Functional role of follicular cells in the generation of osmolarity-dependent Cl^- currents in *Xenopus* follicles

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1. Osmolarity-dependent (osmo-dependent) ionic currents from follicle-enclosed *Xenopus* oocytes (follicles) were studied using the two-microelectrode voltage-clamp technique, combined with intra-oocyte pressure injection of sucrose or polyethylene glycols (PEGs).

2. Intra-oocyte injections of sucrose or PEG (3–25 nmol) generated inward membrane currents (follicles held at −60 mV) associated with an increase in membrane conductance. These currents were carried mainly by chloride ions ($I_{Cl(osm)}$), and were strongly attenuated by increasing the tonicity of the external medium, or by external application of La^{3+} (0·1–1 mM).

3. The ability to generate $I_{Cl(osm)}$ depended on the molecular weight of the injected PEG. Injections of PEG 200 or 300 generated $I_{Cl(osm)}$ in 95% of the follicles tested, PEG 600 generated comparable currents in only 20% of the follicles, while similar injections of PEG 1000 did not elicit $I_{Cl(osm)}$.

4. Octanol (1–1·5 mM), a gap junction channel blocker, reversibly inhibited 50–90% of the $I_{Cl(osm)}$ generated by injections of sucrose or PEG 300. Moreover, sucrose or PEG injections did not elicit $I_{Cl(osm)}$ in defolliculated oocytes.

5. It is concluded that an increase in the internal osmolarity of the follicular cells activates a mechanism, probably involving cellular swelling, which leads to the opening of $I_{Cl(osm)}$ channels most probably located in the follicular cell membrane.

Exposure of follicle-enclosed *Xenopus* oocytes (follicles) to hyposmotic media generates ionic membrane currents that are carried mainly by potassium and chloride ions. Osmolarity-dependent (osmo-dependent) Cl^- currents are also elicited when follicles are exposed to acetylcholine or follicle-stimulating hormone (FSH); the later seems to act through a mechanism involving cAMP synthesis, while the acetylcholine mechanisms remain unknown. Moreover, all the osmo-dependent currents are eliminated after removal of follicular cells by either enzyme (collagenase) treatment or by manual dissection (Arellano & Miledi, 1993, 1994). These results strongly indicate that the receptors and channels involved in osmo-dependent responses, as well as the necessary elements for their activation and modulation, are located in the follicular cells and not in the oocyte itself. The strong electrical coupling between the oocyte and the follicular cells, via gap junction channels (Browne, Wiley & Dumont, 1979; Miledi, Parker & Sumikawa, 1989), explains why ionic currents through the membrane of the follicular cells can be monitored from within the oocyte.

Some of our findings were independently confirmed in a study describing in detail the chloride current generated by hyposmotic shock (Ackerman, Wickman & Clapham, 1994, where this is named $I_{Cl,swell}$). In that study, manual defolliculation did not fully suppress the chloride current, although it was strongly reduced. To explain this, it was proposed (Ackerman *et al.* 1994) that current activation ocurrs in the oocyte itself, and that follicular cells may play some regulatory role. The nature of this positive regulatory process, however, remained unknown.

The characterization and localization of the various elements involved in the osmo-dependent ionic currents are important for understanding oocyte–follicular cell physiology, and relevant also to work on exogenous osmo-dependent proteins expressed in the oocytes (e.g. Gründer, Thiemann, Pusch & Jentsch, 1992; Preston, Carroll, Guggino & Agre, 1992; Krapivinsky, Ackerman, Gordon, Krapivinsky & Clapham, 1994). Therefore, the main aim of this study was to investigate the role of the follicular cells in the generation of the responses to hyposmotic shock in *Xenopus* follicles.

