J. Physiol. (1987), **393**, *pp.* 1–17
With 7 text-figures
Printed in Great Britain

EFFECTS OF CELL VOLUME CHANGES ON MEMBRANE IONIC PERMEABILITIES AND SODIUM TRANSPORT IN FROG SKIN (*RANA RIDIBUNDA*)

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(*Received 16 June 1986*)

SUMMARY

1. Membrane potential and conductances and short-circuit current were continuously measured with microelectrodes and conventional electrophysiological techniques in a stripped preparation of frog skin epithelium. The effects of the removal of chloride or sodium ions and the concentration or dilution of the serosal (inner) bathing solution were studied.

2. Chloride- or sodium-free solutions produced a cell depolarization of about 30 mV in parallel with a fall in the short-circuit current. Mucosal and serosal membrane conductances both decreased and the sodium permeability of the mucosal barrier was calculated to fall to about one-half its value in standard Ringer solution. The observed decrease in the short-circuit current is probably related to the combined effect of the decrease in sodium permeability and the decrease in the driving force across the mucosal membrane.

3. The removal of chloride or sodium ions reduced the depolarization caused by serosal perfusion with high-potassium solutions (50 mM-KCl). The ratio of the change in cell membrane potential under short-circuit conditions to the change in the potassium equilibrium potential ($\Delta E^{c(s.c.)}/\Delta E_K$), was 0·59 in standard Ringer solution and 0·26 and 0·24 after the removal of chloride or sodium respectively. The depolarizing effect of barium-containing solutions (2 mM-$BaCl_2$) was also markedly reduced in chloride- or sodium-free solutions, suggesting a decrease of the potassium selectivity of the serosal membrane in these conditions.

4. Increasing the osmolality of the serosal bathing solution produced similar effects, i.e. cell depolarization, fall in the short-circuit current and membrane conductances and reduction of the depolarizing effect of high-potassium and barium solutions. On the contrary, dilution of the serosal bath produced the opposite effects, consistent with an increase in the serosal permeability to potassium.

5. The effects of chloride- or sodium-free solutions were reversed by the dilution of the serosal bath. Cells repolarized when exposed to low-osmolality solutions *after* being in the absence of serosal chloride or sodium. The repolarization ran in parallel with the restoration of the short-circuit current and the potassium selectivity of the serosal membrane.

6. The results show that the effects produced by the removal of sodium or chloride ions from the serosal bathing solution are most probably mediated by a reduction in cell volume. Cell volume changes would lead to changes in the serosal membrane selectivity to potassium and thus to changes in cell membrane potential and sodium transport.

INTRODUCTION

The removal of sodium or chloride ions from the solution bathing the serosal membrane of the frog skin epithelium leads to a reduction in cell volume and to a decrease in the short-circuit current (Ferreira & Ferreira, 1981). The effect on cell volume is probably the result of the net salt loss which takes place when the uphill accumulation of chloride through a sodium-dependent mechanism is stopped (Ferreira & Ferreira, 1981; Giraldez & Ferreira, 1984). The origin of the fall in short-circuit current, however, remains obscure. The idea that the fall in current is related to cell shrinkage is suggested by the observation that short-circuit current also falls when the serosal solution is made hypertonic and it rises if diluted (MacRobbie & Ussing, 1961; Ussing, 1965). However, the effects caused by cell volume changes cannot be due to simultaneous changes in cell sodium concentration because they ought to produce the opposite effect, i.e. swelling, should decrease sodium pump activity and shrinkage should increase it. The observed changes in the short-circuit current are therefore more probably related to changes in the rate of sodium entry across the mucosal barrier. Due to the relatively low cell sodium concentration, the electrochemical potential gradient for sodium ions across the mucosal barrier is dominated by the mucosal membrane potential, which is in turn determined by the ion selectivity of both mucosal and serosal cell membranes. Volume-induced modifications of the permeability to ions are well documented in some other tissues (Kregenow, 1981; Hoffman, 1983; Sarkadi, Mack & Rothstein, 1984; see Siebens, 1985, for review). An interesting possibility, therefore, would be that a decrease in cell volume induces a change in membrane selectivity to ions, leading to depolarization of the cells and consequent reduction in sodium entry. Here we provide some evidence for this view by studying the changes in cell membrane potential and conductances after the removal of chloride or sodium ions and the concentration or dilution of the solution bathing the serosal membrane. This was done using intracellular microelectrodes and the isolated epidermis of the frog skin (Giraldez & Ferreira, 1984). The results show that the effect of chloride or sodium removal and serosal hypertonicity is to induce a cell depolarization by the loss of the potassium selectivity of the serosal membrane. A preliminary account of this work has been reported to the Physiological Society (Ferreira, Ferreira & Giraldez, 1985).

METHODS

General. Experiments were done on abdominal skins of *Rana ridibunda*. The epidermis was isolated from the chorion according to the method described by Ferreira & Swenson (1979) with two modifications. First, only a small piece of chorion of about 5 mm in diameter was removed. This improved the mechanical stability of the preparation while still allowing the recording of the short-circuit current from a larger surface area of skin. The second modification was that the short-

circuit current was continuously monitored throughout the incubation period. This showed the innocuity of the treatment with collagenase (80 units ml^{-1}, type I collagenase, Sigma) and served to assess the state of the skin at the beginning of the experiment. Skins were mounted internal side upwards in a modified Ussing chamber based on a design by Frömter (1972) and suitable for microelectrode work. The total area of exposed skin was 0·78 cm^2. Both sides of the skin could be continuously superfused by gravity with fresh solutions.

Solutions. The standard Ringer solution had the following composition (mM): Na^+, 112; Cl^-, 124; K^+, 2·5; Ca^{2+}, 1; Mg^{2+}, 1; Tris, 5. The osmolarity was 230 mosmol kg^{-1}. Chloride-free (0 Cl^-) Ringer solution was made by replacing Cl^- by gluconate, and Na^+-free (0 Na^+) Ringer solution by replacing Na^+ by choline. Hypertonic Ringer solution (2 osM) contained the same salts as that of the standard Ringer solution plus 200 mM-mannitol. Hypotonic Ringer solution (0·5 osM) was made by reducing the major salt (NaCl, choline chloride or sodium gluconate) to one-half, the other ions being kept constant. Exceptionally (Fig. 5), 100 mM-sucrose was added or removed from a Ringer solution containing 100 mM-sucrose, so as to produce smaller changes in tonicity at a constant NaCl concentration; those are labelled +100 mM-sucrose and −100 mM-sucrose, respectively. High-potassium solutions (50 mM-K^+ Ringer solution) were made by equimolar replacement of the major cation (sodium or choline) by potassium to a final concentration of 50 mM and in a few cases to 25 mM. Solutions referred to as Ba^{2+} solutions were prepared by adding 2 mM-$BaCl_2$. All solutions were aerated and titrated to a pH of 8. The osmolality was 230 mosmol kg^{-1} except when it was deliberately altered.

