Endogenous Na^+–K^+ (or NH_4^+)–$2Cl^-$ cotransport in *Rana* oocytes; anomalous effect of external NH_4^+ on pH_i

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1. In *Rana* oocytes, measurements with chloride-sensitive microelectrodes show that the mean intracellular chloride activity (34·8 ± 6·3 mM, $n = 79$) is three times higher than that expected for the passive distribution of chloride ions across the outer membrane (12·4 mM, mean membrane potential −43 ± 8·8 mV, $n = 79$).

2. Reuptake of chloride into oocytes depleted by prolonged exposure to chloride-free saline takes place against the electrochemical gradient.

3. Chloride reuptake does not take place in sodium-free solution or in a sodium-substituted potassium-free solution. It is inhibited by bumetanide (10^{-5} M) in the bathing medium.

4. The overall stoichiometry of the transport mechanism deduced from simultaneous measurements of intracellular sodium and chloride using ion-selective electrodes is $1Na^+ : 1K^+ : 2Cl^-$.

5. Ammonium ions substitute for potassium on the cotransporter.

6. In oocytes smaller than 0·9 mm in diameter, exposure to external ammonium causes an alkaline shift in intracellular pH as the NH_3 enters and takes up H^+ to form NH_4^+. We propose that chloride-dependent NH_4^+ transport contributes to the accumulation of NH_4^+ and causes the 'postexposure' acidification as the intracellular NH_4^+ releases H^+ to form NH_3 which is then lost from the cell.

7. In larger oocytes ammonium exposure produces a rapid reduction in pH_i which may be explained in part by cotransport-mediated uptake of NH_4^+. Evidence is also provided for a second chloride-dependent NH_4^+ transport mechanism and a chloride-independent process.

Amphibian oocytes are commonly chosen as vehicles to express ion channels and ion carriers from foreign mRNA because the product can be assessed by membrane current and flux measurements on single cells. However, oocytes possess endogenous transport systems that influence their growth, maturation and fertilization (Dascal, 1987). Their intracellular chloride is maintained at a high level (Kusano, Miledi & Stinnakre, 1982; Barish, 1983; Jaffe & Schlichter, 1985) and flux measurements have demonstrated an endogenous capacity for chloride transport (Richter, Jung & Passow, 1984). Because intracellular chloride is high, the increased chloride conductance at fertilization depolarizes the cell and prevents polyspermy (Cross & Elinson, 1980). Here we report the use of ion-selective microelectrodes to show that ovarian *Rana* oocytes have a Na^+–K^+–Cl^- cotransporter capable of accumulating internal chloride. We provide evidence that the cotransporter accepts NH_4^+ in place of K^+ and hence contributes to an intracellular acidification which is observed in fully grown oocytes exposed to ammonium salts. We suggest that ammonium cotransport may account for hitherto unexplained exceptions to the general assumption that NH_3-containing solutions invariably make cytoplasm more alkaline (Jacobs, 1924).

METHODS

Experiments were performed throughout the year on ovarian oocytes from *Rana temporaria* and *Rana pipiens*. Animals were kept at room temperature, allowed free access to water and fed once a week with meal-worms. Individuals were killed by decapitation and pithing; their ovaries were quickly removed and washed thoroughly in normal saline buffered with 20 mM Hepes (adjusted to pH 7·5 with NaOH) and containing (mM): NaCl, 82; KCl, 2; $CaCl_2$, 1·8; $MgCl_2$, 1; glucose, 5. The larger oocytes were separated into small clusters of approximately twenty to thirty and stored in 200–300 ml saline at 5 °C. The saline solution was changed each day and no antibiotic was required. Oocytes survived for at least a week with no sign of depigmentation and no significant change in their recorded resting potentials.

Prior to the experiment, individual oocytes were mechanically separated and allowed to recover for at least 1 h at room temperature before being transferred to the experimental chamber. Most experiments were performed on

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oocytes 1·4–1·8 mm in diameter but some experiments were done on smaller cells for comparison. The oocytes were, in most cases, enclosed in their follicles and we cannot exclude the possibility that the effects we observed were associated with the follicular cells (which are connected by way of gap junctions; Miledi & Woodward, 1989) rather than the oocytes themselves. We think this unlikely because oocytes that were partially defolliculated (manually) behaved in the same way as normal oocytes. However, we performed no scanning electron microscopy and cannot estimate the number of follicle cells remaining (see Miledi & Woodward, 1989).

During experiments the oocytes were superfused continuously with nominally CO_2-free saline buffered to pH 7·5. In chloride-free solution chloride was replaced with gluconate. Saline calcium levels were measured with a calcium-sensitive electrode (Unicam Ltd, Cambridge, UK) and 10 mM calcium gluconate was added to compensate for Ca^{2+}-binding by gluconate ions (Kenyon & Gibbons, 1977). In sodium-free solution *N*-methyl-D-glucamine was substituted for sodium.

Double-barrelled chloride-sensitive microelectrodes were prepared according to the method of Schwiening & Thomas (1990) using a Corning 477315 anion exchanger (Ciba Corning Analytical, Essex, UK). They were calibrated using a method similar to that described by Thomas (1978; p. 93) in which the calibration solutions were prepared by mixing appropriate amounts of normal saline and isotonic chloride-free sodium gluconate solution. Chloride concentrations in the calibration solutions were converted into activities using an activity coefficient of 0·76 which is equivalent to that of NaCl solution with the same ionic strength as normal saline (Parsons, 1959).

A major difficulty with using electrodes of this type is the interference that arises from normally occurring intracellular anions (Alvarez-Leefmans, Giraldez & Russell, 1990). Thus we found that significant levels of intracellular chloride appeared to remain even in oocytes exposed to chloride-free saline for several hours. Reference electrodes filled with saturated K_2SO_4 were used for all chloride-depletion experiments. The oocytes were bathed in bicarbonate-free saline and so intracellular levels of bicarbonate (to which the electrodes are known to be sensitive) were limited to that produced as CO_2 by cell metabolism.

Results from chloride-depletion experiments with eight different electrodes are shown in Fig. 1*A*. The apparent change in intracellular chloride activity ($\Delta a\mathrm{Cl_i}^*$) during the period in chloride-free saline is plotted against the apparent chloride activity measured initially ($a\mathrm{Cl}^*_{i,t=0}$). The intercept on the abscissa suggests that chloride-free saline would have no effect on oocytes in which $a\mathrm{Cl_i}^*$ was initially 13 mM, as if the background interference was equivalent to 13 mM chloride. However, loss of chloride, (with Na^+ or K^+) is likely to be associated with a reduction in cell volume which would cause the level of background interference to be overestimated. Furthermore, as Fig. 1*B* shows, the final apparent chloride activity ($a\mathrm{Cl}^*_{i,t=\infty}$) was an inverse function of ($a\mathrm{Cl}^*_{i,t=0}$). As this implies that the level of interference varied between oocytes, we have used the line plotted through the points in Fig. 1*B* to correct the data for individual cells. Except where indicated otherwise, the values of intracellular chloride activity ($a\mathrm{Cl_i}$) given in the text have been corrected for interference in this way. Estimates derived in a similar way for chloride-sensitive electrodes constructed from the same Corning anion exchanger and used on other preparations are, in most cases, in the range 3·1–5·2 mM (see Alvarez-Leefmans *et al.* 1990 for review). We cannot account for this discrepancy but a small underestimate of $a\mathrm{Cl_i}$ on our part would not affect our conclusions. In the figures we show uncorrected values as the apparent intracellular chloride activity ($a\mathrm{Cl_i}^*$).

