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A NEAR-ZERO MEMBRANE POTENTIAL IN TRANSPORTING CORNEAL ENDOTHELIAL CELLS OF RABBIT

BY STUART HODSON AND CHRIS WIGHAM

From the Laboratory of Experimental Optometry, University of Wales College of Cardiff, PO Box 905, Cardiff CF1 3YJ

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SUMMARY

1. When rabbit corneal endothelial cells are impaled with 3 M-KCl-filled microelectrodes (R_t = 20–70 MΩ) a stable membrane potential of $-28{\cdot}7 \pm 4{\cdot}8$ mV (mean ± S.D., $n = 400$) is measured.

2. Varying the [KCl] of the filling solution causes a change in measured membrane potential; 154 mM gives typically −2 mV, 10 mM typically +37 mV.

3. Variation in membrane potential with different [KCl] cannot be ascribed to tip potential. Double-barrelled microelectrodes containing a different [KCl] in each barrel both give the same membrane potential when inserted into a cell.

4. Microelectrodes filled with a reference liquid ion exchanger (RLIE) give a membrane potential of +2 mV.

5. Impaling a cell with a double-barrelled microelectrode, one barrel containing KCl and the other RLIE, showed that the ion exchanger is not having a toxic effect on the cell and does not affect membrane potential measured by the KCl-filled barrel.

6. We suggest that microelectrodes containing non-isotonic concentrations of KCl generate a significant and artifactual change in membrane potential of corneal endothelial cells caused by the movement of excess KCl (originating from the microelectrode tip) across the plasma membrane where a liquid-junction potential is generated.

7. We further suggest that the physiological membrane potential of corneal endothelial cells is around zero. This could result from a solution of the constant field equation where:

$$0{\cdot}9\, P_{Na} \simeq P_K \simeq 3{\cdot}2\, P_{Cl}.$$

INTRODUCTION

When cell membrane potentials are measured by penetrating the interior of the cell with a glass microelectrode filled with a salt solution the recorded membrane potential will include two sources of error.

The first is microelectrode tip potential (Adrian, 1956), which is generated when salt diffuses out of the tip and when the ionic mobilities of the cation and anion are different. It is a significant source of error when measuring physiological membrane potential only if it changes on entering the cell, a situation that might arise for example when the ionic mobilities are different in the bathing solution and

cytoplasm. A partial solution to the problem of tip potentials has been to fill the microelectrode with hypertonic salt solutions where the mobilities of the anion and cation are similar, for example 3 M-KCl, and where the Donnan potentials generated by the negative charges on the inside glass surface of the tip are 'salted out'.

The use of hypertonic filling solutions can introduce a second source of error in measurement of membrane potential. Salt continuously diffuses out of the tip into the cell. The higher the concentration of salt in the barrel the greater the efflux from the tip. The rate of change of intracellular KCl resulting from the presence of the microelectrode will depend on the size of the tip and the size of the cell. The corneal endothelial cell, whose role is to regulate corneal hydration by pumping bicarbonate ions out of the cornea into the aqueous humour (Hodson & Miller, 1976; Wigham & Hodson, 1985), is a small hexagonal cell with an approximate area of 400 μm^2, a thickness of 5 μm and a volume of 2000 μm^3. When impaled with a 3 M-KCl-filled microelectrode with a tip resistance of 20 MΩ the KCl will diffuse out of the tip at a rate of 6×10^{-14} mol s^{-1} (Nastuk & Hodgkin, 1950; Giesler, Lightfoot, Schmidt & Francisco, 1972) changing the intracellular KCl by 30 mM s^{-1}. Clearly, although corneal endothelial cells are osmotically tolerant, such a rate of increase cannot be sustained for long. At steady state, KCl must be diffusing out of the tip and out of the cell through the plasma membrane. If the plasma membrane were equal in its permeability to K^+ and Cl^- then the phenomenon would have no effect on membrane potential. However, if the membrane permeability to K^+ and Cl^- is different then it should be possible to record a component of membrane potential which is present only when the tip is inside the cell. This component of the recorded membrane potential would be a liquid-junction type potential.