Electrodes and electrical arrangements. Skins were short-circuited except for periods of 20 s when they were clamped at 10 mV in order to measure transepithelial conductance and fractional resistance. Microelectrodes for intracellular recording were pulled from borosilicate glass tubing (1·5 mm o.d.) in a two-stage microelectrode puller and filled with 4 M-potassium acetate solution. Tip resistances ranged from 40 to 100 MΩ, usually about 60 MΩ. Cells were impaled from the chorial side using a Huxley-type micromanipulator. Conventional impalement criteria were used (Nagel, 1978; Giraldez & Ferreira, 1984): briefly, sharp deflection upon entry, stability of the recorded potential, constancy of the fractional resistance, and no change in the electrode tip potential within 5 mV after withdrawal from the cell. The general electrophysiological parameters of stripped skins, bathed in the standard Ringer solution, will be given throughout the text (see Table 1, for example) and they can be seen to be in good agreement with recent work done on whole skins (Nagel, 1976; Helman & Fisher, 1977). The microelectrode was connected to a conventional high-impedance amplifier through a Ag–AgCl silver wire, and the signal displayed on an oscilloscope and recorded continuously on a three-channel potentiometric pen recorder together with the transepithelial potential and clamping current. Cell and transepithelial potentials were referred to the mucosa which was kept at virtual ground. Under short-circuit conditions the potential difference across the serosal and mucosal barriers are by definition symmetric; therefore $E^m = E^s = E^{c(s.c.)}$, where E^m and E^s represent the electrical potential differences across the mucosal and serosal membranes respectively, and $E^{c(s.c.)}$ the electrical potential difference between the cell and the mucosal bath under short-circuit conditions.

Symbols, units and definitions. Symbols and units used throughout the text are those of Lew, Ferreira & Moura (1979). They are E^t, E^m, E^s for transepithelial, mucosal and serosal potentials, $I^{s.c.}$ for the short-circuit current and I^t for transepithelial current density (A cm^{-2}). The terms G^t, G^c, G^{sh}, G^m, G^s represent transepithelial, transcellular, shunt, mucosal and serosal membrane conductances respectively (S cm^{-2}), and R^t, R^c, R^{sh}, R^m, R^s are the corresponding resistances (Ω cm^2). The fractional resistance of the mucosal barrier, $f(R^m)$, is defined as $f(R^m) = R^m/(R^m + R^s)$. The terms E^t, $E^{c(s.c.)}$, E^m, E^s, $I^{s.c.}$ and I^t were measured values. G^t was calculated from $G^t = \Delta I^t/\Delta E^t$; the term G^{sh} was calculated from experiments in which amiloride (10^{-4} M) was added to the mucosal side. Under the assumption that amiloride has no effect on the shunt pathway and that other ionic permeabilities apart from sodium are negligible in the mucosal barrier, it holds that in the presence of the drug $G^t = G^{sh}$. From here G^c can be also calculated: $G^c = G^t - G^{sh}$. Individual membrane conductances were estimated from the values of $f(R^m)$ and G^c. The value of $f(R^m)$ was obtained from $f(R^m) = \Delta E^m/\Delta E^t$ for small current pulses clamping E^t at +10 mV for 20 s. It can be easily shown that $G^m = G^c/f(R^m)$ and $G^s = G^c/(1 - f(R^m))$. The constant-field equation was used to calculate the mucosal sodium permeability from values of $I^{s.c.}$, $E^{c(s.c.)}$ and intracellular and extracellular concentrations. The expression for the slope conductance of a single ion (Ferreira & Ferreira, 1983) was

used to calculate ionic permeabilities from conductance measurements and to estimate the degree of rectification due to the change in cell membrane potential:

$$G_{ion}^{m,s} = P_{ion}^{m,s} \frac{(zF)^2}{RT} \left[\frac{(e^{V^{m,s}} - 1) - V^{m,s} e^{V^{m,s}}}{(e^{V^{m,s}} - 1)^2} (C_{ion}^{m,c} e^{V^{m,s}} - C_{ion}^{c,s}) + \frac{V^{m,s}}{(e^{V^{m,s}} - 1)} C_{ion}^{m,c} e^{V^{m,s}} \right], \quad (1)$$

where $G_{ion}^{m,s}$ is the partial ionic conductance of the ion across the barrier m or s, $P_{ion}^{m,s}$ its permeability coefficient and $V^{m,s} = (j)\ (zF/RT) E^{m,s}$, where $j = 1$ for E^m and $j = -1$ for E^s; $C_{ion}^{m,c}$ is the concentration of the ion in the mucosal (m), or cell (c) compartment, and $C_{ion}^{c,s}$ is its concentration in the cell (c) or serosal (s) compartment.

RESULTS

Effects of the removal of sodium or chloride ions from the solution bathing the serosal barrier

In the following experiments the serosal bathing solution was rendered chloride-free or sodium-free while cell membrane potential, mucosal fractional resistance and short-circuit current were continuously monitored. Typical experiments are shown in Fig. 1*A* (for chloride) and 1*B* (for sodium). The effects of chloride or sodium removal were very similar and consisted of a marked cell depolarization (lower trace in Fig. 1*A* and *B*) and a parallel fall in the short-circuit current (middle trace, Fig. 1*A* and *B*) that followed a slow time course (half-times about 10 min). Table 1 shows the results of several experiments. It can be seen that from a value of about −80 mV in Ringer solution the cell membrane potential decreased by approximately 30 mV in chloride-free and sodium-free solutions and the short-circuit current fell to about one-half in experiments with chloride-free solutions and to two-thirds with sodium-free solutions. Knowing that the membrane potential is the dominant factor in determining the electrochemical gradient for sodium across the outward-facing membrane, we were interested in determining to what extent the observed cell depolarization could account for the decrease in the short-circuit current. This was done by using plots like those of Fig. 2 that show the results of the two experiments of Fig. 1 for which the values of $I^{s.c.}$ recorded along the transient were plotted against the function: $zFV(C_{Na}^m - C_{Na}^c e^V/(1 - e^V)$, according to the Goldman–Hodgkin–Katz equation. Since, as a first approximation, it can be considered that C_{Na}^m and C_{Na}^c remain constant, the function on the abscissa is only dependent on V and hence on $E^{c(s.c.)}$. Taking $I^{s.c.}$ as identical to the sodium current (see below), it follows that the points on the line give the ratio $I^{s.c.}/zFV(C_{Na}^m - C_{Na}^c e^V)/(1 - e^V)$, i.e. the sodium permeability of the mucosal membrane, P_{Na}^m. Figure 2 shows that the fall in $I^{s.c.}$ is parallel to the depolarization of the cell, indicating that the decrease in the sodium current after

Fig. 1. Effects of the removal of chloride or sodium from the serosal bathing solution on short-circuit current and membrane potential. *A*, recording from an experiment in which serosal chloride was replaced by gluconate. The upper trace shows the transepithelial potential, middle trace short-circuit current and the lower cell membrane potential. Note that the skin was short-circuited except for brief periods when the transepithelial potential was clamped at 10 mV to allow direct measurement of transepithelial conductance and $f(R^m)$. Since the same current flows across the mucosal and serosal barriers, the voltage pulses across the mucosal barrier are proportional to its fractional resistance. The bars at the bottom indicate the different solutions in the serosal superfusate, the mucosal side being always standard Ringer solution. *B*, experiment as *A* but in which serosal sodium was replaced.

