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ION-SELECTIVE MICRO-ELECTRODE STUDIES OF THE ELECTROCHEMICAL POTENTIALS IN TROUT URINARY BLADDER

BY B. J. HARVEY AND B. LAHLOU*

*From the Physiology Department, University College Dublin, Earlsfort Terrace, Dublin 2, Ireland and the *Laboratoire de Physiologie Cellulaire et Comparée, Faculté des Sciences, Parc Valrose, 06034 Nice Cedex, France*

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SUMMARY

1. Intracellular micro-electrode techniques were used to measure the electrical resistances of the cell membranes and the shunt pathway and intracellular ionic activities in trout urinary bladder when the tissue was incubated in Ringer solution and in the presence of the polyene antibiotic ionophore amphotericin B.

2. In control conditions the transepithelial potential was zero and the intracellular potential was -56 mV. The intracellular ionic activities measured with single- and double-barrel ion-sensitive micro-electrodes for the first time in a fish bladder ($a^{\mathrm{i}}_{\mathrm{Na}} = 16$ mM, $a^{\mathrm{i}}_{\mathrm{K}} = 87$ mM, and $a^{\mathrm{i}}_{\mathrm{Cl}} = 21$ mM) indicate an active accumulation of K and Cl ions and an active extrusion of Na ions by the cell. The maintenance of intracellular Cl activity above its equilibrium value depended on the presence of Na ions in the mucosal medium, but was independent of the presence of K ions.

3. Flat cable analysis yielded values for transepithelial, apical, basolateral and shunt resistances of 197, 2790, 1986 and 205 Ω cm^{-2} respectively. Equivalent circuit analysis using amphotericin B yielded similar values for shunt resistance. The paracellular pathway accounts for 96 % of transepithelial current flow and this epithelium may be classified as 'leaky'. The cells are electrically coupled with a space constant of 354 μm.

4. Amphotericin B when added to the mucosal solution induced an immediate serosa positive transepithelial potential of about 9 mV and a short-circuit current of 64 μA cm^{-2}. The V_{t} was ouabain sensitive and dependent on mucosal Na concentration. The origin of the antibiotic induced transepithelial potential was an increase in the sum of the cell membrane electromotive forces. The apical membrane potential depolarized to -7 mV and its resistance fell to 433 Ω cm^{-2}. During the first 10 min of exposure $a^{\mathrm{i}}_{\mathrm{Na}}$ increased to 80 mM and $a^{\mathrm{i}}_{\mathrm{K}}$ decreased to 7 mM with only a small change in $a^{\mathrm{i}}_{\mathrm{Cl}}$. The changes in cellular Na^{+} and K^{+} activities were in accordance with their passive redistribution down their electrochemical gradients.

INTRODUCTION

The mechanism of Na and Cl^- transport in the monolayered epithelium of trout urinary bladder was first studied by Lahlou & Fossat in 1971. Isotope flux studies established the mucosal membrane as the site of electroneutral Na:Cl^- co-transport and as the rate-limiting barrier to transepithelial active NaCl transport (Fossat & Lahlou, 1979*a*). Na entry across the mucosal cell membrane was strictly dependent on the presence of Cl^- in the mucosal solution and vice versa. This mechanism of neutral coupled NaCl absorption has also been found in rabbit ileum (Nellans, Frizzell & Schultz, 1973) and rabbit gall-bladder (Cremaschi & Henin, 1975). In trout urinary bladder, coupled NaCl co-transport is associated with an undetectable transepithelial potential difference (V_t) and short-circuit current (I_{sc}). However, in the presence of the antibiotic ionophore amphotericin B, in the mucosal Ringer, the trout bladder produces a transient serosa positive V_t and I_{sc} (Fossat & Lahlou, 1982). These effects of the antibiotic were found to be strictly Na dependent and were reduced in the presence of ouabain.

The trout bladder may be classed as 'leaky' on account of its low transepithelial resistance R_t (about 200 Ω cm^{-2}). Trout bladder, however, does possess unusual characteristics which distinguish it from other leaky epithelia. The paracellular pathway in this tissue does not exhibit the cation-selectivity ordinarily displayed by similar epithelia studied under the same conditions (Fossat & Lahlou, 1979*b*), it also displays a higher over-all permeability for Cl^- than for Na^+ and transports NaCl as a highly hypertonic solution. The important physiological role of the urinary bladder in the maintenance of osmo-regulation of fish in fresh and salt waters has already been discussed (Lahlou, 1967; Lahlou & Fossat, 1984).