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METHODS

Adult *Xenopus laevis* frogs were killed by decapitation and pithing. Follicles (stage VI; Dumont, 1972) were dissected from the ovaries and normally used with the epithelium removed, in which the inner epithelium was dissected out manually, leaving the basal membrane (collagen layer) protecting the follicular cells. The follicles were stored at 16–18 °C in a sterile modified Barth's medium (composition (mM): 88 NaCl, 0·2 KCl, 2·4 $NaHCO_3$, 0·33 $Ca(NO_3)_2$, 0·41 $CaCl_2$, 0·82 $MgSO_4$, 0·88 KH_2PO_4, 2·7 Na_2HPO_4, 5 glucose; pH 7·4; supplemented with 70 μg ml^{-1} gentamicin and 0·1 % fetal bovine serum). Electrical recordings were made over a period of 1–3 days following dissection, and after stabilizing the follicles for at least 5 h in the medium. In some experiments, oocytes were defolliculated by enzymatic treatment (1 mg ml^{-1} collagenase in normal frog Ringer solution for 1–2 h, at 20–22 °C) or manually (by rolling them on a poly-L-lysine-coated surface) as described previously (Kusano, Miledi & Stinnakre, 1982; Miledi & Woodward, 1989).

Membrane currents were monitored using a conventional two-microelectrode voltage clamp (Miledi, 1982) at 20–22 °C. Follicles and oocytes were routinely held at −60 mV and drugs were applied via the superfusing medium. Bath solutions were normal frog Ringer solution (NR) (composition (mM): 115 NaCl, 2 KCl, 1·8 $CaCl_2$, 5 Hepes; pH 7·0), or hyposmotic Ringer solution (HR) in which the concentration of NaCl was reduced to either 88 mM (HR_{88}) or 50 mM (HR_{50}). In some cases, 50 % of NaCl in HR_{88} was replaced by Tris-HCl or 10 mM NaCl by KCl. The low Cl^- HR_{88} solution contained (mM): 28 NaCl, 30 Na_2SO_4, 30 sucrose, 2 KCl, 1·8 $CaCl_2$, 5 Hepes; pH 7·0. In several experiments, potassium currents were blocked using the non-specific potassium channel blockers Ba^{2+} or tetraethylammonium-chloride (TEA). $BaCl_2$ (1 mM) was added to the superfusing solutions, and TEA (0·25–2 nmol) was co-injected intracellularly with sucrose (see below). All data are given as means ± S.E.M.

Intra-oocyte injections were made by pneumatic pressure ejection from micropipettes (Miledi & Parker, 1984). All the injected solutions were made in 5 mM Hepes and 50 μM EGTA, and pH was adjusted to 7·0 with KOH. Injection solutions were either 0·5 or 2 M sucrose; 2 M sucrose plus 0·5 M TEA (sucrose + TEA); 0·5 M polyethylene glycol (PEG) with an average molecular weight (MW) of 200, 300 or 600; or 0·2 M PEG 1000. The volumes injected were estimated from the diameter of a drop ejected with the tip pipette in the air. This was measured before introducing the injection micropipette into the oocyte, and confirmed at the end of the experiment. Stock solutions of 1-octanol (1 M) were prepared in DMSO.

PEG 200–1000, sucrose, 1-octanol, DMSO, fetal bovine serum, collagenase (Type I) and gentamicin were purchased from Sigma Chemical Co. Porcine FSH was from Calbiochem (La Jolla, CA, USA). TEA-Cl was from Eastman Kodak Co. (Rochester, NY, USA).

RESULTS

Follicle Cl^- currents elicited by intra-oocyte injections of sucrose

Follicles exposed to an external hyposmotic solution are known to generate ionic membrane currents that are deactivated by prolonged incubation in this medium (Arellano & Miledi, 1993). To examine this process further we tested whether these currents could be elicited through a direct increase of the tonicity of the follicle after injecting osmo-active substances into the oocyte.

In the follicles used for this study, the currents elicited by HR_{88} were small (Fig. 1*A*), and were deactivated by pre-incubating the follicles in Barth's medium. Therefore, we decided to carry out the injection experiments with the follicles bathed in HR_{88} because this allowed us to change the external osmolarity in the range used in previous studies (Arellano & Miledi, 1993; Ackerman *et al.* 1994). Intra-oocyte injections of sucrose elicited inward membrane currents ('sucrose currents') that were associated with an increase in membrane conductance. After injecting 25 nmol the peak current in follicles from different frogs varied from a few nanoamps to 500 nA, but was more consistent among follicles from a given donor. The amplitude of the sucrose current was dose dependent (not shown), and in highly responsive follicles (i.e. where injection of ~15 nmol elicited currents of > 250 nA), the currents could be detected after injecting only 3·8 ± 2·3 nmol (5 follicles, 2 frogs). Assuming a follicle volume of 1 μl, at equilibrium this would correspond to a sucrose concentration of *ca* 5 mM. In general, sucrose (15–25 nmol) generated peak currents 4–6 min after injection, and remained stable for more than 15 min (Fig. 1*A*). The sucrose currents elicited in follicles in HR_{88} were attenuated when the bathing solution was switched to NR (Fig. 1*A*), or to HR_{88} + 55 mM sucrose (not shown), indicating that these currents were osmo-dependent. The attenuation produced by NR was partially reversible upon returning to HR_{88}.