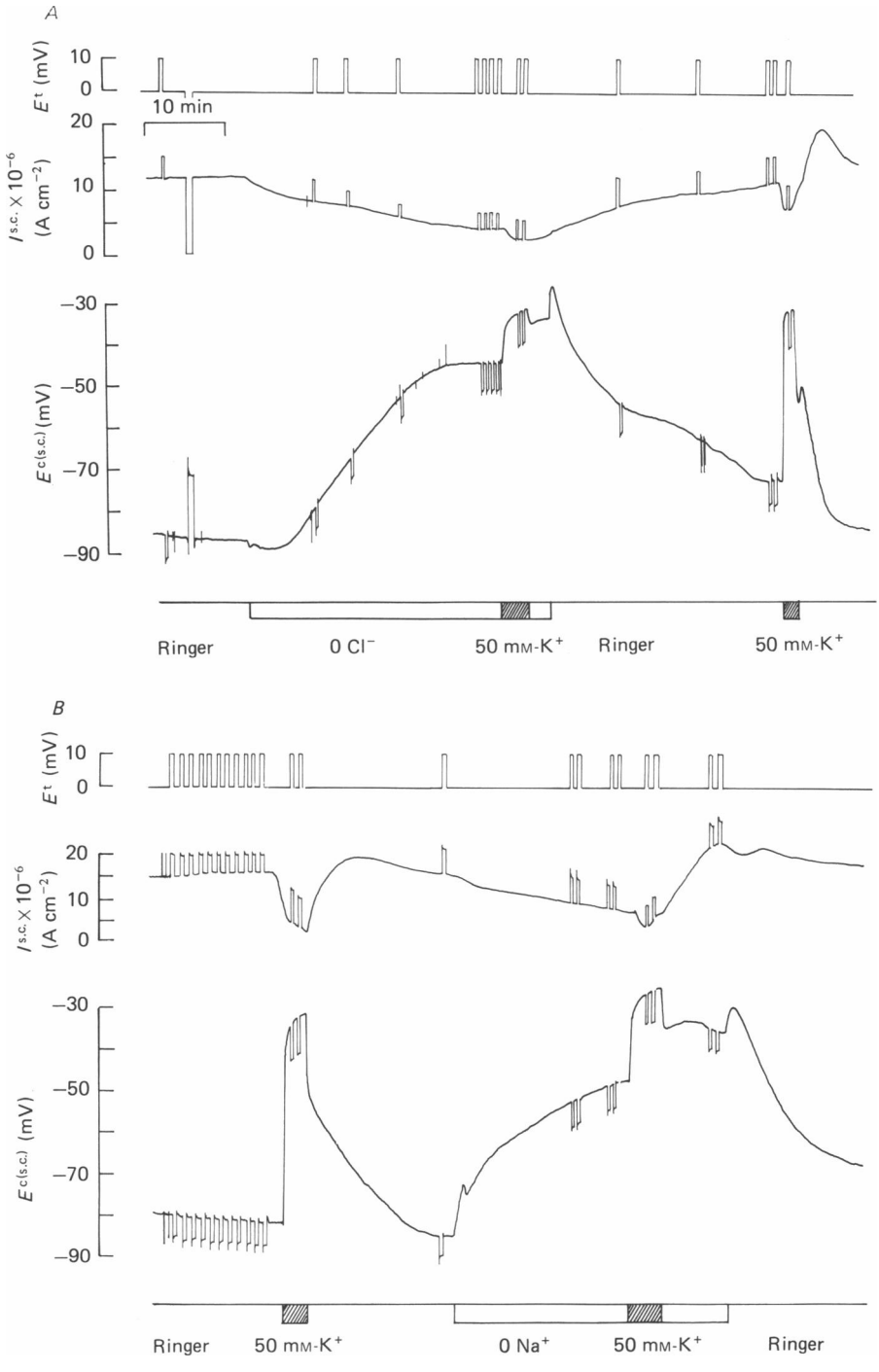

Fig. 1. For legend see opposite.

sodium- or chloride-free solutions was produced by the decrease in the electrochemical gradient for sodium across the mucosal membrane. This would be strictly true if P^{m}_{Na} remained constant throughout the transient. However, it was consistently observed that the values of P^{m}_{Na} at lower potentials (to the left in Fig. 2) tended to

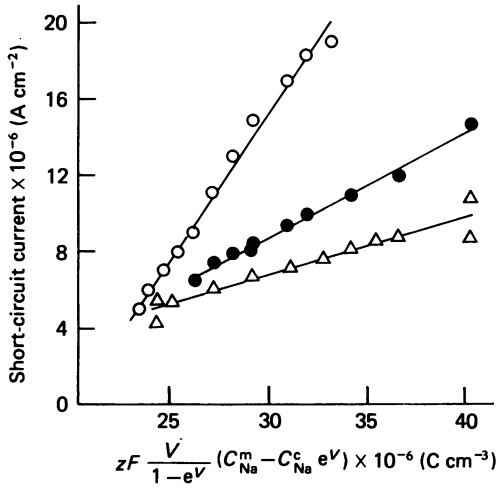

Fig. 2. Relation between short-circuit current and electrochemical driving force across the mucosal membrane. Values of short-circuit current during the transient after 0 Cl^- (△), 0 Na^+ (●) or hypertonic solutions (○) were plotted against the corresponding value of the driving-force term in the Goldman–Hodgkin–Katz equation. C^{c}_{Na} was taken as 20 mM (Nagel, García-Diaz & Armstrong, 1981); C^{m}_{Na} = 120 mM; $V = (zF/RT)\,E^{c(s.c.)}$.

be smaller than those at more negative potentials. Averaged values of P^{m}_{Na} calculated in normal and chloride-free Ringer solutions, in the steady state, revealed an apparent decrease in the mucosal sodium permeability of about 30% (the value of P^{m}_{Na} was $0{\cdot}31 \times 10^{-6}$ cm s^{-1} in standard Ringer solution and $0{\cdot}19 \times 10^{-6}$ cm s^{-1} in chloride-free solution). Apart from the decrease in the cell membrane electrical potential, a change in the sodium permeability of the mucosal barrier must be considered, therefore, as an additional contributor to the fall in $I^{s.c.}$ after the removal of chloride. After sodium removal the value of P^{m}_{Na} tended also to be decreased, but by a much smaller amount ($0{\cdot}28 \times 10^{-6}$ cm s^{-1} in Ringer solution and $0{\cdot}24 \times 10^{-6}$ cm s^{-1} in sodium-free solution).