In this paper we attempt to open for the first time the 'black box' of fish urinary bladder epithelium using intracellular electrophysiological methods. We used two independent methods to determine the electrical resistance of the apical and basolateral cell membranes and of the paracellular shunt pathway; (a) flat cable analysis of radial voltage spread in the epithelium and, (b) equivalent circuit analysis using amphotericin B to change transepithelial resistance and the ratio of apical and basolateral cell membrane resistances. To describe the cellular electrochemical ion gradients across this epithelium we have used ion-sensitive micro-electrodes and have analysed the mechanism of the cellular transport of Na, K and Cl ions and the origin of the amphotericin B induced transepithelial potential.

METHODS

Rainbow trout (*Salmo gairdneri*), of weight 200–250 g were maintained in the laboratory at 11 °C in plastic tanks continuously perfused with tap water. The animals were killed by a sharp blow to the head and the urinary bladder excised immediately and placed in a Petri dish containing oxygenated Forster's Ringer. Serosal fat was removed by careful dissection under microscopic observation. The bladder was then mounted as a horizontal 'flat' sheet; the exposed area of epithelium was 0·6 cm^2. The degree of stretch was controlled by measuring transepithelial resistance during stretching. When the total bladder resistance showed signs of decreasing, stretching was stopped. Both mucosal and serosal chambers were perfused with modified Forster's Ringer solution, the composition of which was (mM): NaCl, 133; KCl, 2·5; $CaCl_2$, 1·5; $MgCl_2$, 1·0; NaH_2PO_4, 0·5; $NaHCO_3$, 15; glucose, 5·5; and gassed with 5% CO_2 in oxygen to give a final pH of 7·3. In some

experiments, Na-free Ringer was used. It contained: choline Cl, 133 mM; KCl, 2·5; $CaCl_2$, 1·5; $MgCl_2$, 1·0; KH_2PO_4, 0·5; choline HCO_3, 15; glucose, 5·5; pH 7·3. When Cl^--free Ringer was used the chloride salt was replaced by equimolar amounts of the corresponding sulphate salt. K-free Ringer was obtained by replacing KCl with NaCl.

Amphotericin B was used as amphotericin B oxycholate (Fungizone, Squibb) at a final concentration of 40 μg ml^{-1} in the mucosal Ringer. Oxycholate is present in the commercial preparation as an emulsifier and is reported to have no effect on the electrical properties of *Necturus* gall-bladder (Graf & Giebisch, 1979) nor trout urinary bladder (Fossat & Lahlou, 1982). Ouabain was obtained from Sigma. All compounds used for solutions were of reagent grade from Merck, Germany.

Measurement of intracellular ionic activities

A solution of 10% w/w of Na Ligand I (Fluka, ETH 227) in *o*-nitrophenyloctyl ether with 0·5% w/w sodium tetraphenyl borate was used as the ion exchanger in Na-sensitive single- and double-barrelled micro-electrodes. Liquid ion exchanger sensitive to K (Corning K 477317) and to Cl^- (Corning Cl 477913) were used in single- and double-barrelled micro-electrodes.

Single- and double-barrel ion-sensitive micro-electrodes were prepared and calibrated as previously described (Harvey & Kernan, 1984*a*, *b*). Double-barrelled micro-electrodes were calibrated with a constant background interference of 100 mM-KCl for Na^+ electrodes, 25 mM-$NaHCO_3$ for Cl^- electrodes and 10 mM-NaCl for K^+ electrodes. The intracellular activity of the main ion species was determined from the appropriate calibration curve.

For single-barrelled ion-sensitive micro-electrodes the selectivity coefficient (K_{jl}) was determined from:

$$K_{jl} = \exp F/RT(E_j - E_l), \tag{1}$$

where E_j and E_l are the potentials recorded by the ion-sensitive electrode in separate solutions of ion j and ion l at a similar activity. In the calculations of activities, the activity coefficient (γ) for Na, K and Cl was assumed to be similar in Ringer and intracellular fluids.

Thus from the tables presented by Moore (1968):

$$\gamma_{Na} = 0{\cdot}76, \quad \gamma_K = 0{\cdot}76, \quad \gamma_{Cl} = 0{\cdot}77.$$

Using single-barrel ion-sensitive micro-electrodes the intracellular activity of the main ion species (a_j^i) was determined from the equation:

$$a_j^i = (a_j^o + K_{jl})\, a_l^o \exp \frac{\Delta E - \psi_{mc} Z_j F}{nRT} - K_{jl} \cdot a_l^i, \tag{2}$$

where '*a*' means activity, i and o intra and extracellular respectively, K_{jl} the selectivity coefficient of the electrode, j and l refer to main and interfering ion species respectively, ΔE is the response of the ion-sensitive electrode on impaling the mucosal membrane. ψ_{mc} is the mucosal membrane potential, *n* is a correction factor for electrodes with non-Nernstian slopes, *Z*, *F*, *R*, *T* have their usual meanings. The characteristics of the ion-sensitive micro-electrodes used are presented in Table 1. The data obtained from single-barrel and double-barrel ion-sensitive micro-electrode measurements were pooled.