Current–voltage (*I–V*) relationships of the sucrose currents were obtained by giving a series of voltage pulses (Fig. 1*B*) before sucrose injection (15–20 nmol, or sucrose + TEA injection; see below), and again during the peak of the current. Current amplitudes were measured at the end of the 2·5 s pulses, and passive currents before sucrose injection were subtracted to obtain the *I–V* relationships. In HR_{88} the *I–V* relationship was linear over the range +40 to −120 mV, and reversed (E_{rev}) at −27 ± 4 mV (5 follicles, 2 frogs). In order to reduce the participation of potassium currents that may also be induced in HR solutions (Arellano & Miledi, 1993) the following experiments were performed: either adding Ba^{2+} to the external solutions or injecting TEA (0·25–2 nmol) (sucrose + TEA). The mean E_{rev} obtained in the latter conditions was −23 ± 2 mV (21 follicles, 6 frogs; Fig. 1*B*), all of which suggests strongly that the sucrose currents were carried mainly by Cl^- ions, since their E_{rev} was close to that for Cl^- in follicles (*ca* −20 mV; Kusano *et al.* 1982). Furthermore, substitution of K^+ or Na^+ ions in HR_{88} had little effect on the amplitude of the currents or their E_{rev} (not shown), while substitution of 60 mM Cl^- in HR_{88} by SO_4^{2-} shifted the E_{rev} of the sucrose current to −8 ± 2 mV (15 follicles, 6 frogs; Fig. 1*B*) (*vs.* ~−6 mV predicted by the Nernst equation). All this is consistent with an ionic current that is carried mainly by Cl^-.

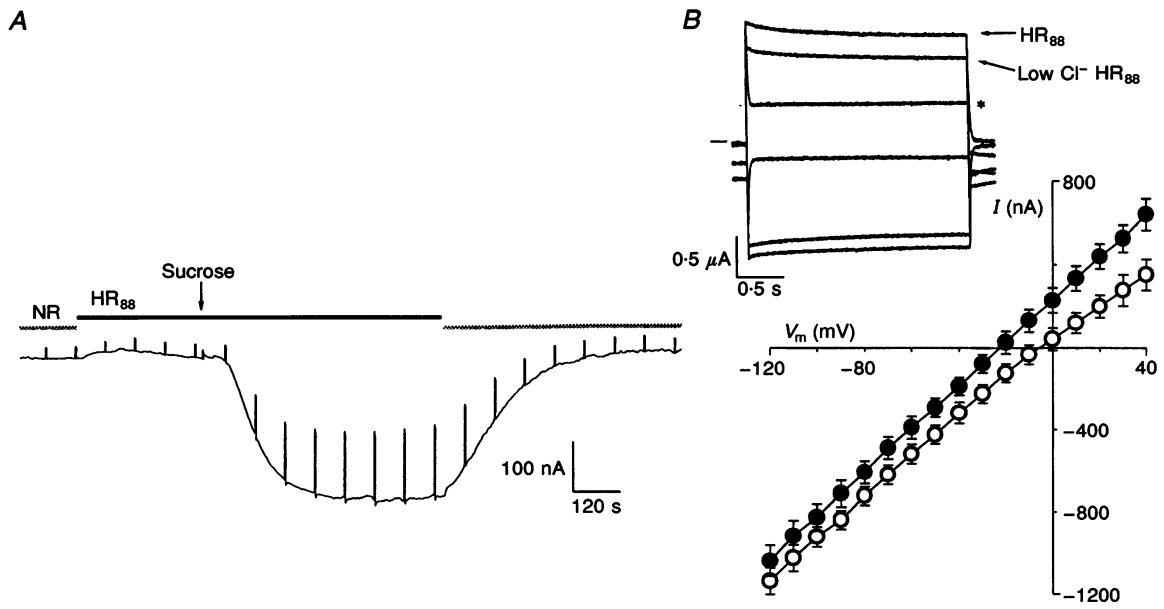

Figure 1. Osmo-dependent currents elicited by sucrose injection in *Xenopus* follicles