Changes in transepithelial and cell conductances were also estimated by clamping the transepithelial potential at 10 mV for short periods and measuring the transepithelial potential and the fractional resistance of the mucosal membrane (Fig. 1*A* and *B*). Since the same current flows across mucosal and serosal barriers the voltage pulses across the mucosal barrier are proportional to its fractional resistance. The results are summarized in Table 1. It can be seen that both in chloride-free and sodium-free solutions the total transepithelial conductance (G^t) decreased and the mucosal fractional resistance ($f(R^m)$) increased. The fall in G^t was at the expense of both shunt and cellular conductances. These conductances were calculated from G^t and $f(R^m)$ as described in the Methods section. It is also apparent that changes in

both mucosal and serosal conductances are more pronounced after chloride removal than after the reduction of sodium. In the first case, G^m and G^s were reduced by about 60 and 50% respectively, whereas in sodium-free solution G^m fell by about 50% and G^s by about 20% of their initial values.

TABLE 1. Effects of chloride-free and sodium-free (serosal) solutions on short-circuit current, membrane potential and cell and shunt resistances. Measurements from continuous recordings like those shown in Figs 1, 4, 6 and 7. The value of G^{sh} was estimated by measuring G^t after amiloride; those of G^m and G^s were calculated from $f(R^m)$ and the total cell conductance ($G^t - G^{sh}$). Numbers are mean ± S.E.M. of nine experiments of 0 Cl^- and eleven of 0 Na^+.

	$I^{s.c.}$ ($\times 10^{-6}$) (A cm^{-2})	$E^{(s.c.)}$ (mV)	G^t ($\times 10^{-6}$) (S cm^{-2})	$f(R^m)$ $\times 100$	G^{sh} ($\times 10^{-6}$) (S cm^{-2})	G^m ($\times 10^{-6}$) (S cm^{-2})	G^s (10^{-6}) (S cm^{-2})
Ringer soln.	12·2 ±2·2	−78 ±3	670 ±140	61 ±3	507 ±114	267	418
0 Cl^-	5·6 ±1·2	−53 ±6	450 ±110	65 ±4	380 ±74	108	200
Ringer soln.	10·3 ±1·2	−82 ±3	620 ±50	62 ±3	471 ±79	240	392
0 Na^+	6·8 ±0·7	−49 ±3	480 ±40	73 ±4	394 ±71	119	319

In order to assess the relative weight of the change in cell potential and in cell permeability in determining the observed decrease in G^m, eqn (1) in the Methods section was used to estimate the fall in G^m_{Na} due to the fall in transmembrane potential (Goldman rectification). Using the values measured for $E^{c(s.c.)}$ in normal and chloride-free Ringer solution and for external and internal sodium concentrations of 120 and 20 mM respectively, eqn (1) shows that a reduction of G^m_{Na} by about 15% can be expected from the change in $E^{c(s.c.)}$. An additional decrease in P^m_{Na} should be needed to explain the fall in G^m. The values for P^m_{Na} calculated from eqn (1) and from the values of G^m in Table 1 were ($\times 10^{-6}$): 0·64 and 0·54 cm s^{-1} in standard NaCl Ringer solution and 0·28 and 0·32 in chloride-free and sodium-free solutions respectively. These figures compare well with those estimated above and indicate a consistent decrease in P^m_{Na}. The decrease in P^m_{Na} is more pronounced when chloride ions are removed from the internal bathing solution than when sodium ions are removed from the same solution.

When eqn (1) is used to describe the diffusional potassium current across the serosal membrane it can be shown that when the cell is depolarized, as in chloride- or sodium-free solutions, potassium conductance (G^s_K) and thus the serosal membrane conductance (G^s) should increase by about 20%. By a similar reasoning it can be shown that G^s_{Cl} should also increase. Table 1, however, shows that the serosal membrane conductance falls in absolute terms.

The serosal barrier of the frog skin epithelium is selective to potassium ions, and the potassium diffusion potential dominates $E^{c(s.c.)}$. The possibility that this selectivity is lost in chloride- or sodium-free solutions was studied in the experiments that follow by examining the effects of high-potassium solutions on cell membrane potentials. Referring back to Fig. 1*A* and *B*, it can be seen that the large cell

depolarization after the increase in potassium concentration of the serosal solution (on average 45±7 mV) is markedly diminished in both chloride-free and sodium-free solutions (21±5 and 19±4 mV respectively). For identical changes in the external potassium concentration from 2·5 to 50 mM, the ratio $\Delta E^{c(s.c.)}/\Delta E_K$, i.e. the induced

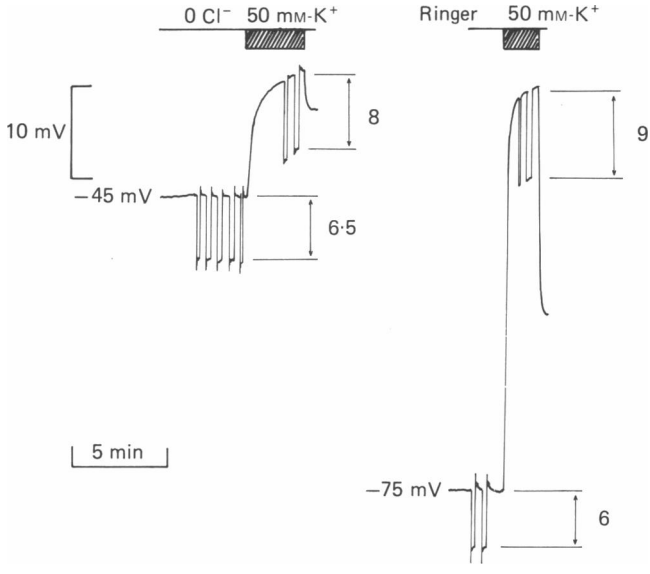

Fig. 3. Effect of chloride-free solutions (serosal) on the potassium selectivity of the serosal membrane. Two regions of the record in Fig. 1*A* were enlarged to show the effect of high-potassium (50 mM-K^+) solutions on cell membrane potential and fractional resistance of the mucosal barrier measured as the voltage drop across this barrier for a constant transepithelial clamp pulse in standard Ringer solution (right) and 0 Cl^- Ringer solution (left). The calibration is shown at the left and the numbers indicate the size of the voltage pulse (in mV) produced by clamping E^t at +10 mV. The bars at the top indicate the solutions superfusing the serosal chamber.

change in the membrane potential for a given change in the potassium equilibrium potential, was 0·59±0·05 in Ringer solution and decreased to 0·26±0·03 and 0·24±0·03 after the removal of chloride or sodium ions respectively. The increase in the fractional resistance of the mucosal barrier when the potassium concentration was raised to 50 mM in absence of chloride was also diminished when compared to Ringer solution. This is more clearly illustrated in Fig. 3 which reproduces traces of $E^{c(s.c.)}$ from the experiment shown in Fig. 1*A* but on a larger scale. It can be seen that although the fractional resistance of the mucosal barrier is in both cases increased in high-potassium solutions, the change in the voltage pulse for a constant transepithelial clamp is 1·5 mV in chloride-free and 3 mV in Ringer solution. This shows that the proportional increase in the fractional conductance of the serosal membrane after high potassium is diminished in absence of chloride, i.e. that the permeability of this membrane to potassium ions decreases in chloride-free solutions.

