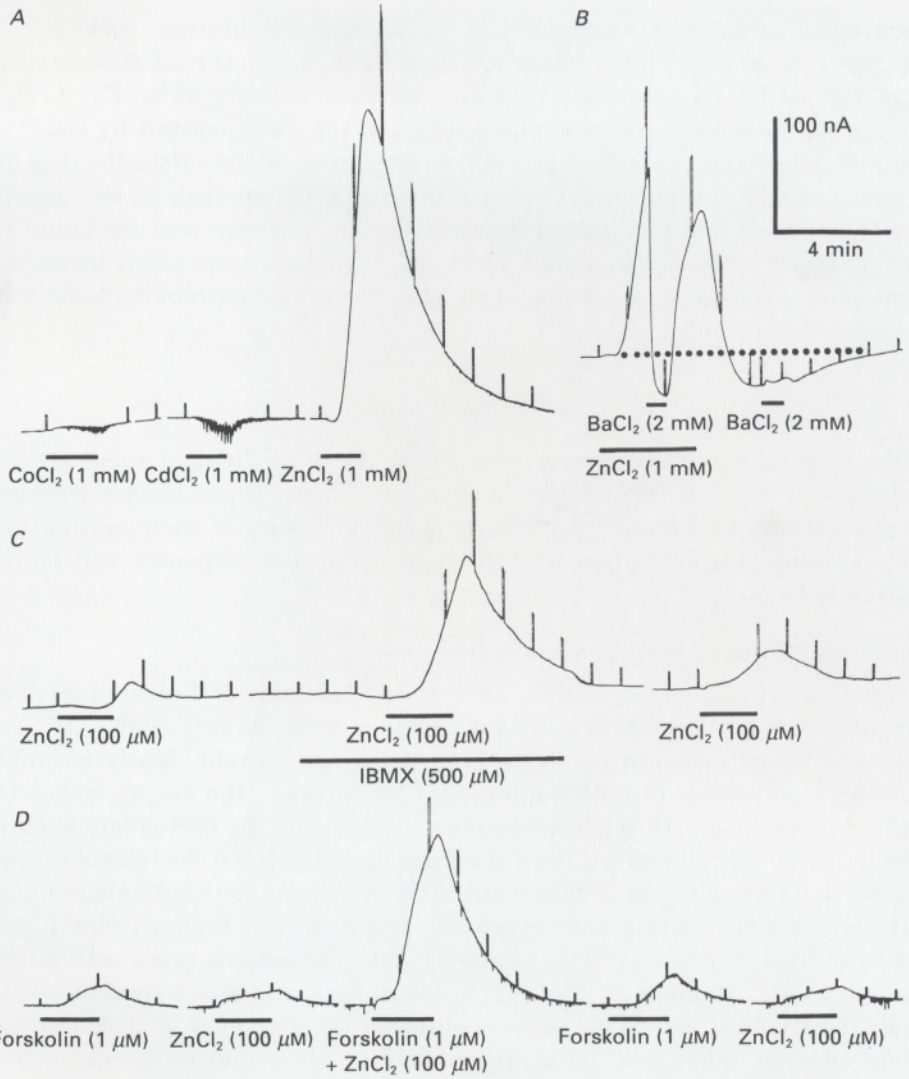

Fig. 11. Smooth outward currents elicited by $ZnCl_2$ seen in follicle-enclosed and ER oocytes voltage clamped at -60 mV. *A*, outward current is evoked specifically by Zn^{2+} ions. Co^{2+} and Cd^{2+} ions elicited only small oscillatory responses, whereas in the same oocyte Zn^{2+} generated a large outward current, associated with an increase in membrane conductance. *B*, $BaCl_2$ selectively blocks the K^+ current, revealing an underlying inward current also associated with an increase in membrane conductance which in this case reversed at -44 mV. Dotted line indicates basal holding current at -60 mV. *C*, potentiation of a K^+ response to Zn^{2+} by the phosphodiesterase inhibitor IBMX. A test exposure to $ZnCl_2$ elicited a small inward current followed by a slowly developing K^+ response. After 6 min pre-incubation in IBMX, re-exposure to $ZnCl_2$ elicited a large K^+ current. On washing out the IBMX for 20 min, the $ZnCl_2$ response returned close to control level. *D*, facilitation between responses to $ZnCl_2$ and to the adenylate cyclase activator forskolin. The first two traces show control responses to forskolin and $ZnCl_2$ applied separately. When applied together, a large K^+ current was generated. The oocyte was then washed for 25 min giving a return to control responses. Records in *A*, *B* and *D* were from ER oocytes taken from a single frog. The record in *C* was from a follicle-enclosed oocyte taken from a different frog.