A, membrane current of a follicle held at −60 mV in NR (dotted bar). HR_{88} was superfused and a single injection of 17 nmol sucrose applied at times indicated by the continuous bar and the arrow, respectively. Voltage steps of +10 mV (3 s) were applied periodically to monitor membrane conductance. *B*, follicle membrane currents at different membrane potentials, 4–6 min after injection with 15–20 nmol sucrose + TEA, and superfused with HR_{88} (●; 7 follicles) or low Cl^- HR_{88} (○; 5 follicles). All follicles from the same frog. Each point indicates mean ± S.E.M. Superimposed traces show examples of responses during voltage pulses to −120 and +40 mV applied to a follicle held at −60 mV. Currents were recorded during superfusion with HR_{88} (*) before injection, and ~5 min after intra-oocyte injection of 20 nmol sucrose + TEA in HR_{88} or in low Cl^- HR_{88}. In this and following records the horizontal lines and the asterisk indicate zero current and passive currents, respectively.

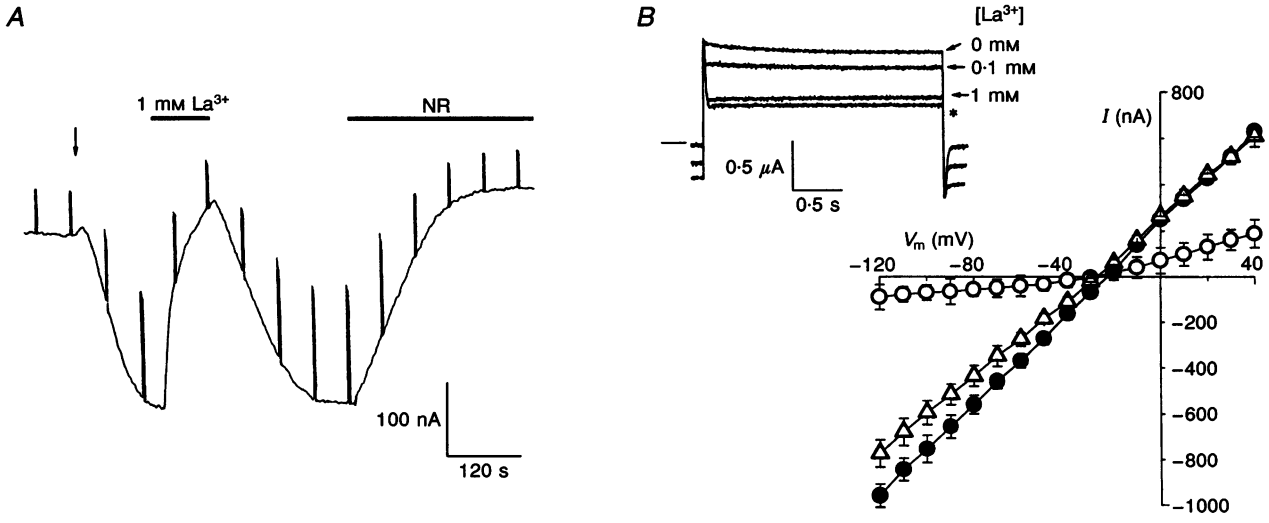

Figure 2. Inhibition of sucrose current by La^{3+}

A, chloride membrane current elicited by intra-oocyte injection of 15 nmol sucrose (arrow) in a follicle held at −60 mV in HR_{88}. La^{3+} (1 mM) added to HR_{88}, or NR applied for the time indicated by the bars inhibited the current. All in the presence of 1 mM Ba^{2+}. *B*, *I–V* relationships in follicles injected with 15–20 nmol of sucrose + TEA, bathed in HR_{88} (●), HR_{88} plus 1 mM La^{3+} (○), and after washing out La^{3+} application for ~8 min in HR_{88} (△). Superimposed records are examples of currents during voltage steps to +40 mV in HR_{88} before injection, and 5–10 min after the injection in HR_{88} containing 0, 0·1 or 1 mM of La^{3+}.