Electrical recording arrangements

The output of conventional and ion-sensitive micro-electrodes was fed via a short screened cable to the input stage of a custom built pre-amplifier (10^{15} Ω input impedance). The output of the pre-amplifier went to a Grass pen recorder and also to a Tektronix dual beam storage oscilloscope. Platinum wires connected to the Ringer solutions via 3 M-KCl agar bridges were used to pass transepithelial current pulses (I_t) (strength $\leqslant 75$ μA cm^{-2}, duration: 300 ms) from a floating constant current source (Argonaut Beaverton, OR, U.S.A.). The duration and cycle time of I_t was controlled by the timing and duration of the voltage output of a digitimer (Devices Instruments Ltd., England).

The transepithelial potential (V_t) and short-circuit current (I_{sc}) were measured with Ag–AgCl wires via 3 M-KCl agar bridges and recorded with a high impedance (10^{12} Ω) electro-meter and displayed by similar methods used for micro-electrodes.

Conventional micro-electrodes used to measure mucosal and serosal membrane potentials were

prepared from borosilicate glass tubing (GC150F Clark Electromedical, England). The tip resistance of micro-electrodes filled with 3 M-KCl was between 20 and 35 MΩ when measured in 3 M-KCl.

Epithelial cells were impaled from the mucosal side under visual control (×200 Olympus stereomicroscope). The micro-electrodes were advanced by manual control using micro-manipulators (Huxley-Goodfellow, England).

TABLE 1. Characteristics of single- and double-barrelled ion-sensitive micro-electrodes used in the present study

Electrode	Slope (mV)	Tip resistance	Selectivity coefficient	
		Single-barrel		
Na^+	53–58	1×10^{10}	K_{NaCa} K_{NaK}	0·09 0·015
K^+	56–60	1×10^{9}	K_{KNa}	0·015
Cl^-	55–58	5×10^{10}	K_{ClHCO_3} $K_{ClH_2PO_4}$	0·09 0·01
		Double-barrel		
Na^+	54–56	5×10^{10}	K_{NaCa} K_{NaK}	0·10 0·019
K^+	55–60	5×10^{9}	K_{KNa}	0·015
Cl^-	55–58	5×10^{10}	K_{ClHCO_3} $K_{ClH_2PO_4}$	0·09 0·01

Cell impalement criteria

The criteria for successful cell impalement by conventional and ion-sensitive micro-electrodes were similar to those previously used by us in studies in frog skin (Harvey & Kernan, 1984*a*). Cell impalements were considered valid in control conditions when on tip advancement from the mucosal solution into the epithelial cell layer, the fractional resistance of the apical membrane (FR_a) remained stable. During cell impalement the micro-electrode tip resistance (R_{tip}) was monitored by the passage through the micro-electrode tip of 1 nA pulses of current.

Artifactual negative potentials may be measured by a micro-electrode tip blocked by cell protein or by pressure against the cell membrane (Nelson, Ehrenfeld & Lindemann, 1978). In this case, the R_{tip} will have extremely high values ($\sim10^9\ \Omega$). We therefore considered that the cell impalement was valid only if R_{tip} measured in the cell was approximately equal to R_{tip} measured in Ringer prior to impalement and that the micro-electrode tip potential was similar before and after cell impalement.

It was technically impossible to impale the same cell with two separate micro-electrodes, however, flat cable analysis revealed that the cells are electrically coupled. In order that the single-barrelled ion-sensitive micro-electrode measured the same value of ψ_{mc} recorded by the conventional 3 M-KCl micro-electrode, the two electrodes must be in the same cell type possessing similar membrane resistances. To this end we compared the $F(R_a)$ values measured by the ion-sensitive and conventional micro-electrodes.