Barium ions selectively block potassium channels in the serosal membrane of frog skin, abolishing the potassium-electrode behaviour of this barrier (Nagel, 1979). A

loss in the selectivity of the serosal barrier to potassium should be manifested as a loss of the effect of barium on the membrane potential as this potential is no longer dominated by the potassium equilibrium potential. The experiment shown in Fig. 4 illustrates the effects of 2 mM-$BaCl_2$ in standard Ringer solution and in chloride-free

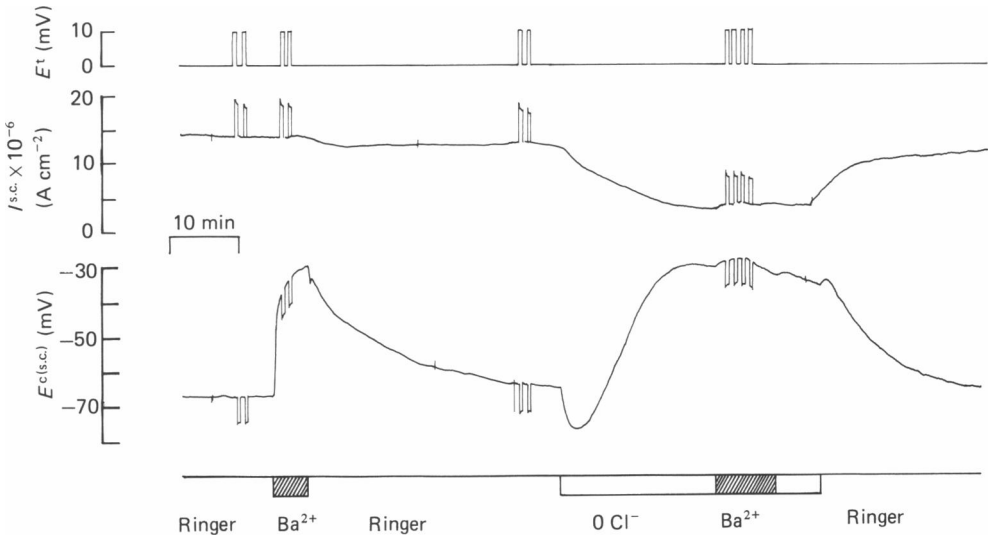

Fig. 4. Effect of barium ions (serosa) on cell membrane potential and short-circuit current. Simultaneous recording of E^t, $I^{s.c.}$ and $E^{c(s.c.)}$ as in Fig. 1. The effect of Ba^{2+} (2 mM) was tested in standard Ringer solution and in 0 Cl^- Ringer solution as indicated by the bars at the bottom.

solutions. It can be observed that the addition of barium ions produces an immediate cell depolarization of about 45 mV if chloride ions are present and that this effect is almost absent in chloride-free solutions. The effect of barium in sodium-free solutions was identical.

Effects of the osmolarity of the serosal bathing solution

The effects of hypertonic solutions were qualitative similar to those just described for sodium or chloride removal and are illustrated by the experiments in Figs 5*A* and 6. Figure 6 shows a recording of $E^{c(s.c.)}$ and $I^{s.c.}$ from an experiment in which the bath osmolarity was increased by the addition of 200 mM-mannitol. It can be seen that the final effect is a marked cell depolarization with a parallel fall in $I^{s.c.}$ and an increase in the fractional resistance of the mucosal barrier. The final size and time course of the depolarization was proportional to the external tonicity, so higher osmolarities produced larger and faster changes (compare Figs 5*A* and 6*A*). Average values for $E^{c(s.c.)}$ and $I^{s.c.}$ are given in Table 2. Again, a large fraction of the decrease in $I^{s.c.}$ is due to the fall in $E^{c(s.c.)}$, as revealed by plots like those in Fig. 2. A decrease in P^m_{Na} was also present and it was more evident after large changes in osmolarity. For the experiment shown in Fig. 6, for example, P^m_{Na} decreased by 22% in the 2 osM solution as compared to Ringer solution. The average value for P^m_{Na} was $0{\cdot}39 \times 10^{-6}$ cm s^{-1} in normal Ringer solution and $0{\cdot}26 \times 10^{-6}$ cm s^{-1} in hypertonic Ringer solution (2 osM),

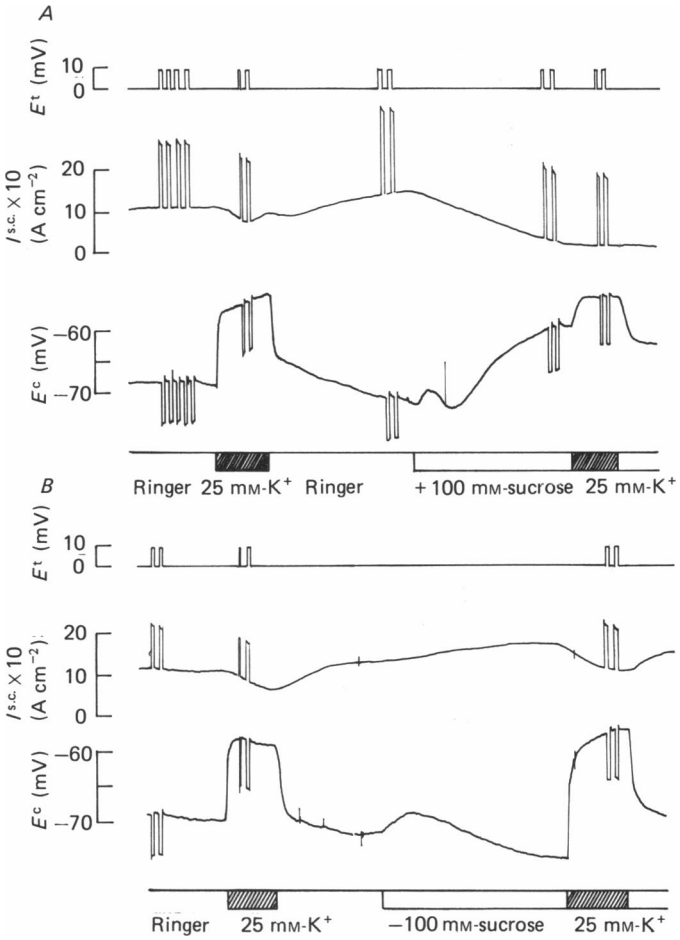

Fig. 5. Effects of changes in tonicity (serosal) on cell membrane potential and short-circuit current. Simultaneous recording of E^t, $I^{s.c.}$ and $E^{c(s.c.)}$. *A*, effects of increasing osmolarity (+100 mM-sucrose Ringer solution) on $E^{c(s.c.)}$ and $I^{s.c.}$, and on the response to high-K^+ solutions. The bars at the bottom indicate the solutions in the internal superfusate. *B*, effects of reducing osmolarity (−100 mM-sucrose Ringer solution) on $E^{c(s.c.)}$, $I^{s.c.}$ and on the response to high K^+.

and this is a 30 % reduction of P^m_{Na}. Accordingly, as shown in Table 2 (middle column), G^m also decreased. The values of P^m_{Na} calculated from (1) and from the values of G^m were 0·61 and $0{\cdot}23 \times 10^{-6}$ cm s^{-1} in normal and hypertonic Ringer respectively.