In addition to its ionic selectivity and its modulation by osmolarity, other results also indicate that the sucrose current is probably the same as that generated in follicles exposed to hyposmotic external solutions. (i) Oocytes that were defolliculated, either manually or by collagenase treatment, did not generate membrane currents after similar injections of sucrose or sucrose + TEA (16 rolled oocytes, 6 frogs; 20 collagenase-treated oocytes, 12 frogs). (ii) Sucrose currents were reduced to 0–5% in the presence of 1 mM La^{3+} in external HR_{88} (Fig. 2), and to 50% by 100 μM La^{3+}, similar to currents generated by superfusion with HR solutions (Ackerman *et al.* 1994; R. O. Arellano & R. Miledi, unpublished results). The effect of La^{3+} was fully reversible after 4–8 min of wash with HR_{88} (Fig. 2). Having established chloride as the main ion carrier of sucrose currents, and the osmolarity change as the signal for activation, we denote this current $I_{Cl(osm)}$.

Follicular $I_{Cl(osm)}$ elicited by PEG injections

When dealing with full follicles, the osmo-dependent mechanism could be located either in the follicular cells or in the oocyte itself. In the former case, our results would be explained by diffusion of sucrose from the oocyte into the follicular cells via gap junction channels. The subsequent increase in tonicity within the follicular cells activates the osmo-dependent mechanism which opens $I_{Cl(osm)}$ channels. If this proposition is correct, a substance injected into the oocyte would be expected to generate $I_{Cl(osm)}$ provided it can permeate through gap junctions. Conversely, if the