Measurements have been made of corneal endothelial cell membrane potentials using hypertonic salt-filled microelectrodes (Wiederholt & Koch, 1978; Lim & Fischbarg, 1979; Jentsch, Matthes, Keller & Wiederholt, 1985; Hodson & Wigham, 1987). These measurements are stable and reproducible, in the range from −20 to −50 mV. We suggest, in this report, that these recorded membrane potentials are generated almost entirely by the presence within these cells of hypertonic salt-filled microelectrodes. Not only do the microelectrodes record the membrane potential, they also, by their presence within the cell, generate it by the liquid-junction type mechanism described above.

METHODS

Tissue preparation and incubation

Eyes were removed from Dutch rabbits weighing approximately 3 kg, killed by intravenous overdose of sodium pentobarbitone whilst they were calm and relaxed. The cornea, endothelial side up, was mounted on a Perspex stub ready for penetration by microelectrodes as described previously (Hodson & Wigham, 1987). The preparation was bathed at 35 °C in Ringer solution containing (mM): NaCl, 106; $NaHCO_3$, 30; KCl, 6·7; $MgSO_4$, 0·6; Na_2HPO_4, 5·55; glucose, 4·5; reduced glutathione, 1·0; $CaCl_2$, 0·56 and bubbled to pH 7·37 with a 5% CO_2, 7% O_2 and 88% N_2 gas mixture. Viability of the preparation was confirmed by observing with a specular microscope the 'cobbled' appearance of the endothelial cell mosaic (Hodson, 1969).

Microelectrode manufacture

Filamented borosilicate glass, 1·5 mm o.d. (Clark Electromedical Ltd), was pulled into microelectrodes using a Narishige PN-3 puller. Microelectrodes filled with 3 M-KCl and with a tip

resistance of less than 20 MΩ failed to hold a stable intracellular potential: microelectrodes with a tip resistance greater than 70 MΩ tended to snap back on entering the cell to around 50 MΩ.

Microelectrodes were filled with a number of different aqueous KCl concentration solutions. Tip resistances were typically: 3 M-KCl, 40 MΩ; 154 mM-KCl, 300 MΩ; 8 mM-KCl, 2·2 GΩ. All microelectrodes were pulled using the same settings on the puller.

Double-barrelled microelectrodes were made by the method of Zeuthen (1980), using double-barrelled 1·5 mm o.d. filamented borosilicate glass (Clark Electromedical, 2GC/150F.) It has been suggested that fused barrels stay together all the way to the tip when pulled. In our experience when pulling sharp needles the two barrels separate at the tip to produce a fork unless twisted before pulling. θ-glass of both normal and thick septum types was unsuitable for twin channel use as the position of the septum in the needle tip varied uncontrollably, sometimes stopping short of the tip. Eccentric double-barrelled microelectrodes (Thomas, 1986) also gave suitable preparations.

The electrodes were back-filled with the appropriate KCl solution. The same technique was used to fill the double microelectrodes when both barrels were filled with aqueous solutions. When ion-selective ligands were used the glass had to be silanized first (Tsien & Rink, 1980). Briefly, the glass is dehydrated at 200 °C, exposed to tri-*n*-butylchlorosilane (Fluka) vapour at 200 °C and then allowed to cool. This technique was used with single- and double-barrelled microelectrodes. With double-barrelled microelectrodes where only one channel contained a ligand a different technique was used. A small stainless-steel tube through which air was blown was inserted into one barrel for 5 min before a second tube, through which tri-*n*-butylchlorosilane vapour was blown, was inserted into the second barrel. After 40 min the silane vapour tube was removed and air was blown through for a further 5 min before this tube was also withdrawn.

The method used to fill these relatively sharp microelectrodes with ligand was critical. If too much was introduced too quickly then small air bubbles appeared at the tip that interrupted the column of exchanger. When a cat's whisker was dipped into exchanger and withdrawn, small beads formed on the whisker, each of which provided a suitable volume, which was inserted into the needle by introducing the whisker to the open end of the glass capillary and touching the bead to the filament. A 10 min interval was left between each addition of exchanger. As the column rose away from the tip larger aliquots were introduced. When the ligand reached a point in the capillary where a whisker could reach, the capillary was back-filled with 154 mM-KCl. When filling the mixed double electrodes the unsilanized barrel was filled first.