These experiments showed also a loss of the selectivity of the serosal barrier to potassium, as revealed by the diminished depolarization after raising the potassium concentration or adding barium to the serosal solution. As referred to before, there was a decrease in G^s to about one-half of its initial value (Table 2), which indicates an absolute decrease of P^s_K.

While an increase in tonicity of the serosal bathing solution produced a cell depolarization and an apparent decrease in P^s_K, dilution of this solution produced the opposite effect. This is illustrated by the experiments in Fig. 5*A* and *B* where the effects of concentration and dilution of the serosal bath are both compared. The

TABLE 2. Effects of hypertonic serosal solutions on short-circuit current, membrane potential and cell and shunt resistances. Measurements from continuous recordings like those in Fig. 6. Numbers are mean ± S.E. of five experiments

	$I^{s.c.}$ ($\times 10^{-6}$) ($A\ cm^{-2}$)	$E^{c(s.c.)}$ (mV)	G^t ($\times 10^{-6}$) ($S\ cm^{-2}$)	$f(R^m)$ $\times 100$	G^{sh} ($\times 10^{-6}$) ($S\ cm^{-2}$)	G^m ($\times 10^{-6}$) ($S\ cm^{-2}$)	G^s (10^{-6}) ($S\ cm^{-2}$)
Ringer	15·0	−80	780	66	593	283	550
soln.	±2·5	±3	±100	±3	±76		
2 OSM	6·2	−42	720	78	650	91	304
	±1·6	±9	±230	±3	±208		

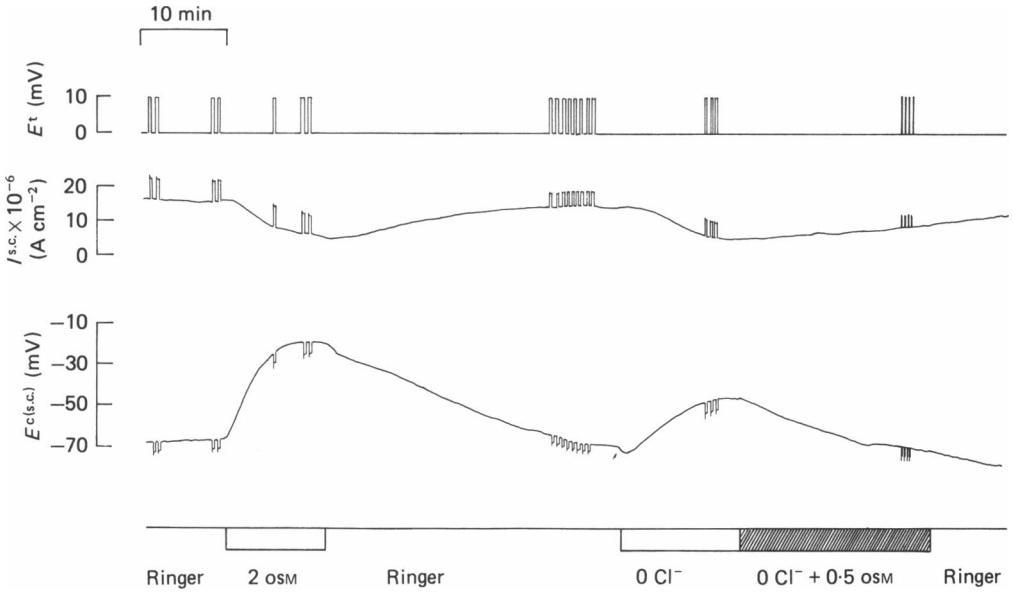

Fig. 6. Effects of combined ionic and osmolarity changes (serosal) on cell potentials and short-circuit current. Simultaneous recording of E^t, $I^{s.c.}$ and $E^{c(s.c.)}$ as in Fig. 1. The serosal bathing solution was first made hypertonic (2 OSM) Ringer solution and after 10 min changed back to standard Ringer solution. Then it was rendered chloride-free (0 Cl^- Ringer solution) for about 10 min after which the chloride-free Ringer was changed for 0·5 OSM, chloride-free Ringer solution. The changes are indicated by the bars at the bottom.

changes in cell potential are apparent around the initial $E^{c(s.c.)}$, and the depolarizing effect of high-potassium solution is diminished by hypertonicity and exaggerated by hypotonicity.

Effects of sodium or chloride replacements combined with osmolarity changes

Given the effects shown for sodium and chloride removal on the one hand and for hypertonic solutions on the other, it seemed interesting to study the effects induced by combined perturbations. Figures 6 and 7 show two experiments in which the cells were exposed to low osmolarity solutions *after* being for some time in chloride- or sodium-free solutions. The changes in $I^{s.c.}$ and $E^{c(s.c.)}$ after removing chloride or