Circuit analysis

The equivalent circuit analysis described by Schultz (1972), Frömter (1972) and Reuss & Finn (1975) was used in calculation of membrane resistances and electromotive forces (e.m.f.). Localization of the micro-electrode tip and assessment of impalement damage was performed by measuring the fractional resistance of the mucosal membrane $F(R_a)$ when a micro-electrode penetrated a cell. $F(R_a)$ was measured as the ratio of mucosal membrane resistance (R_a) to the transcellular resistance (R_c), and was calculated from changes in ψ_{mc} as V_t was clamped between 5 and 15 mV:

$$F(R_a) = \Delta\psi_{mc}/\Delta V_t = R_a/R_c = R_a/(R_a+R_b). \tag{3}$$

The ratio of mucosal and basolateral membrane resistances ($\alpha = R_a/R_b$) was determined from changes in ψ_{mc}, ψ_{cs} (the basolateral membrane potential) and V_t response to transepithelial current pulses. From Kirchhoff's current law, the amount of current entering and leaving the cell must be equal, therefore:

$$\alpha = \Delta\psi_{mc}/(\Delta V_t-\Delta\psi_{mc}) = \Delta\psi_{mc}/\Delta\psi_{cs} = R_a/R_b. \tag{4}$$

When trout urinary bladder was bathed on both sides with Forster's saline, the spontaneous V_t was zero. Under these conditions total tissue resistance R_t was determined from Ohm's law when the V_t was clamped by ± 10 mV. The current–voltage relationship of trout urinary bladder was found to be linear over the V_t range of ± 15 mV.

In the equivalent circuit analysis the Thévenin equivalents at both cell membranes and in the paracellular junction represent the lumped resistances and e.m.f.s of all permeating ion species at the particular barrier. The Thévenin equivalents were calculated from the following equations (Schultz, 1972; Reuss & Finn, 1975):

$$E_a = \psi_{mc} + V_t(R_a/R_j) \quad \text{if} \quad E_j = 0, \tag{5}$$

$$E_b = \psi_{cs} - V_t(R_b/R_j) \quad \text{if} \quad E_j = 0, \tag{6}$$

$$V_t = \frac{(E_a - E_b)\, R_j}{R_a + R_b + R_j} \quad \text{if} \quad E_j = 0, \tag{7}$$

$$R_t = (R_a + R_b)\, R_j/(R_a + R_b + R_j). \tag{8}$$

During exposure to amphotericin B the R_a, R_b and R_j were calculated as the reciprocal of the conductances G_a, G_b and G_j by the following method (Lewis, Eaton, Clausen & Diamond, 1977; Henin & Cremaschi, 1978; Wills, Lewis & Eaton, 1979). E_a, E_b, E_j and G_a, G_b, G_j are the electromotive force and electrical conductance respectively of the apical and basolateral cell membranes and paracellular pathway.

When several pairs of measurements of transepithelial conductance, G_t, and of $\alpha = G_b/G_a$ corresponding to different I_{sc} values are made in the same amphotericin B treated bladder, then a plot of the relationship G_t *versus* $(1+\alpha)^{-1}$ yields a line with slope G_b and y-intercept G_j. The equation relating G_t and $(1+\alpha)^{-1}$ is:

$$G_t = G_b(1+\alpha)^{-1} + G_j. \tag{9}$$

Flat cable analysis

The values of R_a, R_b and R_j were also determined by voltage spread in the epithelium as reported for newt gut epithelium (Shiba, 1971) and *Necturus* gall-bladder (Frömter, 1972). For this analysis the trout bladder was mounted mucosal side up in a perspex chamber on the fixed stage of an inverted phase-contrast microscope (Leitz, Germany). Cellular impalements were done under visual control at $\times 200$ or $\times 320$ magnification. The interelectrode distance was measured with a micrometer eyepiece.

For calculation purposes the epithelium is considered as a flat thin sheet and current injected into a cell through a micro-electrode will flow radially away from the source through the internal resistance (R_i) of the cytoplasm and intracellular junctions and will leak out of the cells through R_a and R_b. The voltage spread into the epithelium may be described by the following differential equation (Shiba, 1971);

$$\frac{d^2V}{dx^2} + \frac{1}{x}\frac{dV}{dx} - \frac{V}{\lambda^2} = 0, \tag{10}$$

where V is the voltage deflexion measured by the exploring micro-electrode at a distance x from the current source and λ is the space constant. The general solution of this equation is:

$$V = AK_0\left(\frac{x}{\lambda}\right) = BI_0\left(\frac{x}{\lambda}\right), \tag{11}$$

where A and B are integration constants and K_0 and I_0 are zero-order modified Bessel functions of the second kind with imaginary argument. When $x \to \infty$; $V \to 0$ and the final solution becomes:

$$V = AK_0\left(\frac{x}{\lambda}\right). \tag{12}$$

Using the method of Shiba (1971) the measured voltage attenuation V was plotted as a semi-log function of radial distance x. The resultant curve was compared by eye to sets of Bessel functions K_0 drawn on transparent paper calculated for values of λ ranging from 200 to 700 μm. The vertical displacement (in mV) on the graph of the best fit Bessel function gave the value of A in eqn. 12.