3-isobutyl-1-methylxanthine (IBMX) or showed facilitation with K^+ currents generated by the adenylate cyclase activator forskolin. In three ER oocytes from two frogs, 100 μM-IBMX potentiated the Zn^{2+} response by 525 ± 95 % (Fig. 11*C*), though for three other cases (two frogs) potentiation of the $ZnCl_2$ current by IBMX was not as pronounced (i.e. < 50 %), the effect appearing to be critically dependent on responsiveness of oocytes and concentrations of $ZnCl_2$ applied. In two oocytes there was 4·0- and 5·2-fold facilitation between the Zn^{2+} current and forskolin responses (Fig. 11*D*). We also noticed that $ZnCl_2$ K^+ responses were often increased by an intervening exposure to forskolin, even when the actual current evoked by forskolin had been washed out.

DISCUSSION

We describe here that extracellular applications of divalent cations to *Xenopus* oocytes and ovarian follicles produce at least three types of electrical response: (1) generation of oscillatory Cl^- currents, (2) a closing of some resting ionic conductances and (3) activation of a K^+ current. These responses will be discussed separately below.

Oscillatory currents evoked by divalent cations

The divalent cations Cd^{2+}, Ni^{2+}, Zn^{2+}, Co^{2+}, Mn^{2+} and Cr^{2+} elicited oscillatory Cl^- currents when bath applied to *Xenopus* oocytes, whereas Sr^{2+}, Ba^{2+}, Ca^{2+} and Mg^{2+} ions were less effective or inactive. This oscillatory current closely resembled that elicited by activation of native muscarinic receptors in the oocyte, suggesting that both responses arise through a common mechanism. In particular, both currents arise in the oocyte membrane itself (i.e. they do not involve the follicular cells); both are due to a flux of Cl^- ions dependent on intracellular, but not extracellular, Ca^{2+}; both show desensitization upon repeated exposures; and both are closely mimicked by intracellular injection of IP_3. Moreover, divalent cations cause activation of IP_3-regulated Ca^{2+} channels in the oocyte membrane and the responses to ACh and divalent cations clearly show cross-facilitation (cf. Parker *et al.* 1987), all of which strengthens the idea that both are coupled to the same intracellular messenger system.

In the case of oscillatory responses to ACh, there is strong evidence that the Cl^- currents are generated through an intracellular messenger pathway involving inositol phosphates, similar to that described in many other cell types (for reviews see Berridge, 1987; Gilman, 1987). Briefly, this cascade is thought to be initiated by M3-muscarinic receptors in the oocyte membrane which activate a class of GTP-binding proteins coupled to the enzyme phosphoinositidase (phospholipase-C) (Dascal, Ifune, Hopkins, Snutch, Lubbert, Davidson, Simon & Lester, 1986; van Wezenbeek, Tonnaer & Ruigt, 1988). The activated enzyme hydrolyses phosphatidyl-inositol 4,5-bisphosphate, forming the intracellular messengers IP_3 and diacylglycerol (DG) (Oron *et al.* 1985). IP_3 and possibly other inositol phosphates cause the release of Ca^{2+} from intracellular stores (Busa, Ferguson, Joseph, Williamson & Nuccitelli, 1985; Parker & Miledi, 1986, 1987*b*), which in turn activates Ca^{2+}-gated Cl^- channels in the

oocyte membrane (Miledi, 1982; Miledi & Parker, 1984; Takahashi *et al.* 1987). The role of DG in oscillatory responses remains unclear but, via activation of protein kinase C, may regulate receptor desensitization (Kato, Kaneko & Nomura, 1988). As divalent cations appear to interact with the same pathway it becomes important to determine at what stage in the cascade, and by what mechanism, this occurs. In order to elicit the characteristic oscillatory Cl^- current it seems likely that cations raise cytosolic levels of inositol phosphates, either by promoting their synthesis, or possibly by inhibiting their degradation (e.g. Storey, Shears, Kirk & Michell, 1984). We do not yet understand the exact mode of action of divalent cations, but our results point to some possibilities and allow others to be ruled out.

Firstly, injections of $CoCl_2$ into oocytes which gave large responses to externally applied Co^{2+} almost entirely failed to elicit the oscillatory response. Thus divalent cations probably act on the external surface of oocyte membranes, rather than evoking responses because they enter oocytes, to interact directly with components of the intracellular coupling mechanism. Regarding sites on the external membrane surface, it was clear when comparing oocytes from different frogs that relative sensitivities to ACh, serum and divalent cations varied independently. It is therefore unlikely that cations generate oscillatory responses through direct interactions either with muscarinic receptors or the putative receptors to a serum protein. Nevertheless, cations might act through an as yet uncharacterized 'receptor' which is independently, and perhaps randomly, expressed in oocytes from some frogs. If so, it would remain unclear as to whether divalent cations are the specific and physiologically relevant agonists of this receptor, or whether the true agonist is a neurotransmitter or hormone, and effects of divalent cations merely incidental. Alternatively, it is possible that cations interact not with a receptor as such, but with another membrane-associated component of the phosphoinositide signalling system; for example with a G protein subunit or with the phosphoinositidase enzyme. However, if this is the case it is difficult to see why sensitivity to divalent cations should vary so greatly between oocytes, particularly amongst those which are sensitive to ACh, and thus demonstrably possess a functional signalling pathway.