The sodium-sensitive electrodes were either double-barrelled or constructed with reversed tips (Thomas, 1978). In the latter case they were used in conjunction with the reference barrel of a chloride-sensitive microelectrode. They were calibrated using isotonic KCl solutions containing 1–10 mM NaCl. Reversed-tip

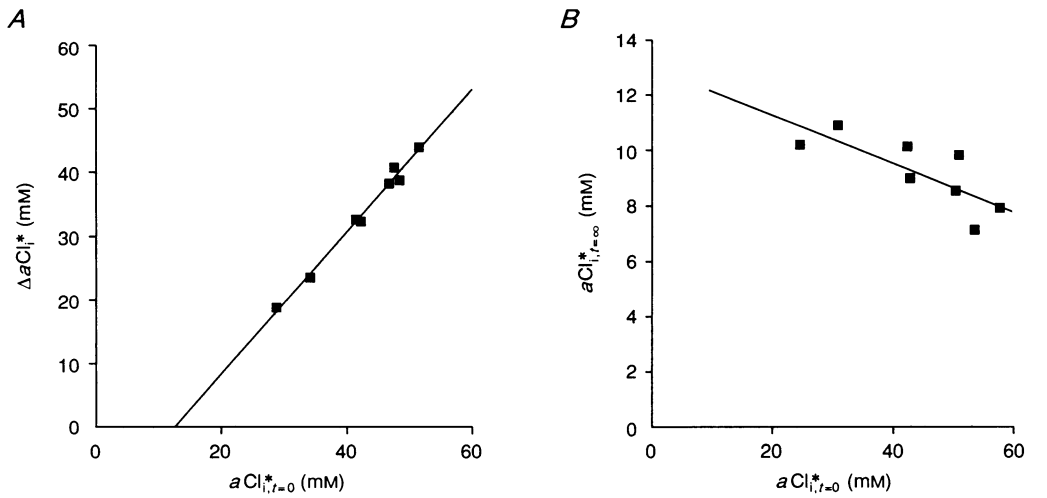

Figure 1. Effect of chloride-free saline on apparent intracellular chloride activity ($a\mathrm{Cl_i}^*$) measured in *Rana* oocytes

Results from eight double-barrelled chloride-selective microelectrodes. *A*, relationship between the initial value, $a\mathrm{Cl}^*_{i,t=0}$ (abscissa) and the maximal change in $a\mathrm{Cl_i}^*$ ($\Delta a\mathrm{Cl_i}^*$; ordinate) during treatment with chloride-free saline. The regression line (correlation coefficient, 0·989) intercepts the abscissa at $a\mathrm{Cl_i}^* = 13$ mM. *B*, relationship between $a\mathrm{Cl}^*_{i,t=0}$ (abscissa) and the minimum value of $a\mathrm{Cl_i}^*$ after prolonged exposure to chloride-free saline ($a\mathrm{Cl}^*_{i,t=\infty}$; ordinate). The line drawn through the points has a correlation coefficient of 0·603.

sodium-sensitive glass (NAS_{11-18}, Corning) electrodes were used in preference to the sodium-sensitive double-barrelled liquid membrane (sodium ion ionophore 1 – cocktail A, Fluka Chemie AG, Buchs, Switzerland) electrodes for quantitative measurements of the change in intracellular sodium activity ($a\mathrm{Na_i}$) because of their better sensitivity in the physiological range (1–10 mM Na^+) and because they were less sensitive to changes in intracellular calcium that may have taken place during the experiment.

The pH-sensitive liquid membrane (hydrogen ion ionophore 1 – cocktail A, Fluka) microelectrodes were prepared according to the method of Schwiening & Thomas (1990). They were calibrated at the beginning and end of each experiment using pH 7·5 Hepes-buffered saline and 20 mM Pipes-buffered saline at pH 6·5.

RESULTS

Measurement of resting intracellular chloride activity

Intracellular double-barrelled microelectrodes were used to measure the chloride activity in *Rana* oocytes. The difference in potential between the chloride-sensitive liquid ion exchanger membrane (barrel 1) and the reference potential (barrel 2) is proportional to the log of the chloride activity. This apparent intracellular chloride activity ($a\mathrm{Cl_i}$*) was corrected as described in Methods. Calculated values of the chloride equilibrium potential (E_{Cl}) are shown in Fig. 2 plotted against the oocyte membrane potential (E_{m}). In each case the calculated E_{Cl} was less than E_{m} and it appeared that chloride ions were accumulated in the oocyte cytoplasm. The mean value of E_{m} was −43 mV (S.D. 8·8; $n = 79$). The mean corrected value of $a\mathrm{Cl_i}$ was 34·8 mM (S.D. 6·3 mM; $n = 79$), a value close to that reported for immature *Xenopus* oocytes (Cl_i^-, 33·4 mM; Barish, 1983) and mature *Rana* eggs (Cl_i^-, 44 mM; Jaffe & Schlichter, 1985) but nearly three times that expected for a passive distribution according to the membrane potential (12·4 mM). These data are summarized in Table 1 together with the mean values of measured intracellular sodium ($a\mathrm{Na_i}$) and potassium ($a\mathrm{K_i}$) activities obtained with sodium- or potassium-sensitive microelectrodes.

Table 1. Intracellular chloride, sodium and potassium activities in *Rana* oocytes measured with ion-sensitive microelectrodes

	Mean (mM)	S.D.	n
$\mathrm{Cl_i}$*	58·0	7·5	82
$a\mathrm{Cl_i}$*	44·1	5·7	82
$a\mathrm{Cl_i}$	34·8	6·2	82
$\mathrm{Na_i}$	4·4	1·6	52
$a\mathrm{Na_i}$	3·4	1·3	52
$\mathrm{K_i}$	109·0	9·3	6
$a\mathrm{K_i}$	82·8	7	6

Recovery of intracellular chloride in depleted oocytes

To investigate how chloride is accumulated against its electrochemical gradient, oocytes were first depleted of chloride by prolonged exposure to chloride-free saline. In the experiment shown in Fig. 3, E_{m}, $a\mathrm{Cl_i}$ and $a\mathrm{Na_i}$ were monitored in a large (1·8 mm diameter) manually defolliculated oocyte superfused at the outset with normal saline. After the oocyte had been penetrated with voltage recording and ion-sensitive electrodes, its membrane potential (top trace) slowly increased to −52 mV; the bathing medium was then changed to a chloride-free solution. There was an 18 min delay, during which the internal chloride appeared to increase slightly (perhaps because of a volume change), but thereafter $a\mathrm{Cl_i}$* declined steadily. A simultaneous measurement of $a\mathrm{Na_i}$ (lowest trace) showed that Na^+ also left the cytoplasm; $a\mathrm{Na_i}$ declined from its resting level (3·1 mM) to 1·1 mM in about 45 min.