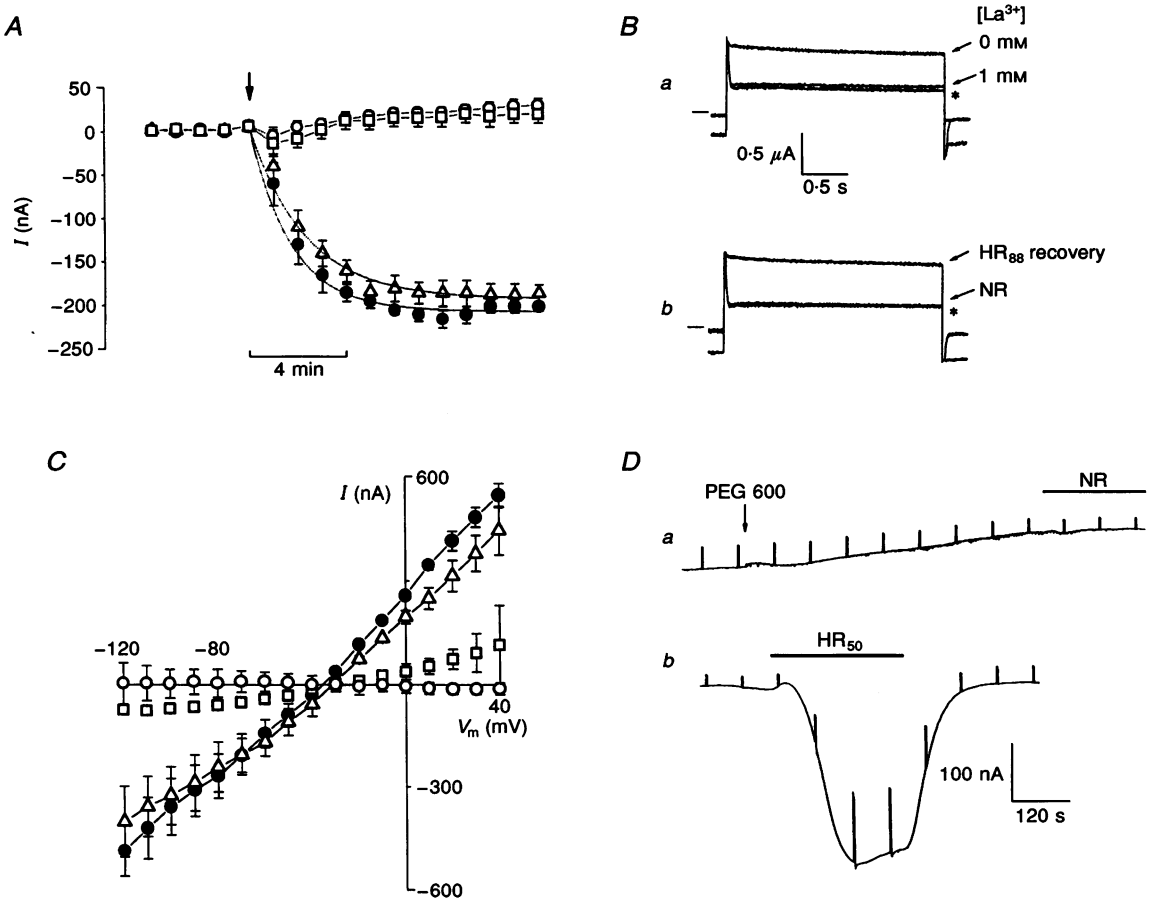

Figure 3. $I_{Cl(osm)}$ elicited by PEG injections into follicles

A, average follicle membrane currents after injection (arrow) of 10–20 nmol of either sucrose (●; 4 follicles), PEG 200 (△; 3 follicles), PEG 600 (□; 3 follicles), or 10–15 nmol PEG 1000 (○; 3 follicles). All follicles from the same frog. *B*, superimposed records of chloride membrane currents during voltage steps applied as in Fig. 2*B*. *a*, follicle before (*) and ~5 min after injection with 11 nmol PEG 300 in absence or presence of La^{3+}. *b*, same injected follicle after La^{3+} application was washed out with HR_{88} (recovery); subsequently the current was inhibited by NR. *C*, *I–V* relationships of follicle currents 6–10 min after injection with (10–25 nmol) sucrose (●), PEG 300 (△), PEG 600 (□), or PEG 1000 (○). Each point represents the average current (± S.E.M.) in 3–6 follicles from different frogs. PEG 600-injected cells that did not give $I_{Cl(osm)}$ were not included. *D*, follicle membrane current in HR_{88} injected with 15 nmol PEG 600 (*a*). Superfusion with NR (*a*) did not produce any change in current, but superfusion with HR_{50} (*b*) elicited a large $I_{Cl(osm)}$ reversed by HR_{88}. All the follicles in this figure were held at −60 mV in the presence of 1 mM Ba^{2+}.

activation mechanism was located in the oocyte itself, $I_{Cl(osm)}$ would be generated regardless of the permeability of the substance through gap junctions channels, which are known to be impermeable to substances with a MW higher than approximately 1000 (Lowenstein, 1981). We examined these possibilities by injecting PEGs of four different MW into follicles.

Injections of PEG 200 or 300 (10–20 nmol) generated follicular inward currents ('PEG currents') of amplitudes similar to those elicited by sucrose injections, in follicles from the same frog (Fig. 3*A*). In general, the characteristics of the currents induced by PEG 200 or 300 were similar to those of $I_{Cl(osm)}$ elicited either by sucrose injections or hyposmotic shock. For example, the PEG currents were abolished in NR (27 follicles, 8 frogs), reduced to 5% by 1 mM La^{3+} (12 follicles, 5 frogs) (Fig. 3*B*), and showed similar *I–V* relationships (Fig. 3*C*). In contrast, similar injections of PEG 1000 (10–20 nmol) did not activate PEG currents (13 follicles, 6 frogs) (Fig. 3*A* and *C*). Though the volume required to reach similar concentrations of PEG 1000 in the follicles was greater (30–70 nl compared with 10–35 nl of sucrose solution), it was clear that even 40 min after injection, PEG 1000 did not generate $I_{Cl(osm)}$ (excessive volume injection drastically decreased the membrane resistance and in some follicles this triggered large oscillatory currents). Injections of PEG 600 (10–25 nmol) gave results intermediate between those observed with PEGs of smaller or greater MWs. In some cases PEG 600 generated small PEG currents that had similar $I_{Cl(osm)}$ *I–V* relationships, and were again inhibited by NR or 1 mM La^{3+} in HR_{88} (5 follicles, 2 frogs; Fig. 3*C*). However, PEG 600 failed to generate $I_{Cl(osm)}$ in about 80% of the follicles tested (23 follicles, 7 frogs), even 10–30 min after their injection (e.g. Fig. 3*A*).