The specific resistance to current flow from the cell to the external fluid compartments (R_z) was calculated from (Frömter, 1972):

$$R_z = \frac{2\pi A \lambda^2}{i}, \tag{13}$$

where i is the applied intracellular current (5×10^{-8} A).

Since

$$\frac{1}{R_z} = \frac{1}{R_a} + \frac{1}{R_b} \tag{14}$$

and

$$R_j = (R_a + R_b)\, R_t / ((R_a + R_b) - R_t) \tag{15}$$

by rearranging eqns. 14 and 15 and substituting $\alpha = R_a/R_b$ the values of R_a, R_b and R_j can be calculated from

$$R_a = (1+\alpha)\, R_z, \tag{16}$$

$$R_b = (1+\alpha)\, \alpha^{-1}\, R_z, \tag{17}$$

$$R_j = \frac{(1+\alpha)^2\, R_t\, R_z}{(1+\alpha)^2\, R_z - \alpha\, R_t}. \tag{18}$$

Finally, the horizontal resistance R_i and the specific resistivity of the cellular conducting material (ρ) are given by (Frömter, 1972):

$$R_i = \frac{2\pi A}{i} \tag{19}$$

and

$$\rho = R_i\, h, \tag{20}$$

where h is the height of the cells.

Values are reported as means ± S.E. of mean. The paired t test was used to evaluate the statistical significance of observed differences as indicated in the text.

RESULTS

Membrane potentials and resistances

The experimentally determined values of apical membrane potential were normally distributed around a mean value of $-55{\cdot}8 \pm 6{\cdot}9$ mV ($n = 180$ impalements in twelve bladders). Since the spontaneous transepithelial potential was nil the e.m.f. produced at both cell membranes must be equal to the corresponding membrane potential.

Flat cable analysis showed that the cells were electrically coupled and the space constant for the intra-epithelial spread of current measured in eight bladders was $354 \pm 33\ \mu$m (range 250–550 μm). The values of cell membrane and paracellular resistances obtained by cable analysis using eqns. 15–18 are summarized in Table 2. It was found that the ratio of the mean resistances of the cell pathway to the shunt $(R_a + R_b)/R_j$ was 23 indicating that 96 % of transepithelial current supplied by an external current source bypassed the cells. The urinary bladder of the fresh water adapted trout is, therefore, a very leaky epithelium, similar to *Necturus* gall-bladder.

The addition of amphotericin B (40 μg ml^{-1}) to the mucosal Ringer caused a rapid appearance of V_t ($8{\cdot}9 \pm 1{\cdot}4$ mV) and I_{sc} ($64 \pm 10\ \mu$A cm^{-2}) in twelve bladders studied. These effects were transient, lasting approximately 30 min but were prolonged when Cl^- was substituted by SO_4^{2-} in the mucosal Ringer, implying that mucosa to serosa Cl^- flux partially shunts the ionophore induced V_t.

The mucosal and basolateral cell membrane potentials depolarized following amphotericin B (Fig. 1). In twelve bladders under open-circuit conditions $\psi_{mc} = -6{\cdot}8 \pm 1{\cdot}3$ mV and $\psi_{cs} = -16{\cdot}2 \pm 1{\cdot}4$ mV and under short-circuit conditions the intracellular potential was $-10{\cdot}4 \pm 1{\cdot}5$ mV. Cable analysis of eight ionophore treated bladders (results summarized in Table 2) revealed a decrease in R_a and R_b of 85 and 58 % respectively following 30 min treatment with amphotericin B. During this period the shunt resistance decreased by 10 % indicating that the effects of the antibiotic were mainly confined to the cell membranes.

The equivalent e.m.f.s at the apical and basolateral membranes (E_a and E_b respectively) were calculated from the measured potentials and resistances according to eqns. 5 and 6. It was found that amphotericin B reversed the polarity of E_a from -56 mV to $+4$ mV and decreased E_b from -56 mV to -37 mV ($V_t = 4{\cdot}5 \pm 0{\cdot}6$ mV).