According to Schlichter (1989) chloride currents recorded from oocytes of *Rana pipiens* were fully inhibited by 0·4 mM SITS (4-acetamido-4′-isothiocyanatostilbene-2,2′-disulphonic acid; BDH Laboratory Supplies, UK). Hence,

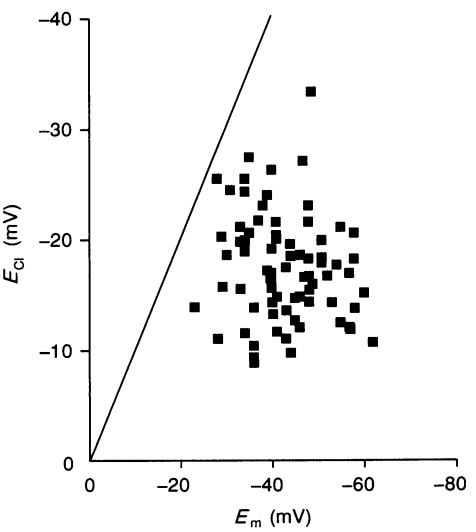

Figure 2. Relationship between the chloride equilibrium potential (E_{Cl}) and the oocyte membrane potential (E_{m})
Values of E_{Cl} calculated from $a\mathrm{Cl_i}$ and $a\mathrm{Cl_o}$ are plotted against the corresponding value of E_{m} for 73 oocytes. Only oocytes with an E_{m} greater than −20 mV were included. The $a\mathrm{Cl_i}$ values were corrected for interference on the chloride-sensitive microelectrode as described in Methods. The line shown is the expected relationship if chloride was passively distributed across the outer membrane.

in the experiment shown in Fig. 3, 0·5 mM SITS was added to the bathing medium in order to block possible passive Cl^- fluxes; active transport of Na^+ was prevented by adding 0·1 mM G-strophanthin (Sigma Chemical Co., UK). These additions to the bathing medium had little effect on the efflux of chloride but E_m increased by 5 mV and aNa_i started to rise more rapidly. When external chloride was eventually reintroduced, aCl_i* began to increase immediately and reached a value of 32 mM (aCl_i, 21 mM). During chloride recovery E_{Cl} decreased from −35 to −30 mV while E_m increased from −34 to −43 mV and so all chloride uptake took place against the chloride electrochemical gradient. The mean final value for intracellular chloride (corrected) in these experiments was 21 mM (S.D. 1; $n = 3$), which was significantly below the mean initial value of 35 mM (S.D. 6). In normal saline the mean final value was 29 mM (S.D. 7; $n = 4$).

Although chloride recovery seemed slightly more complete in the absence of SITS and G-strophanthin, there was no further increase in aCl_i once SITS had been removed from the external solution (see Fig. 3). Normal pH_i regulation, at this stage of development, is totally blocked by Na^+-free saline (authors' unpublished observations) and is insensitive to SITS (1 mM) and so it is unlikely that there is significant Cl^-–HCO_3^- exchange in these cells. In fact in two out of seven experiments, aCl_i appeared to decrease once SITS had been removed from the medium. This could arise if the cotransporter was operating in the face of a sustained SITS-sensitive passive leak of chloride ions out of the cell.

A possible explanation for the incomplete recovery of chloride is that it was caused by high levels of intracellular sodium. In the experiment shown in Fig. 3, chloride recovery is associated with an increase in aNa_i and Fig. 4, which shows the final levels of intracellular chloride (corrected) and intracellular sodium from ten similar experiments, demonstrates that there is a clear inverse correlation between the two.

Effect of the loop diuretic bumetanide

The recovery of internal chloride after depletion was found to require external sodium and to be inhibited by the loop diuretic, bumetanide in the experiment shown in Fig. 5. As before, the oocyte was first depleted of its internal chloride

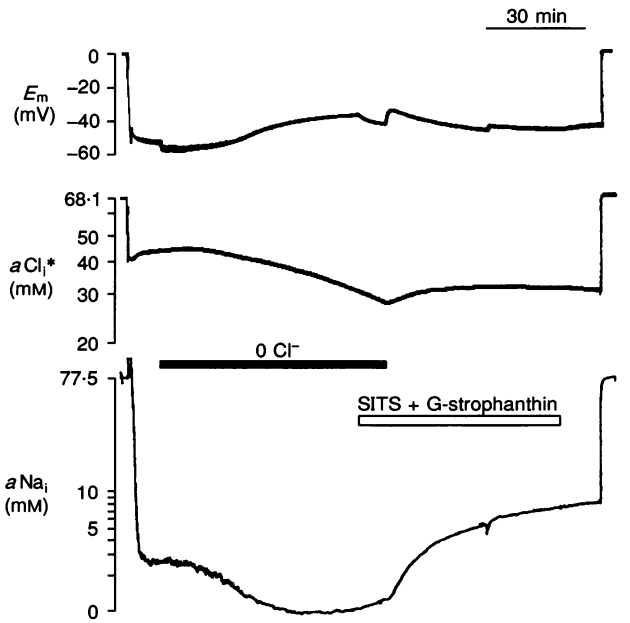

Figure 3. Depletion of intracellular chloride in chloride-free saline and its recovery upon return to normal conditions

The upper trace shows the membrane potential (E_m) taken from the reference channel of the electrode; the centre trace shows the apparent intracellular chloride activity (aCl_i*) monitored with a double-barrelled chloride-sensitive microelectrode and the lower trace shows the intracellular sodium activity (aNa_i) measured with a sodium-sensitive glass microelectrode. After the insertion and stabilization of the two electrodes, the solution in the bath was changed from standard control saline to chloride-free medium. For the first 15 min the internal chloride remained steady or increased slightly but after further exposure aCl_i* declined steadily to 28 mM. After 2 h SITS (4-acetamido-4′-isothiocyanatostilbene-2,2′-disulphonic acid; 0·5 mM) and G-strophanthin (0·1 mM) were added to the bathing medium. There appeared to be little change in the rate of loss of intracellular chloride. Upon readmission of external chloride, aCl_i* recovered rapidly against the driving force for passive distribution. The increase in chloride was associated with an increase in aNa_i. Manually defolliculated oocyte (*Rana temporaria*), 1·8 mm diameter at room temperature.

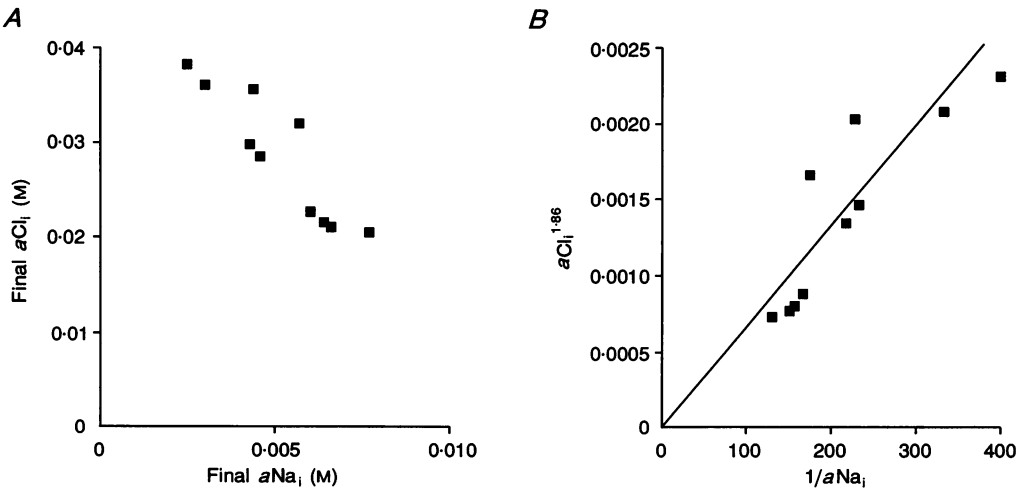

Figure 4. Relationship between intracellular chloride activity (aCl_i) and intracellular sodium activity (aNa_i)
A, intracellular chloride activity (aCl_i; ordinate) plotted against intracellular sodium activity (aNa_i; abscissa). *B*, $aCl_i^{1·86}$ (ordinate) against $1/aNa_i$ (abscissa). The straight line is drawn with a slope equal to $6·6 \times 10^{-6}$ mol^3.