Sometimes, PEG 600 or 1000 injections elicited outward membrane currents (E_{rev} between −20 and −38 mV; 11 follicles, 5 frogs), that were associated with a decrease in membrane conductance (Fig. 3*B–D*). The failure of injections of PEG 600 or 1000 to evoke $I_{Cl(osm)}$ does not appear to be due to the blocking of $I_{Cl(osm)}$ or gap junction channels, or to any impairment of the osmo-dependent mechanism, since the follicles still generated $I_{Cl(osm)}$ when they were superfused with HR_{50} (8 follicles, 3 frogs; Fig. 3*D*).

Thus, the activation of $I_{Cl(osm)}$ is clearly dependent on the MW of the substance injected, most probably consequent to the ability of the injected molecules to cross gap junctions and act within the follicular cells.

Effect of 1-octanol on the follicular $I_{Cl(osm)}$

Further evidence supporting the notion that the osmo-dependent mechanism is located in the follicular cells was provided by the demonstration of an inhibitory effect of octanol on $I_{Cl(osm)}$ evoked by intra-oocyte injections of sucrose or PEG 300. Octanol blocks gap junctions (Johnston, Ramón & Simon, 1980), and has been previously used to uncouple oocytes and follicular cells in *Xenopus* (Sandberg, Bor, Ji, Markwick, Millan & Catt, 1990; Supplisson, Kado & Bergman, 1991). We tested the effect of octanol on $I_{Cl(osm)}$ generated by injections of sucrose + TEA (total ~20 nmol). External application of 1–1·5 mM octanol in HR_{88} (0·05–0·1% DMSO) during the peak of the sucrose current caused a rapid decrease that was dose dependent, and was not mimicked by

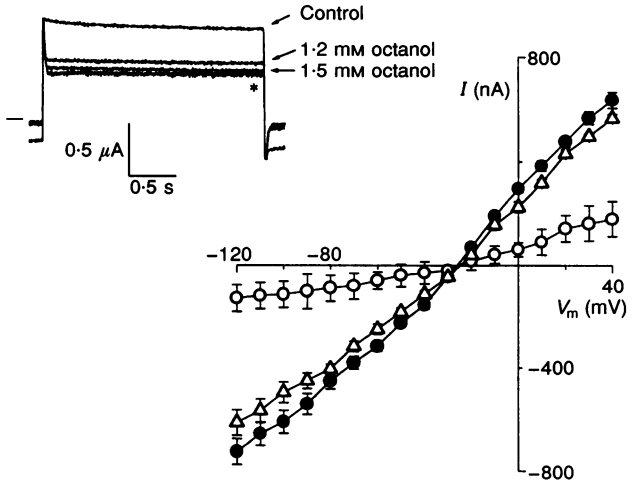

Figure 4. Octanol blockage of follicular $I_{Cl(osm)}$

I–V relationship of follicular $I_{Cl(osm)}$ elicited by sucrose (15–20 nmol) injection, during control superfusion with HR_{88} (●), in the presence of 1·2 mM octanol (○), and recovery after wash (△) for 5 min. Each point represents the average (± S.E.M.) from 3–5 follicles (2 frogs) tested in every condition. Superimposed records show an example of the currents during voltage steps to +40 mV, in a follicle in HR_{88} before (*), ~6 min after sucrose injection (Control) and 1·2 and 1·5 mM octanol in HR_{88} applied subsequently.

corresponding applications of DMSO alone. For example, 1 mM octanol reduced $I_{Cl(osm)}$ by 48 ± 6% (4 follicles, 3 frogs), while 1·2 mM reduced it to 14 ± 5% (12 follicles, 5 frogs; Fig. 4). In all follicles the inhibitory effect of octanol on $I_{Cl(osm)}$ was largely reversible after washing with HR_{88} for 2–4 min. Octanol (1·2 mM) also prevented the generation of $I_{Cl(osm)}$ when it was applied to follicles 2–3 min before injection of sucrose + TEA (4 follicles, 2 frogs). Furthermore, octanol produced a similar inhibition of the $I_{Cl(osm)}$ generated by injections of PEG 300. In follicles from the same frogs, 1·2 mM octanol inhibited approximately 90% of the potassium current activated by applications of 1 μg ml^{-1} FSH (Woodward & Miledi, 1987) (not shown). In some follicles, application of octanol (> 1·2 mM) elicited an additional inward current that appeared to originate in the membrane of the oocyte itself (4 of 24 follicles, 3 frogs). This effect of octanol still remains to be studied in detail.