Microelectrode calibration

Potentials measured in the calibrating solutions with KCl-filled microelectrodes never varied by more than ±2 mV. Throughout this paper, we expect ±2 mV to be the probable error in our determinations. With reference liquid ion exchanger (RLIE, potassium tetrakis (*p*-chlorophenyl) borate, obtained through R. Thomas, Bristol), which is equiselective for Na^+ and K^+ filling the microelectrodes, calibration was achieved by immersion into mixtures of KCl + NaCl. Potential changes measured were similar to the KCl-filled electrodes. When a 3 M-KCl-filled microelectrode gave a resistance of approximately 40 MΩ, a similar electrode filled with RLIE gave 30 GΩ.

Calibration traces had to be reversible and reproducible to within ±2 mV both before and after cell impalement before the recording was considered satisfactory.

RESULTS

Corneal endothelial cells impaled with microelectrodes filled with 3 M-KCl demonstrate a membrane potential (Fig. 1) of $-28{\cdot}7 \pm 4{\cdot}8$ mV (mean ± S.D., $n = 400$). The traces were stable for at least 20 min; membrane resistance (R_m) was also stable and in the range of 200–2000 MΩ (Hodson & Wigham, 1987). Recorded membrane potential altered if the microelectrode was filled with a different [KCl]. Reducing the [KCl] in the filling solution resulted in the membrane potential becoming more positive (Fig. 2). Double-barrelled microelectrodes where both barrels contained the same [KCl] gave the same membrane potential in each barrel as the single-barrelled equivalent. Tip potentials were never more than ±2 mV.

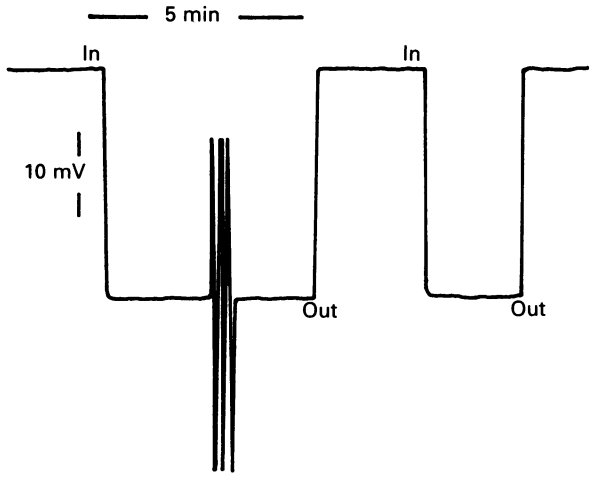

Fig. 1. Impaling a corneal endothelial cell with a 3 M-KCl-filled microelectrode produces a stable measurement of membrane potential and R_m (indicated by the pulses during impalement). It is routinely possible to withdraw the electrode and re-impale the cell.

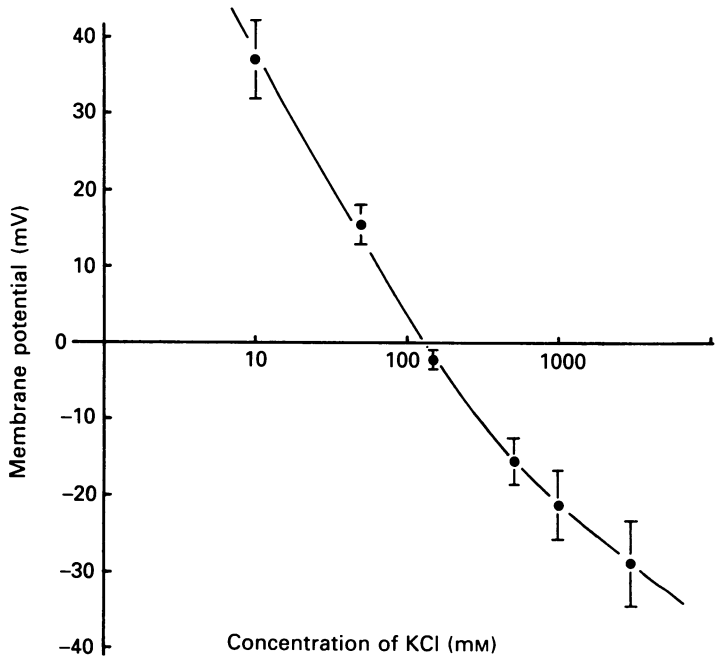

Fig. 2. When the concentration of KCl filling the microelectrode is changed, different membrane potentials are recorded. Bars indicate standard deviations about the mean. At concentrations around and below isotonic, membrane potential is a linear function of log[KCl]. At higher concentrations the relationship deviates but does not saturate.