TABLE 2. Results of cable analysis in control bladders and in the presence of amphotericin B ($n = 8$ bladders)

R_t (Ω cm^2)	α (R_a/R_b)	A (mV)	λ (μm)	R_z (Ω cm^2)	R_i (kΩ)	R_a (Ω cm^2)	R_b (Ω cm^2)	R_j (Ω cm^2)	ρ (Ω cm)
Control									
197 ± 5	$1{\cdot}41 \pm 0{\cdot}09$	$7{\cdot}9 \pm 1{\cdot}1$	354 ± 33	1149 ± 149	998 ± 141	2790 ± 417	1986 ± 252	207 ± 5	2994
Amphotericin B									
164 ± 7	$0{\cdot}53 \pm 0{\cdot}03$	$4{\cdot}5 \pm 0{\cdot}8$	235 ± 19	285 ± 35	570 ± 100	433 ± 55	843 ± 105	186 ± 6	1710

It must be remarked that the cable analysis took at least 30 min to perform and the values of resistances and e.m.f.s are for a period of prolonged action of the ionophore. We therefore used a circuit analysis technique using changes in total tissue conductance (G_t) and voltage divider ratio (α) to determine the rapid effects of amphotericin B on R_a, R_b and R_j.

A plot of G_t *versus* $(1+\alpha)^{-1}$ yielded a linear relationship of slope G_b (basolateral conductance) and y-intercept G_j (shunt conductance). The results are summarized in Fig. 2 and Table 3. Using this data to calculate the membrane e.m.f.s from eqns. 5 and 6 it was found that following 10 min of ionophore action $E_a = +18$ mV, $E_b = -68$ mV and after 30 min $E_a = +8$ mV, $E_b = -39$ mV. This result confirms the results from cable analysis that E_a reverses polarity and that after prolonged action of amphotericin B, the E_b depolarizes. In the short-term, however, there appears to be a transient hyperpolarization of E_b which lasts for approximately 10 min. During this period R_a was reduced to 29% of its control value while the paracellular and basolateral resistances decreased by 7% and 20% respectively. Following 30 min exposure to the ionophore, R_j and R_b were 81% and 61% of their control values respectively with no further decrease in R_a.

Intracellular ionic activities

The intracellular ionic activities of Na, K and Cl ions determined in twelve urinary bladders are summarized in Table 4A. It was found under control conditions that a^i_{Na} was 58 times less than that predicted for passive distribution, indicating that Na ions are actively extruded from the cell. However, both a^i_K and a^i_{Cl} were higher than their equilibrium value, implying an active accumulation of these ions by the cell. Cl and K ion exit across the serosal membrane occurs down their electrochemical gradients. When the mucosal side of the urinary bladder was perfused with Na-free Ringer, there was a rapid decrease in a^i_{Na} (from $15{\cdot}7 \pm 1{\cdot}9$ mM to $3{\cdot}8 \pm 0{\cdot}9$ mM, $n = 25$ cells) accompanied by a fall in a^i_{Cl} (from $21{\cdot}2 \pm 3{\cdot}4$ mM to $15{\cdot}9 \pm 2{\cdot}3$ mM, $n = 18$ cells), both of these effects were reversed on restoration of mucosal Na Ringer (Fig. 3). The value of a^i_{Cl} measured in Na-free Ringer is close to that predicted for passive distribution Cl ions across the apical membrane ($E_{Cl} = -48$ mV). Furthermore, when all Cl^- was replaced by SO_4^{2-} in the mucosal Ringer a rapid decrease occurred in a^i_{Na}

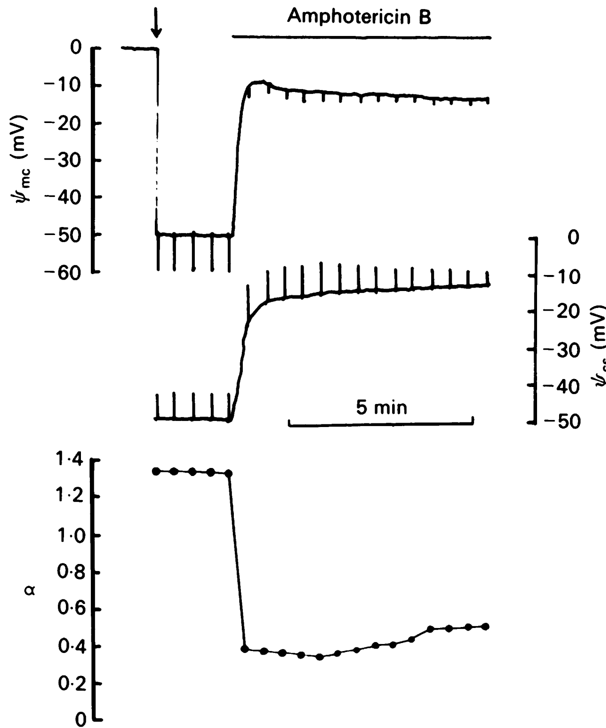

Fig. 1. Recording of mucosal membrane potential (ψ_{mc}), basolateral membrane potential (ψ_{cs}) and calculated ratio of apical to basolateral membrane resistance (α) during the addition of amphotericin B (40 mg ml^{-1}) to the mucosal solution. The ionophore caused a rapid depolarization of ψ_{mc} and ψ_{cs} and a decrease in α. The spikes on the membrane potential traces are in response to the passage of transepithelial current pulses, 70 $\mu A\ cm^{-2}$ in strength. Since α is calculated from $\Delta\psi_{mc}/\Delta\psi_{cs}$ due to these current pulses it can be observed from the recording that the fall in α on the addition of amphotericin B was due mainly to changes in $\Delta\psi_{mc}$ and therefore to changes in mucosal membrane resistance.