DISCUSSION

Our results show that, in follicles, $I_{Cl(osm)}$ can be generated by an increase in internal tonicity produced by the intra-oocyte injection of various substances, as well as by a decrease in the tonicity of the bathing solution. The ability of the injected substances to generate $I_{Cl(osm)}$ decreased sharply when their MW increased beyond about 600. This effect may be explained by the permeability of these compounds through the gap junction channels and, accordingly, $I_{Cl(osm)}$ was inhibited by exposing follicles to octanol in order to block the gap junctions. Therefore we propose that injection of low (≤ 600) MW osmo-active substances increases the internal tonicity of the follicular cells due to diffusion of these molecules, from the oocyte cytoplasm into the follicular cells, via gap junctions. The increase in internal tonicity in the follicular cells activates the mechanism that generates $I_{Cl(osm)}$.

It still remains unclear whether the channels that mediate $I_{Cl(osm)}$ are located in the membrane of the follicular cells or in the oocyte membrane itself, since it is possible that channel protein and the molecule which confers osmo-dependence are different, and located in separate compartments. For example, activation of the osmo-dependent mechanism in the follicular cells could induce the production of a second messenger which, after diffusing into the oocyte, would activate channels in the oocyte membrane itself. Such a mechanism could explain the ability to regulate $I_{Cl(osm)}$ of exogenous cytoplasmic molecules expressed in the oocyte (Krapivinsky *et al.* 1994). However, we consider this possibility unlikely because there is no clear evidence of the involvement of any second messenger in the generation of $I_{Cl(osm)}$ (Ackerman *et al.* 1994; Arellano & Miledi, 1994). Instead, we propose that in the native follicle both the activation mechanism and the $I_{Cl(osm)}$ channels are located mainly in the membrane of the follicular cells. This proposal is based on our results on defolliculated oocytes which failed to generate osmo-dependent currents by intra-oocyte injections of osmo-active substances. Also, we have shown previously that defolliculation eliminates the currents generated after exposure to HR solutions, or by application of different agonists in HR (Arellano & Miledi, 1993, 1994). Furthermore, although increases of cellular volume by inflation have been succesfully used in various systems to activate osmo-dependent Cl^- channels (e.g. Hagiwara, Masuda, Shoda & Irisawa, 1992), we failed to generate $I_{Cl(osm)}$ after increasing directly the oocyte volume by injecting substances such as paraffin oil (R. O. Arellano & R. Miledi, unpublished results).

The osmo-dependent mechanisms linking the increase of tonicity and activation of $I_{Cl(osm)}$ channels are still unknown, but the increase in follicular cell volume is probably one of the intermediary steps. If this is correct, the fact that injections of osmo-active molecules of high molecular weight, like PEG 1000, were not able to activate $I_{Cl(osm)}$ suggests that the rate of increase of oocyte volume is lower than that of the follicular cells. However, we still do not know if this is simply because of a smaller surface area : volume ratio of the oocyte (that will be more than one order of magnitude smaller considering oocyte macrovilli), or is also due to a lower water permeability of the oocyte membrane. Thus, effects caused by cellular volume changes in defolliculated oocytes by hyposmotic solutions would have a slower time course compared with that of follicles. Some results suggest that this is correct; for example, in our experiments $I_{Cl(osm)}$ is activated soon (0·5–1 min) after HR_{50} application has started, and current reached a peak in approximately 3 min, while the enhancer effect of hyposmotic solutions on the activity of the chloride channels named CIC-2 expressed in oocytes (Gründer *et al.* 1992) is a slow process which begins to be apparent only after ~10 min of a similar change in external osmolarity.

Besides, the ability of PEG 600 to generate $I_{Cl(osm)}$ in some follicles suggests that this molecule may just permeate the oocyte–follicular cells gap junctions. The efficiency of PEG 600 in generating $I_{Cl(osm)}$ would be dependent on the degree of intercellular coupling (i.e. number of gap junction channels open), and perhaps on the folding structure of the PEG 600 molecules.

The results presented here will be important for a comprehensive understanding of follicle physiology. The mechanisms involved in the activation of $I_{Cl(osm)}$ channels remain unknown. Nevertheless, our results show the important role that the coupling of the oocyte with the follicular cells may have in the volume control of the gamete during its development and growth, and they provide important information which must be taken into consideration when *Xenopus* oocytes are used to express and study osmo-dependent molecules and channels.

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