by exposure to chloride-free solution. After about 80 min when aCl_i* had decreased to near 20 mM (aCl_i, 10 mM), the bathing medium was changed to a chloride- and sodium-free solution containing *N*-methyl-D-glutamine in place of sodium. Chloride was then returned to the external solution but, in the absence of sodium, aCl_i did not recover and, in fact, decreased even further. When sodium was reintroduced there was an abrupt increase in aCl_i much as occurred when external chloride was restored in Fig. 3.

For a short period during the sodium-dependent aCl_i recovery shown in Fig. 5, the cell was exposed to bumetanide (10^{-5} M; Leo Pharmaceutical Products, Denmark), a known inhibitor of cation-coupled chloride fluxes in many different tissues (Ellory, Dunham, Logue & Stewart, 1982). Bumetanide strongly inhibited chloride recovery and slowed sodium uptake (not shown) but had no effect on E_m. Upon removal of bumetanide the oocyte once again began to regain chloride. Subsequent further exposure to chloride-

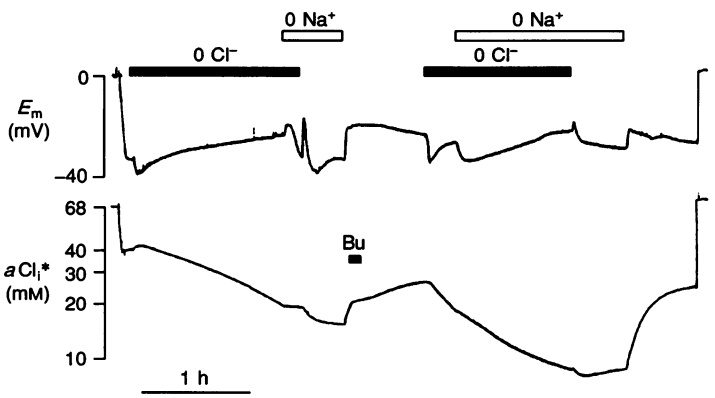

Figure 5. Effect of the loop diuretic bumetanide on sodium-dependent chloride accumulation
The lower trace shows the apparent intracellular chloride activity (aCl_i*) monitored with a double-barrelled chloride-sensitive microelectrode. The upper trace shows the membrane potential (E_m) taken from the reference channel of the electrode. Changes in the composition of the bathing medium are shown at the top of the figure. Intracellular chloride was depleted in chloride-free saline and then, after a brief exposure to a saline free of both chloride and sodium, chloride was restored to normal. The aCl_i* remained low until sodium was returned to the bathing medium, when it increased rapidly. Bumetanide (10^{-5} mM) completely inhibited aCl_i* recovery. There was no effect on membrane potential. In the second part of the experiment the effect of prolonged exposure to chloride- and sodium-free saline was found to enhance chloride loss. Manually defolliculated oocyte 1·4 mm in diameter at room temperature.

free and sodium-free solution caused renewed depletion of internal chloride. With the reintroduction of chloride there was little change in aCl_i until the oocyte was returned to sodium-containing solution at the end of the experiment.

Similar effects on the recovery of both aCl_i and aNa_i were recorded in four other oocytes . Bumetanide (10^{-5} M) produced 87 % inhibition (S.D. 5 %; $n = 4$) in the rate of aCl_i recovery and 75 % inhibition (S.D. 5 %; $n = 4$) in the rate of aNa_i recovery. In three experiments in which bumetanide was applied in chloride-free solution, 10^{-5} M caused a clear inhibition in the rate of aCl_i recovery when external chloride was returned to normal levels; 10^{-7} M bumetanide had little effect.

Stoichiometry of chloride transport.

Further analysis of the simultaneous measurement of aNa_i and aCl_i measured during the transport of chloride into chloride-depleted oocytes showed that each sodium ion that entered the cytoplasm was associated with two chloride ions, i.e. the $Na^+ : Cl^-$ ratio was close to 1 : 2. In the experiment shown in Fig. 3 the recovery of aCl_i and the concomitant increase in aNa_i were compared during the first 20 min after the reintroduction of external chloride. The increase in both ions followed an exponential time course, each with similar rate constants (aCl_i, 0·077 min^{-1}; aNa_i, 0·047 min^{-1}) as expected if Na^+ and Cl^- were accumulated via the same mechanism. Assuming that there was no rapid uptake of ions by intracellular stores, examination of their initial rate of rise provided a means of separating any direct coupling from secondary effects that follow changes in intracellular concentration. Measurement of aNa_i and aCl_i at 2 min intervals during the first 6 min of the recovery period and calculation of ΔaNa_i and ΔaCl_i from the corresponding rate constant, showed that the average $Na^+ : Cl^-$ ratio was 1 : 1·8. In five other experiments the average rate constant of aCl_i recovery was 0·11 min^{-1} (S.D. 0·05) and that for the associated increase in aNa_i was 0·09 min^{-1} (S.D. 0·04). The initial rate of change of aCl_i was calculated to be 0·54 mol min^{-1} (S.D. 0·14) while the initial rate of change of aNa_i was 0·27 mol min^{-1} (S.D. 0·1) so that the coupling ratio between Na^+ and Cl^- was 1 $Na^+ : 2Cl^-$.

The absence of a significant change in membrane potential when chloride uptake was inhibited by bumetanide (Fig. 5) suggested that transport is electroneutral and hence that other ions are involved in the process. Experiments using potassium-free solution (see Fig. 6) showed that oocytes would not regain chloride unless both sodium and potassium ions were present in the external solution. Thus the overall stoichiometry of the Na^+–K^+–Cl^- cotransporter is likely to be $1Na^+ : 1K^+ : 2Cl^-$ as found by flux measurements in Ehrlich cells (Geck, Pietrzyk, Burckhardt, Pfeiffer & Heinz, 1980) and in renal epithelia (Greger, 1985) rather than the $2Na^+ : 1K^+ : 3Cl^-$ ratio proposed for perfused squid axons (Russell, 1983) and ferret red cells (Hall & Ellory, 1985).