Experiments were also made with double-barrelled microelectrodes where the two barrels contained different KCl concentrations. Independence of the two barrels was confirmed by measuring the tip resistance of the two separate barrels individually and in series. θ-needles give a series resistance less than the individual values when

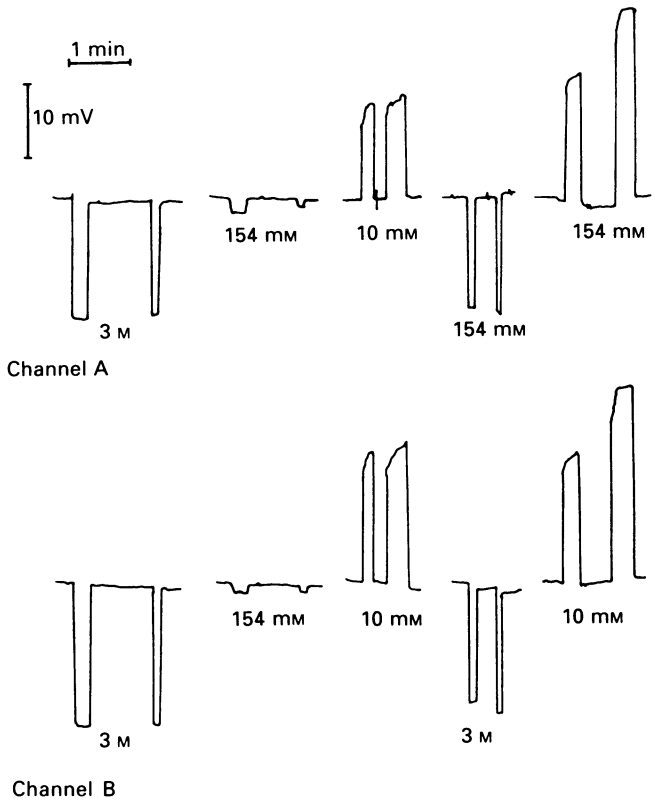

Fig. 3. A composite trace showing measurements of membrane potential made using double-barrelled microelectrodes where the two barrels contain various concentrations of KCl. Channels A and B indicate the simultaneous response from the individual barrels. The KCl concentration in each barrel is shown underneath the trace; five combinations are shown. Two impalements are shown for each combination. Both barrels always showed the same membrane potential, even when the filling concentrations were different.

the septum stops short of the mouth. In all cases, both barrels gave the same value for membrane potential. The magnitude of membrane potential was determined by the filling solution furthest away from 154 mM (Fig. 3).

Corneal endothelial cells were also impaled with microelectrodes containing RLIE. Values of membrane potential recorded with single-barrelled RLIE microelectrodes were $+2{\cdot}2 \pm 0{\cdot}2$ mV (mean $\pm$ S.E.M., $n = 27$). A typical trace is shown in Fig. 4. The validity of measurements made using RLIE can be questioned on the grounds that the solvent, *n*-octanol, might affect cell function (Oxford & Swenson, 1979). To check for any effect, cells were impaled with double-barrelled microelectrodes where one barrel contained various concentrations of KCl and the other RLIE. Measured membrane potential in the KCl-filled barrel of the mixed microelectrode was never