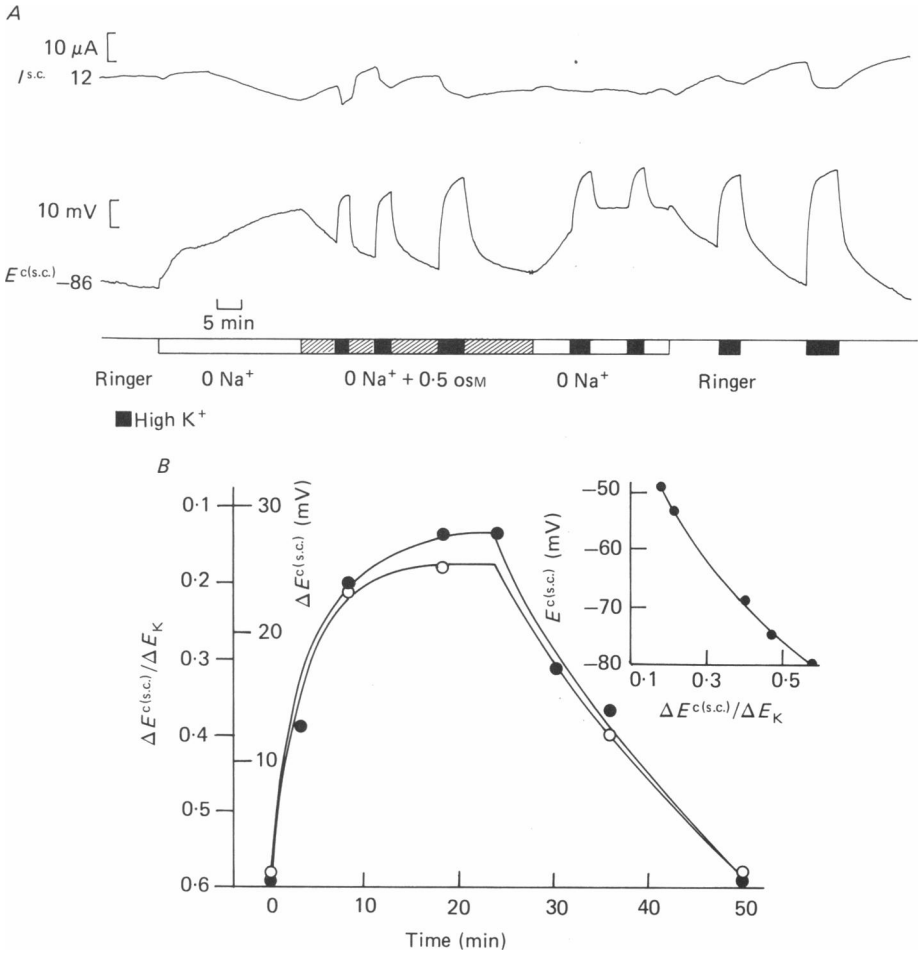

Fig. 7. Effects of combined ionic and osmolarity changes of the serosal bathing solution on cell potentials and short-circuit current. *A*, experiment similar to that of Fig. 6 showing a simultaneous recording of $I^{s.c.}$ and $E^{c(s.c.)}$. The serosal bathing solution was first changed to sodium free (0 Na^+) for about 20 min and then changed again to a half-strength sodium-free solution. The change to 0 Na^+ was then repeated and reverted by Ringer solution. The changes in the serosal solution are indicated at the bottom. *B*, time course of the change in potential and potassium selectivity of the serosal membrane after 0 Na^+ serosal solution. Data from the experiment shown in *A*. The values of $\Delta E^{c(s.c.)}$ (●) and $\Delta E^{c(s.c.)}/\Delta E_K$ (○) throughout the change in 0 Na^+ are plotted on the same time scale, which was arbitrarily set to zero at the time of the last high-K^+ pulse in 0 Na^+ 0·5 osM Ringer solution. $E^{c(s.c.)}$ is the value of the membrane potential immediately before each high-K^+ pulse and $\Delta E^{c(s.c.)}$ the change in $E^{c(s.c.)}$ from this value to the peak of the pulse. The inset shows the same data plotted as $E^{c(s.c.)}$ against $\Delta E^{c(s.c.)}/\Delta E_K$.

sodium from the serosa are reversed by dilution. This suggests a common link between sodium and chloride effects and those of hypertonic solutions, this most probably being the induced changes in cell volume.

Figure 7*A* illustrates the time course of the membrane permeability change as successive high-potassium pulses were given at different times during the transient depolarization and repolarization upon sodium removal, dilution and restoration.

The values of $E^{c(s.c.)}$ and the fraction $\Delta E^{c(s.c.)}/\Delta E_K$ for this experiment are plotted in Fig. 7*B* to show the parallel between the time course of the change in permeability and the change in potential. The relation between $E^{c(s.c)}$ and $\Delta E^{c(s.c.)}/\Delta E_K$ is shown in the inset. This experiment suggests that the fall in $E^{c(s.c.)}$ is due to the loss in potassium selectivity of the serosal membrane.

DISCUSSION

The experiments described in this paper were aimed at studying the origin of the decrease in the short-circuit current that takes place when sodium or chloride are removed from the solution bathing the serosal membrane of frog skin epithelium. The results show that (1) the removal of either sodium or chloride leads to cell depolarization, (2) the change in potential is probably caused by a decrease in the selectivity of the internal barrier to potassium ions and (3) the changes in membrane permeability and potential are mimicked by increasing the tonicity and reverted by dilution. These observations suggest the presence of volume-dependent ionic permeabilities in the serosal membrane of frog skin.

The results provide a reasonable explanation for the observation that the short-circuit current is decreased by removal of sodium or chloride ions (Ferreira, 1968; Ferreira & Ferreira, 1981) as well as after increasing the osmolarity of the serosal bathing solution (MacRobbie & Ussing, 1961; Ussing, 1965). As shown here, the decrease in the sodium current is parallel to the fall in the driving force for sodium ions across the mucosal membrane, brought about by the fall in membrane potential. A decrease in the mucosal membrane sodium permeability was also detected by plots such as that of Fig. 2 and by calculating individual conductances from circuit analysis. The agreement between these two methods was good. Both gave a fractional reduction of the mucosal sodium permeability of about 30–40% after the removal of chloride or increasing the tonicity of the serosal solution and 10–20% in the absence of sodium. The fall in P^m_{Na} after chloride-free and hypertonic solutions can be related to the increase in intracellular sodium concentration that is known to occur under these conditions (Ferreira & Ferreira, 1981). As postulated by Erlij & Smith (1973) the sodium entry step may be regulated by the level of intracellular sodium in such a way that an increase in cell sodium produces a decrease in P^m_{Na}. Since P^m_{Na} also decreases in experiments with sodium-free solutions in which the cell concentration of sodium is decreased, another mechanism might be also responsible for the decrease in P^m_{Na}.

The decline in the short-circuit current produced by the reduction in the influx of sodium across the mucosal barrier implies that the net efflux of sodium ions across the serosal membrane is also reduced. This could be brought about, in principle, by a reduced pump rate or by an increased recirculation of sodium ions across the serosal barrier. Present experiments do not allow us to discern between these two possibilities. A situation somewhat similar to the present case, in which the net sodium extrusion rate and the potassium ion permeability of the serosal membrane seem to be varying in parallel, has been found by others in different epithelia (Schultz, 1981; Brown & Sepúlveda, 1985), the nature of this link remaining obscure.

In this preparation, the intracellular sodium concentration appears to be increased

by about 20% in chloride-free Ringer solution (Ferreira & Ferreira, 1981). This means that a reduced activation of the sodium pump is not likely to be the origin of the decreased short-circuit current. Alternative mechanisms would be a decrease in the number of active sites or an increased recycling of sodium due to an increase in the permeability to sodium ions of the serosal barrier. The latter possibility is consistent with the loss of the potassium-electrode behaviour of the serosal membrane and the changes in its conductance that occur in chloride-free solutions. In low-sodium experiments, however, the 'recycling' mechanism could never work because there is no external sodium. In this case, it seems that the intracellular sodium ion concentration is reduced to about two-thirds of its control value (Ferreira & Ferreira, 1981).

Major changes in ionic permeabilities seem to occur, however, at the serosal membrane, as revealed by the results of experiments with high-potassium and barium. The fact that the current density across the serosal barrier is not zero and, moreover, that it may change under the different experimental conditions, makes it difficult to analyse these experiments in terms of diffusion potentials without a number of simplifying assumptions (see below, and Lew, Ferreira & Moura, 1979; Ferreira & Ferreira, 1983). Nevertheless we can use the results of high-potassium solutions to estimate the importance of the potassium gradient in determining the serosal membrane potential (Hodgkin & Horowitz, 1959) and therefore the cell potential. The depolarizing effect of high-potassium solutions (and hence the ratio $\Delta E^{c(s.c.)}/\Delta E_K$) decreased dramatically in sodium- or chloride-free and hypertonic solutions, going from 0·6 to 0·3, indicating that the serosal membrane became less permeable to potassium ions. In accordance with this, the addition of barium to chloride- or sodium-free solutions induced a depolarization which was 10% of that obtained with normal Ringer solution. A decrease in cell potassium concentration to about one-fifth could, in principle, produce a cell depolarization similar to the one observed in these experiments. Despite a net loss of potassium ions, however, the intracellular potassium concentration appears to remain constant under the conditions considered here (Ferreira & Ferreira, 1981). Moreover, the time courses of the changes in permeability revealed by the effects of high-potassium solutions and the change in membrane potential are very similar, as shown in Fig. 7*B*. The suggestion is, therefore, that a change in the membrane selectivity to ions takes place.