Effect of ammonium exposure on chloride transport and intracellular pH

Although chloride recovery normally required the presence of external potassium, ammonium was found to substitute for K^+ and appeared to be transported into the oocyte cytoplasm in combination with Cl^- and Na^+. In Fig. 6 the oocyte was depleted of intracellular chloride as before; the K^+ in the bathing medium was then substituted by Na^+

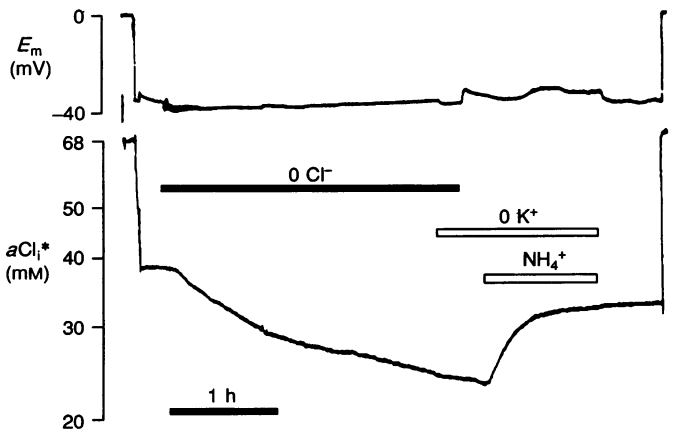

Figure 6. Reaccumulation of chloride with NH_4^+ substituting for K^+ in the bathing medium
The lower trace shows the apparent intracellular chloride activity (aCl_i*) monitored with a double-barrelled chloride-sensitive microelectrode. The upper trace shows the membrane potential (E_m) taken from the reference channel of the electrode. Changes in the composition of the bathing medium are shown by the horizontal bars. Intracellular chloride was depleted in chloride-free saline and then, after a brief exposure to a saline free of both chloride and potassium, chloride was restored to normal. The aCl_i* remained low until ammonium chloride (2 mM) was added to the bathing medium. It then increased rapidly and no further increase was observed when the normal saline solution was perfused again. Follicle-enclosed oocyte (*Rana pipiens*); 1·8 mm diameter at room temperature.

and 12·5 min later chloride was returned to the solution. The recorded aCl_i* remained at a low level for a further 15 min until 2 mM NH_4Cl was added to the bathing medium. This observation was repeated on three separate oocytes. In other experiments 2 mM tetraethylammonium (TEA) chloride or 2 mM tetramethylammonium (TMA) chloride were substituted for potassium, but in each case there was no chloride recovery until either K^+ or NH_4^+ was added to the bathing medium.

In order to confirm that the chloride was transported by the same sodium-dependent cotransporter we monitored aNa_i and aCl_i together and recorded an 8·9 mM increase in aCl_i associated with a 5·2 mM increase in aNa_i (Na^+:Cl^-, 1:1·7). Undepleted oocytes also gained chloride when exposed to NH_4^+ (see below) but this was only partly dependent on external sodium. In two oocytes 20 mM NH_4^+ produced an initial rate of chloride increase in sodium-containing solution of 1·22 mM min^{-1} (S.D. 0·55); in sodium-free saline the rate of rise was reduced to 0·59 mM min^{-1} (S.D. 0·07) which suggested the presence of a sodium-independent NH_4^+–Cl^- transport system in addition to Na^+–NH_4^+–Cl^- cotransport.

In rabbit kidney, entry of NH_4^+ via the Na^+–K^+–Cl^- cotransporter acidifies the cells of the ascending limb of the loop of Henle (Kinne, Kinne-Saffran, Schutz & Scholermann, 1986) and so we examined the effect of ammonium exposure on the intracellular pH (pH_i) of *Rana* oocytes. In the smallest oocytes, less than 0·9 mm diameter, application of 20 mM NH_4Cl (pH 7·5) caused a rapid alkaline shift in pH_i (lower trace in Fig. 7) together with a large reduction in the cell membrane potential. In thirteen experiments the mean change in pH_i was 0·43 pH units (S.D. 0·12); the mean decrease in resting potential was 45 mV (S.D. 19·6). According to Jacobs (1924) the most likely explanation for the change in pH_i is that cells are many times more permeable to NH_3 than NH_4^+, and that NH_3, having entered the cell, takes up a proton to form NH_4^+ and so makes the cell alkaline.

Figure 7 shows that upon removal of NH_4Cl, pH_i returned to a significantly more acidic value (pH 6·6) than at the start of the experiment. In thirteen experiments the mean displacement was 0·20 pH units (S.D. 0·14). This 'postexposure' acidification was abolished if the oocyte was perfused with chloride-free solution. In Fig. 7, after a second exposure to ammonium, this time in chloride-free conditions, pH_i again returned to near 6·6, but following another chloride-free ammonium treatment pH_i returned to a more alkaline value. When the bathing medium was returned to normal and the effect of ammonium was tested for a fourth time, a large 'postexposure' acidification was once again recorded. It is possible that the 'postexposure' acidification was the result of the intracellular accumulation of NH_4^+ via chloride-dependent cotransport. The NH_4^+ that remains when the oocyte is returned to normal saline releases a proton to form NH_3 and hence acidifies the cell.

In growing oocytes that had developed grey pigmentation a different response to 20 mM NH_4Cl was observed. In

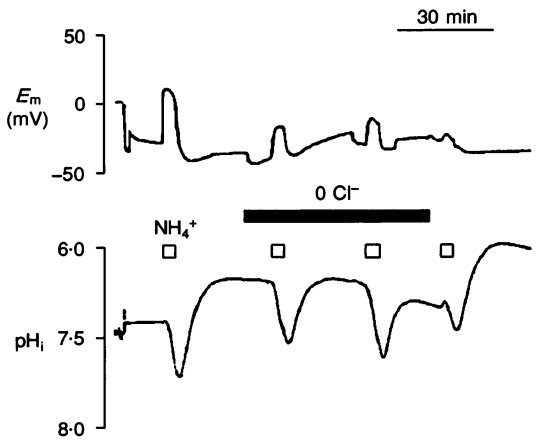

Figure 7. Effect of ammonium on a small-diameter oocyte
Effect of ammonium application on the membrane potential (E_m; top trace) and the intracellular pH (pH_i; bottom trace) in a small-diameter (0·9 mm) oocyte. Exposure to 20 mM NH_4Cl resulted in a rapid increase in pH_i and depolarization of E_m. When the NH_4Cl was removed, pH_i decreased to pH 6·6 and remained at this value. The bathing medium was changed to a chloride-free solution and the oocyte was once again exposed briefly to 20 mM NH_4^+ (added as $(NH_4)_2SO_4$). The observed increase in pH_i was approximately the same as before but there was little change in the resting level once the NH_4^+ had been removed. Following a third brief exposure to NH_4^+, which also caused an increase in pH_i, there was a slow recovery towards normal pH_i levels. The oocyte was then returned to normal saline and exposure to NH_4^+ once again led to a substantial reduction in pH_i once it had been washed off. Each NH_4^+ exposure caused a large membrane depolarization due mainly to the activation of a chloride conductance.

these cells exposure to ammonium induced a rapid decrease in pH_i which recovered slowly when the NH_4Cl was washed from the bath. The lower section of Fig. 8 shows pH_i (together with calibrations before and after the experiment) in a 1·7 mm diameter oocyte during five successive periods of exposure to 20 mM NH_4^+. Each period of exposure lasted 1·5 min. At the start of the experiment the resting cytoplasmic pH was about 7·5 but as soon as the oocyte was exposed to ammonium, pH_i fell by almost 0·2 pH units. There was little immediate recovery upon removal of the ammonium and a second period of exposure lead to a further reduction in pH_i. This result was reproduced in all of the thirty oocytes tested.