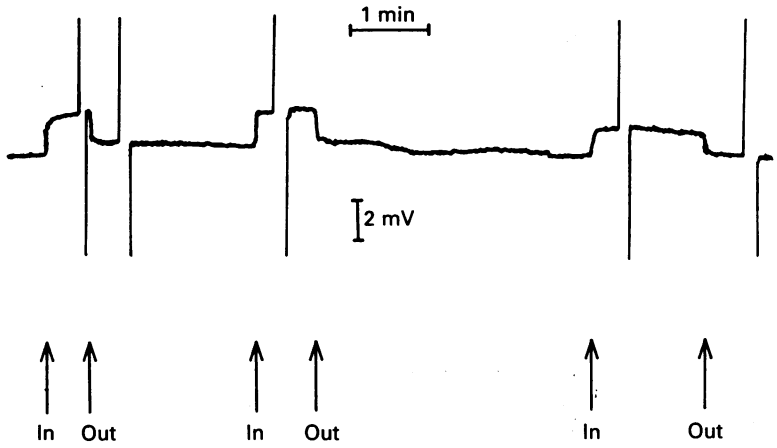

Fig. 4. Corneal endothelial cell membrane potential measured using RLIE-filled microelectrodes which respond equally to the major cations, Na^+ and K^+. Measured membrane potential will be unaffected by an electrolyte leakage from the needle tip.

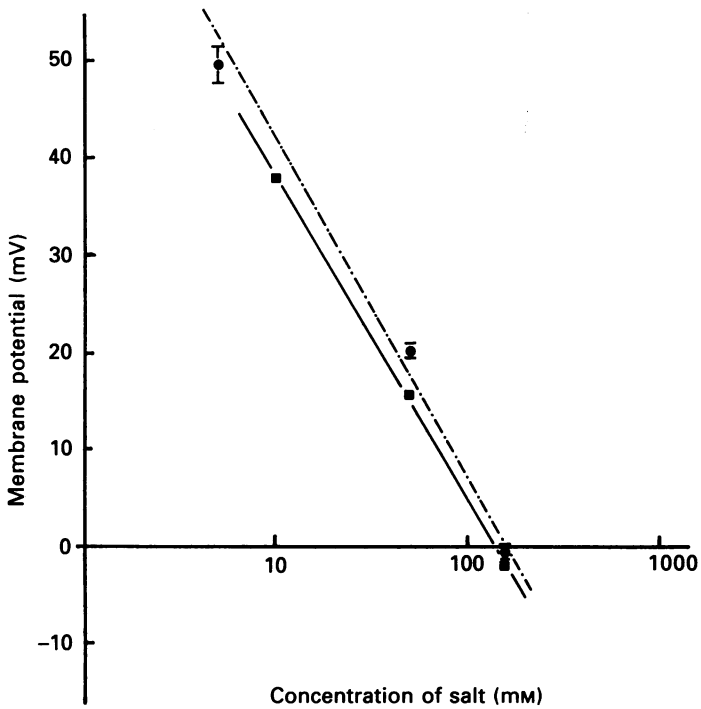

Fig. 5. Measurements of membrane potential using microelectrodes filled with different concentrations of KCl (■) and NaCl (●) show a similar electrolyte-concentration-induced artifact. The slightly different slope indicates that the cell membrane is very slightly more permeable to K^+ than Na^+.

significantly different from that recorded with a KCl-alone microelectrode impaling the same preparation. (For example, KCl (10 mM)–RLIE double-barrelled microelectrodes recorded 27·2±2·0 mV (mean±S.E.M., $n = 13$) in the KCl channel; KCl (10 mM) single-barrelled microelectrodes recorded 26·0±1·7 mV (mean±S.E.M., $n = 13$).) This suggests that, in these preparations, *n*-octanol within an impaling microelectrode has no significant effect upon membrane potential and, consequently, that the membrane potentials recorded with RLIE microelectrodes (+2·0 mV) are correct.

When we impaled cells with microelectrodes filled with various [NaCl] we recorded similar membrane potentials to those found in microelectrodes filled with the same concentration of KCl (Fig. 5).

We repeated the data reported here with microelectrodes filled with various concentrations of potassium sulphate, potassium acetate or potassium citrate. When the concentration of the filling solution was 500 mM, recorded membrane potential was about equal to that seen with 3 M-KCl-filled microelectrodes. These membrane potentials also showed an approximately linear dependence on the concentration of the filling solution and all (sulphate, acetate and citrate) showed a stable positive membrane potential when their concentration was reduced to 50 mM. At concentrations isotonic to 154 mM-KCl, 159 mM-potassium acetate-filled microelectrodes indicated a membrane potential of −3·5 mV, 134 mM-potassium sulphate indicated a membrane potential of −5·0 mV, and 110 mM-potassium citrate indicated a membrane potential of −2·0 mV.