The potential difference across the serosal membrane may be estimated by (Lew *et al.* 1979):

$$E^s = \frac{RT}{zF} \ln \frac{V^s Y^s + (\phi^p - I^{s.c.}/F)}{V^s X^s + (\phi^p - I^{s.c.}/F)}, \tag{2}$$

where $V^s = (zF/RT)E^s$; $Y^s = (P^s_{Na} C^s_{Na}) + (P^s_K C^s_K) + (P^s_{Cl} C^c_{Cl})$

and $X^s = (P^s_{Na} C^c_{Na}) + (P^s_K C^c_K) + (P^s_{Cl} C^s_{Cl})$.

Given the large value of the product $(P^s_K C^c_K)$ as compared to any other of the parameters (it is in the order of 10 times larger than $(\phi^p + I^{s.c.}/F)$), eqn (2) can be simplified to give an expression which is similar to the one obtained for zero-current conditions:

$$E^s = \frac{RT}{zF} \ln \frac{Y^s}{X^s}. \tag{3}$$

This expression implies the assumption that $V^sX^s \gg (\phi^p - I^{s.c.}/F)$ and hence that the fraction $(\phi^p - I^{s.c.}/F)/V^sX^s$ tends to zero.

The change in the potential of the serosal membrane after changing the potassium and sodium concentration in the external solution from $C^s_{Na(1)}$, $C^s_{K(1)}$ to $C^s_{Na(2)}$, $C^s_{K(2)}$ is then given by

$$\Delta E^s = \frac{RT}{zF} \ln \frac{(P^s_{Na} C^s_{Na(1)}) + (P^s_K C^s_{K(1)}) + (P^s_{Cl} C^c_{Cl})}{(P^s_{Na} C^s_{Na(2)}) + (P^s_K C^s_{K(2)}) + (P^s_{Cl} C^c_{Cl})}. \quad (4)$$

This equation shows that the observed change in potential in high potassium is independent of cell potassium concentration, reinforcing the idea that the observed changes are due to the alteration of the normal ionic permeabilities. Equation (4) allows the calculation of the ratio $\beta = P^s_{Cl}/P^s_K$ knowing the intracellular concentration of chloride, and assuming that in standard Ringer solution P^s_{Na} is close to zero. For typical values of C^c_{Cl} of 30 mM and concentrations of potassium in the solution of 2·5 and 50 mM, the value of β is 0·33, in excellent agreement with previous estimates (Giraldez & Ferreira, 1984).

The case of barium inhibition of potassium channels can be also treated starting from eqn (3) although additional simplifications are needed. In standard Ringer solution C^s_K is very low and the product $(P^s_K C^s_K)$ is small compared to $(P^s_{Na} C^s_{Na}) + (P^s_{Cl} C^c_{Cl})$; actual values are close to 2·5 and 11 mol cm^{-2} s^{-1} respectively. The effect of barium ions is to reduce P_K; therefore, we can write eqn (3) for $P^s_K = P^s_K$ in Ringer solution and $P^s_K = 0$ in barium Ringer solution and subtract to obtain the change in E^s upon adding barium, which is

$$\Delta E^s = \frac{RT}{zF} \ln \frac{(P^s_{Na} C^c_{Na}) + (P^s_{Cl} C^s_{Cl})}{(P^s_{Na} C^c_{Na}) + (P^s_K C^c_K) + (P^s_{Cl} C^s_{Cl})}. \quad (5)$$

Now, since in the standard Ringer solution $(P^s_{Na} C^c_{Na})$ is very small, eqn (5) can be further simplified to give:

$$\Delta E^s = -\frac{RT}{zF} \ln\left(1 + \frac{1}{\beta^s} \frac{C^c_K}{C^s_{Cl}}\right). \quad (6)$$

This gives a relation between the change in potential and the intracellular potassium concentration for a given value of β assuming that the sodium permeability is very low. For $C^c_K = C^s_{Cl} = 120$ mM and $\beta = 0{\cdot}33$, the value of ΔE^s from eqn (6) is -36 mV, in good agreement with experiments (see Fig. 4).

A loss of the potassium-electrode behaviour of the serosal membrane could be brought about by either a decrease in P^s_K, an increase in the permeability to other ions, or both. The total conductance of the serosal membrane decreased in absolute terms both in chloride-free, in sodium-free and in hypertonic solutions. This suggests that an absolute decrease in the permeability to potassium ions occurs.

Since the common effect of chloride-free, sodium-free and hypertonic solutions is to decrease the cellular volume (MacRobbie & Ussing, 1961; Ussing 1965; Ferreira & Ferreira, 1981), it is tempting to suggest that the observed permeability changes are induced by changes in cell volume. This is also reinforced by the fact that dilution of the serosal bath counteracts the effects of low sodium or chloride (Figs 6 and 7). The time course of the events is also very similar. Comparing the time constant of the

change in membrane potential (and potassium selectivity) measured here with that of the changes in cell volume after similar changes in tonicity (Table IV of MacRobbie & Ussing, 1961, and Fig. 7 of Ussing, 1965) it can be seen that they are approximately the same, about 4–10 min for step changes of about 200 mosM. The slower time course after chloride-free or sodium-free solutions is also in agreement with the slower shrinkage that is found in these situations (see Ussing, 1982, Fig. 2 for example). In summary, the time course and final magnitude of the depolarization and of cell shrinkage seem to vary in parallel, and in agreement with the hypothesis that the cell permeabilities are volume dependent.

Changes in cell volume are known to initiate events leading to the activation of ionic fluxes in a variety of cell types (Kregenow, 1977; Hoffman, 1983; Sarkadi, Mack & Rothstein, 1984), including epithelia (Ussing, 1982; London, Cohen, Guggino & Giebisch, 1983). Increased potassium and chloride fluxes after increasing cell volume, for instance, are well documented (Kregenow, 1977; Bakker-Grunwald, 1978; Hoffman, 1982; Grinstein, Dupre & Rothstein, 1983. In frog skin, Ussing (1982) has also postulated an increased permeability to KCl in hypotonic media which would operate as a volume-regulatory mechanism to restore isotonic volume by losing KCl salt. A situation like the one analysed in this paper, i.e. a reduced P_K, and possibly increased P_{Na} and P_{Cl} in hypertonic media, has a precedent in the work of Kregenow (1977) in nucleated red cells, Hoffman (1983) in Ehrlich cells and Adragna & Tosteson (1984) in human red cells, and it may be considered as a regulatory adjustment that prevents solute depletion by reducing potassium loss.

We thank Dr A. E. Hill for reading the manuscript and J. Honorato, U. Santos, Lourdes Santos and Josefina Revuelta for their assistance in the experiments and in preparing the typescript. The work was partially supported by CAICYT project No. 2873/83.

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