One explanation for the fall in cytoplasmic pH is that the larger oocytes accumulate NH_4^+ more actively than the smaller ones. The extra NH_4^+ might enter by way of K^+ channels, via the Na^+ pump in place of K^+ or as a substitute for H^+ in the Na^+–H^+ exchanger. However, as Fig. 8 shows, neither the potassium channel blocker TEA (82 mM) nor the Na^+, K^+-ATPase inhibitor G-strophanthin (1 mM) have any effect on the NH_4^+-induced acidification. Inhibition of Na^+–H^+ exchange by 1 mM amiloride was also ineffective (authors' unpublished observations) and it seems unlikely that NH_4^+ enters by any of these routes. Another possibility, entry via chloride channels, was excluded by observations on two oocytes of NH_4^+-induced acidification in the presence of SITS (see Schlichter, 1989). In these oocytes the standard NH_4^+ exposure produced a decrease in pH_i of 0·092 (S.D. 0·04); in the presence of 0·5 mM SITS the decrease in pH_i was 0·122 (S.D. 0·04). When measuring aCl_i during NH_4^+ exposure we found an increase of 1·93 mM (S.D. 0·66; $n = 10$) associated with a pH_i decrease of 0·08 (S.D. 0·04; $n = 8$).

To assess the contribution of chloride-dependent NH_4^+ transport to the fall in cytoplasmic pH, large oocytes were exposed to ammonium in chloride-free saline. At the start of the record shown in Fig. 9, the oocyte was bathed in normal saline and two initial applications of ammonium caused pH_i to decrease to near 7·0. Subsequent exposure of the oocyte to ammonium in chloride-free saline produced a greatly reduced pH_i decrease (0·05 pH units or less) as if external chloride was required for much of the ammonium entry. On return to normal saline, treatment with ammonium once again produced a large decrease in pH_i (0·29 pH units). The experiment was repeated a second time before the pH-sensitive double-barrelled electrode was withdrawn and calibrated. In seventeen experiments on twelve large-diameter oocytes, exposure to chloride-free saline reduced the peak acidification by an average of 59 % (S.D. 17 %).

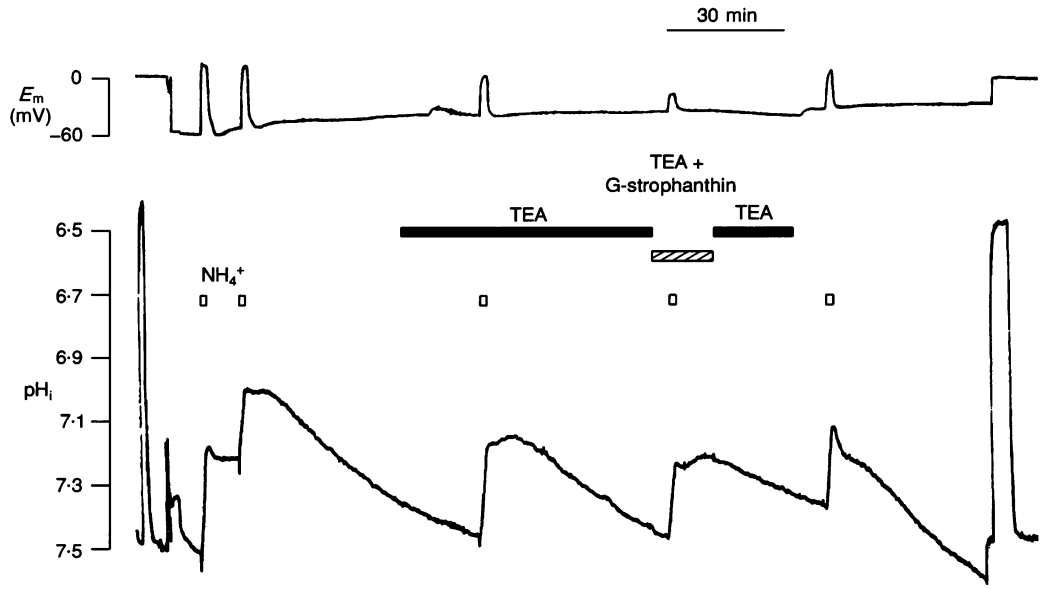

Figure 8. Effect of ammonium on a large-diameter oocyte under control conditions and in the presence of G-strophanthin and/or TEA

Effect of ammonium application on the membrane potential (E_m; top trace) and the intracellular pH (pH_i; bottom trace) of a large-diameter (1·7 mm) oocyte. Exposure to 20 mM ammonium chloride for 1·5 min resulted in a rapid acidification of pH_i and depolarization of E_m and a second exposure took pH_i to near 7·0. After about 40 min the bathing medium was changed to one containing 82 mM TEA but this had little effect on the response of the oocyte to a third exposure to NH_4^+. Addition of G-strophanthin (1 mM) to the TEA-containing bathing medium also had little effect on the cell's response to NH_4^+ although the change in membrane potential was less. At the end of the experiment the oocyte was returned to normal saline and the effect of a fifth exposure to NH_4^+ was tested.

A comparison between the rate of change of pH_i in normal as against chloride-free saline provided further evidence for the role of chloride. In normal oocytes aCl_i increased by 1·21 mM min^{-1} (S.D. 0·68; $n = 6$) immediately after NH_4^+ exposure while pH_i decreased by 0·104 units min^{-1} (S.D. 0·066; $n = 6$). In chloride-free saline NH_4^+ exposure decreased pH_i by 0·048 units min^{-1} (S.D. 0·027; $n = 6$); a 54 % reduction in the rate of change of pH_i. If the chloride-independent pH change is excluded from the calculation, there is a 0·046 unit change in pH_i per millimolar increase in aCl_i. The intracellular buffering power in CO_2-free saline may be taken to be about 10 mequiv H^+ per pH unit per litre (see for example Meech & Thomas, 1980). Consequently each millimolar increase in aCl_i is associated with about 0·5 mequiv H^+ or a ratio of $2Cl^-:1NH_4^+$.

As reported above there was a 52 % reduction in rate of NH_4^+-induced chloride uptake in sodium-free solution. There was also a 43 % (S.D. 8 %; $n = 4$) reduction in acidification in sodium-free saline. Measurements of aNa_i during NH_4^+ exposure in normal chloride revealed a relatively slow rate of increase; 0·07 mM min^{-1} (S.D. 0·02; $n = 4$) but in two oocytes which were tested in chloride-free saline there was an overall fall in aNa_i.

Although prolonged depletion of internal chloride or sodium increased the NH_4^+-induced acidification it is clear that the cotransporter does not provide the only pathway for NH_4^+ entry because intracellular acidification was not fully blocked by either chloride-free or Na^+-free conditions. What is more, bumetanide and furosemide were relatively ineffective even at high concentrations. Bumetanide (10^{-4} M) produced 23 % inhibition ($n = 2$) and furosemide (10^{-3} M) 22 % inhibition (S.D. 10 %; $n = 4$); in both cases these high levels of inhibitor proved irreversible.