DISCUSSION

Stable membrane potentials are routinely found on penetrating rabbit corneal endothelial cells with microelectrodes filled with solutions of KCl (Fig. 1). The magnitude of the potential is a function of the concentration of KCl in the microelectrode (Fig. 2). It seems to us that physiological membrane potential cannot be estimated from such data and that some artifact is predominating. We considered that the artifact was either a tip potential or an induced membrane potential. A tip potential results from the diffusion of salt out of the tip. If the mobility of the cation (u^+) is greater than the mobility of the anion (v^-) then the inside of the microelectrode will rapidly drive negative until the EMF is of sufficient magnitude to promote the efflux of anions and impair the efflux of cations so that both ions exit at the same rates from the microelectrode tip and a stable tip potential, which is a liquid-junction potential, is recorded. The potential is given by the liquid-junction equation:

$$V_t = \frac{(u^+ - v^-)}{(u^+ + v^-)} \frac{RT}{zF} \ln \frac{C_b}{C_m}, \tag{1}$$

where V_t = tip potential, R = the gas constant, T = temperature in absolute units, z = the valency of the ion, F = the Faraday constant and C is salt concentration; suffix b indicates the bulk phase in the barrel of the glass microelectrode and suffix m indicates the medium surrounding the tip. An induced membrane potential is generated by a differential mobility of anion and cation not at the tip but at the plasma membrane as salt diffuses out of the tip and then out of the cell. If the

permeability of the plasma membrane to K^+ is equal to its permeability to Cl^-, then the phenomenon might have no effect upon recorded membrane potential. If the membrane permeabilities of K^+ and Cl^- are different, then it should be possible to record a component of membrane potential which is present only when the tip is inside the cell. This membrane potential would be a liquid-junction type potential but mobility, u, would need to be replaced by permeability, P, in eqn (1). The first derivative of the equation is given by:

$$\frac{dV_P}{d\log C} = 2{\cdot}303\frac{RT}{zF}\frac{(P_{Cl}-P_K)}{(P_{Cl}+P_K)}, \tag{2}$$

where, at 37 °C, $2{\cdot}303RT/zF = 61{\cdot}5$ mV.

To distinguish between the two possibilities, we introduced two microelectrodes into one cell, each microelectrode filled with a different concentration of KCl. If the artifact is a tip potential, then each microelectrode should record, independently, the same potential as it would if it were in a cell on its own. This never happened. Both channels always recorded the same electrical potential, indicating that the entire content of the cell was actually at this potential and that the artifact was a membrane potential generated by the presence of the microelectrode in the cell. It was of interest to note that both channels recorded the membrane potential appropriate to one of the two filling solutions and it was the potential of that solution furthest away from 154 mM-KCl. It was also clear that the spread of values of membrane potentials was least with 154 mM-KCl filling solution. It could be argued from these observations that the artifact was minimal with 154 mM-KCl filling solution, which gave membrane potentials of $-2{\cdot}0$ mV, but these arguments are not conclusive and we still wished to know the physiological membrane potential of corneal endothelial cells. We next penetrated the cells with microelectrodes filled with RLIE (Thomas & Cohen, 1981). RLIE is a liquid ion exchanger with equal selectivity for sodium or potassium. As it enters the cell, it experiences little change in total $[Na^+]+[K^+]$ and the ion-selective component of the recorded potential should be a small positive component of a few millivolts (depending mainly on the concentration of fixed negative charge in the metabolites of these cells). The remainder of the signal should be physiological membrane potential. When we penetrated corneal endothelial cells with RLIE microelectrodes, we recorded membrane potentials always slightly positive, $+2{\cdot}2\pm0{\cdot}2$ mV, suggesting that the physiological membrane potential is near zero. There was a doubt over the RLIE result as RLIE includes as its solvent *n*-octanol. It seemed possible that *n*-octanol could leach out of the tip into the cytoplasm, inducing a change in cell function (Oxford & Swenson, 1979). This was potentially another source of error. To check this possibility, we penetrated the cells with double-barrelled microelectrodes, one barrel filled with RLIE, the other with various concentrations of KCl. The experiment was designed to see if the presence of an RLIE tip in the cell had any effect upon membrane potentials recorded with the KCl barrel. They had none. Any particular KCl-filled tip gave similar values for membrane potential whether or not an RLIE tip was in the same cell. The simplest interpretation of this is that RLIE, by its presence in a cell, does not alter membrane potential.