Though overall responsiveness varied greatly between oocytes from different frogs, it was clear that sensitivity to the various cations always ran in parallel. Moreover, there were no clear variations in the relative potencies of different cations, the potency sequence being similar whether in low, medium or highly responsive oocytes. This, together with cross desensitization, all suggests that the different cations elicit responses in the same way and through the same site, though some (e.g. Cd^{2+}, Ni^{2+} and Zn^{2+}) have markedly higher activity. Because high-sensitivity oocytes responded to Ni^{2+} and Cd^{2+} at concentrations as low as 5 μM, this appears to rule out simple changes in membrane surface potential as the mechanism which initiates oscillatory currents. Nevertheless, it is interesting that the potencies of various cations to shift sodium channel activation in nerve (e.g. Hille, Woodhall & Shapiro, 1975) follow a similar sequence to their activity in inducing oscillatory responses in oocytes. It therefore remains possible that generation of oscillatory currents is dependent on the abilities of different divalent cations to bind fixed charges on the membrane.

Under some ionic conditions, divalent cations have been shown to induce

maturation of *Xenopus* oocytes *in vitro* (e.g. Kofoid, Knauber & Allende, 1979; Wallace & Misulovin, 1980). There is no obvious correlation between the reported efficacy of different cations to induce maturation and their potency in eliciting oscillatory currents.

Blocking of resting currents by divalent cations

In oocytes where oscillatory currents were not evoked, divalent cations elicited smooth membrane currents associated with a decrease in membrane conductance, presumably due to the blocking of resting 'leakage' currents. Reversal potentials of these cation-sensitive currents ranged between 0 and -80 mV, suggesting the presence of a variety of underlying conductances which were expressed to different extents in oocytes from different frogs. Given the respective reversal potentials in oocytes for Na^+ (*ca* $+60$ mV), Cl^- (*ca* -20 mV) and K^+ (*ca* -100 mV) (Kusano *et al.* 1982; Barish, 1983), it is likely that when cation-sensitive currents reversed at strongly negative potentials they were carried substantially by K^+. In oocytes where reversal potentials were more positive their ionic basis remains unclear, but may involve channels permeable to Na^+ or Cl^-. Since these closing effects remained after removal of extracellular calcium or loading with intracellular EGTA, it does not appear that Ca^{2+} influx contributes appreciably to the cation-sensitive currents. Furthermore, these currents were not dominated by reductions in conductance to Cl^- ions, as would be expected if there was any significant resting influx of Ca^{2+}, in turn leading to activation of Ca^{2+}-gated Cl^- channels. The maintained Cl^- current described by Robinson (1979) might therefore arise because resting intracellular Ca^{2+} levels are high enough to activate some Ca^{2+}-gated Cl^- channels, or may be due to Ca^{2+}-independent Cl^- channels (cf. Parker & Miledi, 1988).

Although we have yet to characterize these complex conductance changes produced by divalent cations, they could provide a useful tool to study 'leakage' currents, which are otherwise rather intractable to experimental observation.

K^+ currents elicited by $ZnCl_2$

Potassium currents elicited specifically by Zn^{2+} ions were abolished by defolliculation, suggesting that they arise in follicular cells. The smaller inward currents, usually associated with K^+ responses, were also reduced by defolliculation, but it remains unclear to what extent these currents are follicular in origin. Potentiation of $ZnCl_2$ K^+ responses by IBMX, and facilitation between these currents and responses to forskolin, support the view that Zn^{2+} ions interact with a follicular cyclic nucleotide-activated K^+ conductance. In which case it is interesting to note that Zn^{2+}, and not Ni^{2+}, Co^{2+} or Mn^{2+}, has also been shown to enhance gonadotrophin-induced cyclic AMP synthesis in rat gonadal tissue (Nishi, Hatano, Aihara, Okahata, Kawamura, Tanaka, Miyachi & Usui, 1984).

To conclude, our experiments suggest that extracellularly applied divalent cations can interact with two major intracellular signalling pathways in *Xenopus* ovarian follicles. Several ions stimulate the phosphoinositide pathway in the oocyte itself, while Zn^{2+} ions also appear to act upon the cyclic nucleotide pathway probably located in follicular cells. We do not know whether these actions are particular to

Xenopus oocytes/follicles or extend to other cell types (see note added in proof). These results imply that some caution is required in the experimental use of divalent cations, as they might have many effects in addition to their well characterized blocking of Ca^{2+} fluxes.

Note added in proof. Extracellular divalent cations (e.g. Cd^{2+}, Ni^{2+}, Co^{2+} and Mn^{2+}) have recently been shown to increase inosital polyphosphate formation and mobilize intracellular Ca^{2+} in human skin fibroblasts, artery muscle and endothelial and neuroblastoma cells. Although in these cases Zn^{2+} acts as an antagonist of cation responses (see Smith, Dwyer & Smith, 1989), cadmium evokes inositol polyphosphate formation and calcium mobilization.

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