All oocytes, at whatever stage of development, became depolarized when exposed to NH_4Cl (see Figs 7, 8 and 9). This depolarization was correlated with the presence of the ammonium rather than with any change in pH_i. The potential change associated with ammonium application was observed even when the oocyte was bathed in 82 mM TEA (Fig. 8). It was larger in chloride-free saline (Fig. 9) and smaller under conditions of depleted internal chloride (small oocyte shown in Fig. 7) and so it may result, at least in part, from an increase in chloride conductance. Pharmacological tests failed to support this conclusion; the change in membrane potential was unaffected by the presence in the bathing medium of 0·5 mM SITS (which is known to block a calcium-activated chloride conductance in *Rana* oocytes; Schlichter, 1989). Furthermore G-strophanthin (1 mM), while having little effect on the change in pH_i, significantly reduced the membrane depolarization (Fig. 8).

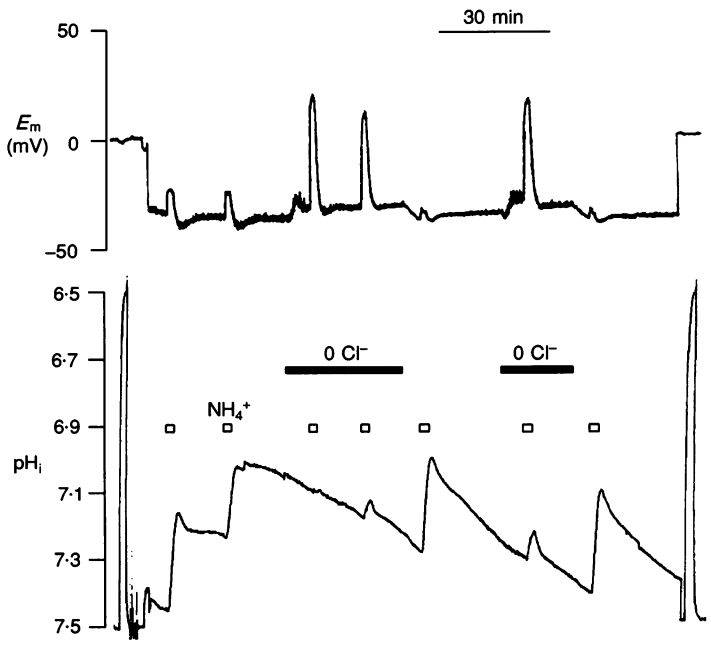

Figure 9. Effect of ammonium on a large-diameter oocyte under control conditions and in chloride-free saline

Effect of ammonium application on the membrane potential (E_m; top trace) and the intracellular pH (pH_i; bottom trace) of a large-diameter oocyte. Exposure to 20 mM ammonium chloride resulted in a rapid acidification of pH_i and depolarization of E_m. When the Na^+–K^+–$2Cl^-$ cotransporter was inhibited by perfusion with chloride-free solution (and 20 mM NH_4^+ was added as $(NH_4)_2SO_4$) the intracellular acidification was significantly reduced. Each ammonium exposure caused a large membrane depolarization due mainly to the activation of a chloride conductance.

DISCUSSION

Boyle & Conway (1941) showed that chloride ions in frog muscle were in Donnan equilibrium with potassium. Subsequent measurements with intracellular chloride-sensitive liquid ion-exchanger-based microelectrodes confirmed that under normal steady-state conditions the chloride equilibrium potential and the membrane potential differed by less than 2 mV (Bolton & Vaughan-Jones, 1977). However, in other muscle types, levels of intracellular chloride are four (quiescent sheep Purkinje fibres; Vaughan-Jones, 1979) and five (guinea-pig vas deferens; Aickin & Brading, 1982) times more than predicted for passive distribution.

In nerve cells a high level of internal chloride has been measured in extruded squid axoplasm by Mauro (1954), using silver–silver chloride electrodes and in certain vertebrate neurons (Alvarez-Leefmans, Gamiño, Giraldez & Noguerón, 1988; using ion-exchanger-based microelectrodes) but chloride was found to be passively distributed in some, but not all, molluscan neurons (Kerkut & Meech, 1966; using micropipettes filled with precipitated silver).

Both ion-exchanger-based and some forms of silver-based chloride-sensitive microelectrodes may overestimate intracellular chloride in some preparations (Alvarez-Leefmans *et al.* 1990) but it has been possible to validate many microelectrode measurements with some independent technique such as ion analysis of squid axoplasm (Keynes, 1963), the reversal potential of chloride-selective cholinergic inhibitory postsynaptic potentials (Kerkut & Meech, 1966) or the depolarizing chloride-selective GABA response (Ballanyi & Grafe, 1985). Calcium-activated chloride conductances provide a means to validate measurements in amphibian oocytes, and in *Xenopus* the chloride equilibrium potential is about −30 mV (i.e. 30–60 mV more depolarized than resting; Barish, 1983).

Intracellular accumulation of chloride in *Rana* oocytes

Inwardly transporting chloride carriers such as the Na^+–K^+–Cl^- cotransporter are found in many different preparations (Chipperfield, 1986). In immature oocytes of the toad, *Bufo bufo*, passive potassium influx is sensitive to frusemide and to reduction of Cl^- or Na^+ (Chipperfield & Fry, 1982) while the intracellular recordings of aCl_i, aNa_i, and pH_i reported here strongly suggest that reaccumulation of chloride in chloride-depleted *Rana* oocytes is also largely mediated by Na^+–K^+–Cl^- cotransport. Experiments were conducted in a bicarbonate-free bathing medium and so we cannot estimate the contribution from chloride–bicarbonate exchange *in vivo*, but in *Xenopus* oocytes $^{36}Cl^-$ exchange is insensitive to the stilbene disulphonate DIDS, a chloride–bicarbonate exchange inhibitor (Bartel, Lepke, Layh-Schmitt, Legrum & Passow, 1989).

The proposal that Na^+–K^+–Cl^- cotransport is responsible for chloride reaccumulation is supported by its dependence on external sodium and potassium, its inhibition by low levels of the loop diuretic bumetanide and the inverse relationship between aNa_i and aCl_i following chloride reuptake (see Fig. 4). Evidence provided here and by others suggests that $1Na^+$, $1K^+$ and $2Cl^-$ are transported together. The process is electroneutral and consequently independent of membrane potential. Thus the free energy available for transport depends simply on the ionic gradients of the three participating ions and at thermodynamic equilibrium $(aNa_i) \times (aK_i) \times (aCl_i)^2$ should equal $(aNa_o) \times (aK_o) \times (aCl_o)^2$. If aNa_o, aK_o and aCl_o are constant and changes in aK_i are small, a graph of $(aCl_i)^2$ against $1/aNa_i$ should be a straight line with a slope equivalent to the value of $(aNa_o) \times (aK_o) \times (aCl_o)^2/(aK_i)$; $6{\cdot}6 \times 10^{-6}$ mol^3. In fact the best fit to the data is obtained when $(aCl_i)^{1{\cdot}86}$ is plotted against $1/aNa_i$ as in Fig. 4*B*. This small discrepancy could arise if the cotransporter was operating in the face of a sustained passive leak of chloride ions out of the cell. It is unlikely to be the result of active sodium transport because the bathing medium contained 0·1 mM G-strophanthin.