We conclude that the physiological membrane potential for corneal endothelial cells incubated under the conditions that we describe here is near zero. How do we explain our conclusion of a near-zero membrane potential? One explanation for our observations could be that the cells are 'dead'. We think this unlikely because, besides our experience of several decades working on the transport characteristics of this preparation, we have conducted a number of further investigations which will be published in full elsewhere. They include (*a*) a measured intracellular sodium concentration of 8–15 mM in cells recording near-zero potentials, (*b*) an intracellular pH of 6·9, far from that expected in bathing solutions of pH 7·4 and (*c*) the observation of 'conventional' membrane potentials in ciliary epithelium cells (R_m = 200–400 MΩ, volume similar to corneal endothelial cells, mounted under the same conditions reported here) which show a membrane potential of −60 to −70 mV when the cells are impaled with microelectrodes filled with 3 M, 154 or 10 mM-KCl (Grigg & Wigham, 1989).

We suggest another explanation for the near-zero membrane potential. From Fig. 2, $dV_P/d\log C$ is measured at −32·5 mV around isotonic values and if this is substituted into eqn (2), at 37 °C we obtain:

$$\frac{P_{Cl}-P_K}{P_{Cl}+P_K} = -\frac{32{\cdot}5}{61{\cdot}5},$$

which has a solution when $P_K = 3{\cdot}2\,P_{Cl}$.

Making the same calculations for NaCl-filled microelectrodes (Fig. 5) indicates that $0{\cdot}85\,P_{Na} = P_K = 3{\cdot}2\,P_{Cl}$.

This gives us a possible explanation of our observations, if we apply these values in the constant field equation:

$$V_m = 61{\cdot}5\log\frac{P_K[K^+]_o + P_{Na}[Na^+]_o + P_{Cl}[Cl^-]_i}{P_K[K^+]_i + P_{Na}[Na^+]_i + P_{Cl}[Cl^-]_o} \qquad (3)$$

V_m is near zero when the logarithm is near to unity. We estimate $[Na^+]_i$ for these cells to be $13{\cdot}1 \pm 2{\cdot}5$ mM, using ion-selective microelectrodes (C. Wigham & S. Hodson, unpublished observation). The logarithm is at about unity when $P_{Na} \simeq P_K > P_{Cl}$.

The data we record here are self consistent. They may be explained by the corneal endothelium having a plasma membrane roughly equipermeable to sodium and potassium and consequently even though it has a transport system entirely dependent upon Na^+–K^+-ATPase and a low intracellular $[Na^+]$ (and we suppose, correspondingly, a high $[K^+]$) because it appears to be not strongly mono-cation selective, it does not develop a significant membrane potential.

The electrogenic current generated by an Na^+–K^+-ATPase membrane activity driving the inside negative on a conventional stoichiometry of 3 Na^+ : 2 K^+ driven per ATP hydrolysed could be neutralized, in part, by the established electrogenic efflux of HCO_3^- out of these cells which would tend to drive the inside positive.

Although ion gradients across cell membranes are essential to cellular life, as they generate the dissipation functions which lead to low intracellular entropy states and the order essential for the system, there seems no pressing reason why the electrochemical gradients should have an electric component in non-excitable cells.

Clearly, in some non-excitable cells there is a membrane potential (for example, it seems to us to be so in ciliary epithelium) but we appear to have discovered a cell type where it is insignificant. For example, sodium ions have an elevenfold concentration difference across the membrane of corneal endothelium and, we estimate, are subject at most to about a 4 mV step across the membrane. At least 94% of the dissipative energy in the sodium ions is driven by chemical forces. Interestingly, the hypothesis indicates that the dissipative energy of the potassium ion across the plasma membrane of corneal endothelium is of similar magnitude.

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