(from 16·1 ± 1·8 to 11·5 ± 1·2 mM, $n = 15$ cells), and in a^i_{Cl} (from 20·0 ± 2·4 to 10·6 ± 1·8 mM, $n = 15$ cells), which was reversed when mucosal Cl^- was restored (Fig. 4).

Voltage clamping of the mucosal cell membrane by ±30 mV (by passing transepithelial d.c.) did not have a significant effect on a^i_{Na} or a^i_{Cl} under normal Ringer conditions. A hyperpolarizing voltage clamp of ψ_{mc} in Cl^--free Ringer did not affect a^i_{Na} nor did a depolarizing clamp restore a^i_{Cl} in Na-free Ringer. These results are consistent with an electroneutral mode of NaCl co-transport.

Effects of amphotericin B on intracellular ionic activities

Following the addition of amphotericin B to the mucosal Ringer a large rapid increase in a^i_{Na} occurred which was accompanied by a decrease in a^i_K of approximately the same magnitude and relative to these changes only a small increase in a^i_{Cl} was measured (Fig. 5). The reciprocal changes in intracellular Na and K ion activities reflect a passive redistribution of these ions across the apical membrane and were such that the electrochemical driving forces ($\Delta\bar{\mu}$) favouring Na^+ entry and K^+ exit were reduced. The results are summarized in Tables 4A, B.

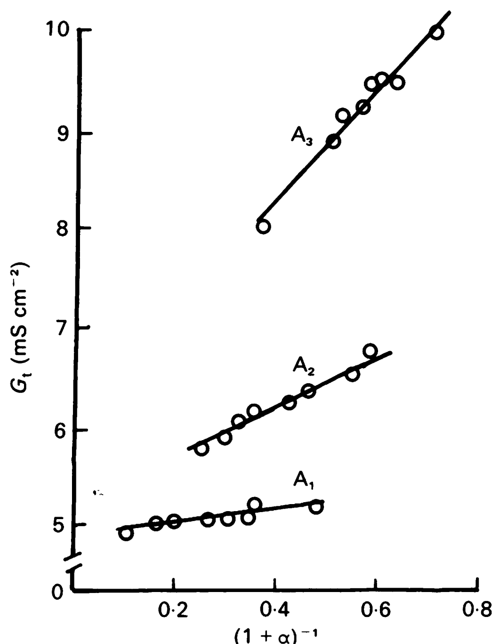

Fig. 2. Linear regression analysis of the relationship between transepithelial conductance (G_t) and $(1+\alpha)^{-1}$, where $\alpha = G_b/G_a$, the ratio of basolateral to apical membrane conductance. The equation relating G_t and $(1+\alpha)^{-1}$ is $G_t = G_b(1-\alpha)^{-1}+G_j$, where G_j is the conductance of the paracellular pathway. Solving this equation for G_t and α during the action of amphotericin B gave:

Amphotericin B (0–10 min) curve A_1: $5{\cdot}32 = 0{\cdot}729\ (0{\cdot}43)+4{\cdot}98$.
Amphotericin B (10–20 min) curve A_2: $7{\cdot}0 = 0{\cdot}91\ (0{\cdot}68)+5{\cdot}26$.
Amphotericin B (20–30 min) curve A_3: $9{\cdot}6 = 1{\cdot}19\ (0{\cdot}61)+6{\cdot}13$.

The linearity of curve A_1 implies that amphotericin B changes G_a only in the first 10 min of its action whereas the changes in the slope and y-intercept of curves A_2 and A_3 indicate an increase in basolateral membrane conductance and paracellular conductance, thus the effects of the ionophore are not confined to the apical membrane during this time period.

The change in $\Delta\bar{\mu}_{Na}$ was much greater than the change in $\Delta\bar{\mu}_K$ (90 mV *versus* 11 mV) and furthermore the e.m.f. of the apical membrane reversed towards the E_{Na}. This implies that the ionophore greatly increased apical Na permeability relative to that of K^+ making the apical membrane behave like a Na 'battery'. The ψ_{mc}, however, remained about 16 mV more negative than E_{Na} and this most likely reflects the contribution of K ion efflux to the membrane potential under these conditions.