Ammonium cotransport in *Rana* oocytes

Since the work of Jacobs (1924), ammonium salts have been assumed to penetrate animal cells largely as NH_3, although there is a limited entry of NH_4^+ by way of K^+ channels (Binstock & Lecar, 1969), via the Na^+ pump in place of K^+ (Post & Jolly, 1957) or as a substitute for H^+ in the Na^+–H^+ exchanger (Kinsella & Aronson, 1981). In *Rana* oocytes potassium channels are fully blocked by levels of TEA (Schlicter, 1989) that have no effect on the NH_4^+-induced acidification (Fig. 8). Inhibition of the Na^+, K^+-ATPase by G-strophanthin (1 mM) or inhibition of Na^+–H^+ exchange by 1 mM amiloride (Fig. 8 and authors' unpublished observations) are also ineffective and it seems unlikely that NH_4^+ enters by these routes.

In most cell types exposed to external ammonium there is an increase in pH_i because when NH_3 enters the cell, it takes up a proton to form NH_4^+. The discovery of NH_4^+ transport by the Na^+–K^+–Cl^- cotransporter in mammalian kidney (Kinne, Kinne-Saffran, Schutz & Scholermann, 1986) and its contribution to pH_i recovery in vascular smooth muscle (Tseng & Berk, 1992) suggest that it may be of widespread occurrence and play a significant role in the cellular response to external ammonium. Inward transport of NH_4^+ leads to intracellular acidification because once inside the cell the NH_4^+ releases a proton to form NH_3. Hitherto unexplained differences in the rates of penetration of ammonium salts into different cell types can perhaps be attributed to differences in cotransporter activity.

In the smallest *Rana* oocytes that we examined, chloride-dependent NH_4^+ cotransport appeared to contribute to pH_i regulation. These oocytes became alkaline in 20 mM NH_4^+ but then exhibited a slowly developing acid transient (see Fig. 7). This acidification was abolished in chloride-free

solution. In larger *Rana* oocytes there was no alkaline transient whatsoever and instead the cells became acidic immediately. This reduction in pH_i was partially dependent on external chloride and the two ions appeared to be transported in a ratio of $2Cl^-:1H^+$. Both the increase in aCl_i and the reduction in pH_i were partially dependent on external sodium. The increase in aNa_i was small but this could be attributed to Na^+ efflux via Na^+, K^+-ATPase. Nevertheless the chloride increase was not fully blocked by sodium-free solution as if there was a contribution from some other form of NH_4^+–Cl^- transport.

Chloride-dependent NH_4^+ transport appears unlikely to be the only mechanism responsible for the NH_4^+-induced acidification as chloride-free saline seemed to be fully effective only when pH_i was quite acidic (a condition that would of itself tend to inhibit ammonium influx). Furthermore the sensitivity of the acidification to bumetanide (10^{-4} M produced 23% inhibition) is significantly less than that of chloride recovery (10^{-5} M produced 85% inhibition). Even in chloride-free saline there appeared to be no underlying alkaline shift in pH_i (see Fig. 9). This may indicate that the larger oocytes have a low permeability for NH_3 but it is more likely that any alkaline shift was hidden by some other ammonium-dependent acidification.

A source of protons in many cell types is calcium–hydrogen ion exchange by the outer membrane (Carafoli, 1991) or intracellular organelles (Meech & Thomas, 1980) following an increase in cytoplasmic calcium. Exposure to ammonium causes a momentary Ca^{2+} influx in sea urchin eggs (Zucker, Steinhardt & Winkler, 1978) and a similar influx in *Rana* oocytes might release protons as well as contributing to the change in membrane potential (see Figs 7, 8 and 9).

The possibility that oocytes become less permeable to NH_3 as they approach maturity seems unlikely for when oocytes are exposed to 20 mM NH_4Cl, the pH of the solution close to the outer membrane surface falls significantly as if the surface layer had lost NH_3 to the cell interior (author's unpublished data). However, there would be an apparent reduction in NH_3 permeability if the diffusion compartments of NH_3 and NH_4^+ change relative to one another. For example, there may be an increase in some acidic internal compartment that takes up NH_3 preferentially and reduces its concentration in the part of the cytoplasm that is accessible to the pH electrode.

Ammonium cotransport in excitable cells

In mouse soleus muscle fibres exposed to NH_4^+ in K^+-free conditions there is a rapid acidification of the cell cytoplasm which is inhibited by the presence of potassium and blocked by ouabain (Aickin & Thomas, 1977). Thus conditions which promote the influx of NH_4^+ via the cotransporter (K^+-free solution) speed up the acidification whereas conditions that inhibit the inward cotransport (ouabain-induced increase in internal sodium) abolish it.

In both vertebrate (Lux, Loracher & Neher, 1970) and invertebrate (Ascher, Kunze & Neild, 1976) central neurons at neutral pH, NH_4^+ exposure produces an increase in internal chloride which returns rapidly to normal when the NH_4^+ is removed; intracellularly injected NH_4^+ promotes chloride efflux. Exposure to NH_4^+ at alkaline pH (where levels of external NH_3 would be expected to be high and levels of NH_4^+ would be expected to be low) produces little change in internal chloride until NH_4^+ is removed. There is then an abrupt fall which is enhanced if the experiment is carried out in K^+-free solution (Ascher *et al.* 1976). It has been proposed that ammonium salts inhibit chloride efflux (Aickin, Deisz & Lux, 1982) but evidence for furosemide-sensitive sodium-dependent K^+–Cl^- transport in rat sympathetic neurons (Ballanyi & Grafe, 1985) indicates a possible contribution from Na^+–NH_4^+–Cl^- cotransport. Thus at neutral pH, high levels of external NH_4^+ do not inhibit chloride efflux but instead promote chloride influx while in alkaline conditions, NH_3 entry leads to chloride loss because of the raised internal NH_4^+.

Physiological role for Na^+–K^+–Cl^- cotransport in oocytes

As a result of the active accumulation of chloride ions, the chloride equilibrium potential in *Rana* oocytes is more positive than the resting membrane potential (see Fig. 2). Consequently the long-lasting increase in chloride conductance seen at fertilization in these cells depolarizes the outer membrane and provides an electrical block to polyspermy (Cross & Elinson, 1980; Jaffe & Schlichter, 1985).

In addition to its likely physiological role in the accumulation of chloride, Na^+–K^+–Cl^- cotransport may play a role in oocyte volume regulation. This may be particularly important during oogenesis as the cells grow rapidly and accumulate considerable quantities of protein. Once the oocyte has been released from the oviduct and is surrounded by fresh water the cotransporter may play an important role in pH_i regulation. *In vivo*, H^+ produced from metabolism may be trapped by NH_3 to give NH_4^+ and then actively extruded from the cell cytoplasm in combination with Na^+ and Cl^-.

Amphibians are a transitional stage (Wood, Munger & Toews, 1989) in the evolution from ammoniotelism (teleost fish) to urotelism (mammals) and they excrete both ammonium and urea. In *Rana* the total ammonia concentration in arterial blood plasma is about 50 mmol l^{-1} and in *Xenopus* it is even higher (Wood, Munger & Toews, 1989). Given that the cotransporter studied in membrane vesicles has an affinity for potassium (in 100 mM NaCl) of about 1 mM and that it may have an equal affinity for ammonium ions (Kinne, Koenig, Hannafin, Kinne-Saffran, Scott & Zierold, 1985) it seems possible that the developing oocyte may actively take up NH_4^+ via the cotransporter and incorporate it into protein. In this way NH_4^+ movement may contribute to the accumulation of chloride.

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