Effects of ouabain on the amphotericin B treated bladder

Pre-treatment of trout urinary bladder for 30 min with 10^{-4} M-ouabain in the serosal Ringer reduced the amphotericin B induced V_t by 35%. Under these conditions the mean maximum V_t obtained was $5{\cdot}8 \pm 1{\cdot}3$ mV and that of I_{sc} was $34 \pm 4{\cdot}8$ μA cm^{-2} ($n = 12$ bladders). These values are significantly different from those measured in separate bladders treated with amphotericin B in the absence of ouabain ($P < 0{\cdot}001$).

Under ouabain conditions immediately prior to amphotericin B addition the $a^{i}_{Na} = 36 \pm 4$ mM and $\psi_{mc} = -44 \pm 3$ mV causing a reduction in the electrochemical driving force for Na entry by 33 mV with respect to control (Fig. 6). The effect of ouabain to reduce the ionophore induced V_t may be due to a decrease in $\Delta\bar{\mu}_{Na}$ favouring passive Na entry and/or an inhibition of the basolateral Na–K exchange pump.

TABLE 3. Results of circuit analysis of membrane e.m.f.s and resistances in trout urinary bladder in Normal Forster's fish Ringer and during the presence of amphotericin B. ($n = 12$ animals)

V_t (mV)	I_{sc} (μA cm^{-2})	R_t (Ω cm^2)	R_a (Ω cm^2)	R_b (Ω cm^2)	R_j (Ω cm^2)	E_a (mV)	E_b (mV)
Control (1 h)							
0	0	188±8	1824±137	1372±137	201±9	−55	−55
Amphotericin B (10–20 min)							
8·9±1·4	64·4±10	143±10	529±71	1100±90	190±9	+18	−68
Amphotericin B (20–30 min)							
4·5±0·6	44·0±4	104±6	529±71	842±72	163±15	+8	−39

In order to investigate the latter possibility we added 10^{-4} M-ouabain to the serosal Ringer 5 min prior to the addition of amphotericin B. In this situation the ionophore induced V_t was reduced by 19 % compared to that produced in the absence of ouabain. (The difference of $1{\cdot}7 \pm 0{\cdot}2$ mV was significant $P < 0{\cdot}01$, $n = 7$ bladders.)

Since ouabain did not affect a^{i}_{Na} nor $\Delta\bar{\mu}_{Na}$ in the short period prior to ionophore addition the reduction in V_t in this case may have been due to the elimination of an electrogenic component of the Na–K pump. Moreover, the calculated $E_b = -58$ mV under these conditions following amphotericin B showed little hyperpolarization above the control E_b value (−57 mV).

The dependence of the amphotericin induced V_t and I_{sc} on $\Delta\bar{\mu}_{Na}$

We examined the effects of changing the Na electrochemical gradient across the apical membrane on amphotericin B induction of V_t and I_{sc}. The $\Delta\bar{\mu}_{Na}$ was altered by perfusing the mucosal side with Ringer containing Na concentrations of 0·1, 1·0, 5·0, 10, 20, 50, 100 and 140 mM. Intracellular Na^+ activity and apical membrane potential were measured using a double-barrel Na-sensitive micro-electrode. The Na electrochemical driving force was calculated from the equation:

$$\Delta\bar{\mu}_{Na}(\text{mV}) = 2{\cdot}3\,\frac{RT}{F}\log\frac{a^{o}_{Na}}{a^{i}_{Na}} - \psi_{mc}. \tag{21}$$

When the sign of $\Delta\bar{\mu}_{Na}$ is positive, passive Na uptake is favoured, whereas with negative $\Delta\bar{\mu}_{Na}$, Na exit is favoured. The relationship between $\Delta\bar{\mu}_{Na}$ and the amphotericin B induced V_t is shown in Fig. 7. The amphotericin B provoked V_t and I_{sc} may be produced when the actual chemical gradient for Na entry is unfavourable, the driving force under these conditions is the membrane potential. Thus Na transfer through the cation channel created by amphotericin B in the apical membrane is by electrodiffusion.

Effects of K and Ba ions on NaCl transport

Recent investigations in renal epithelia have shown a K dependence of Na and Cl uptake (Greger & Schlatter, 1983). We wished to investigate the effects of K^+-free Ringer and Ba^{2+} (a known blocker of K channels in epithelia (Nagel, 1979)) on the uptake of Na^+ and Cl^- in the fish urinary bladder.

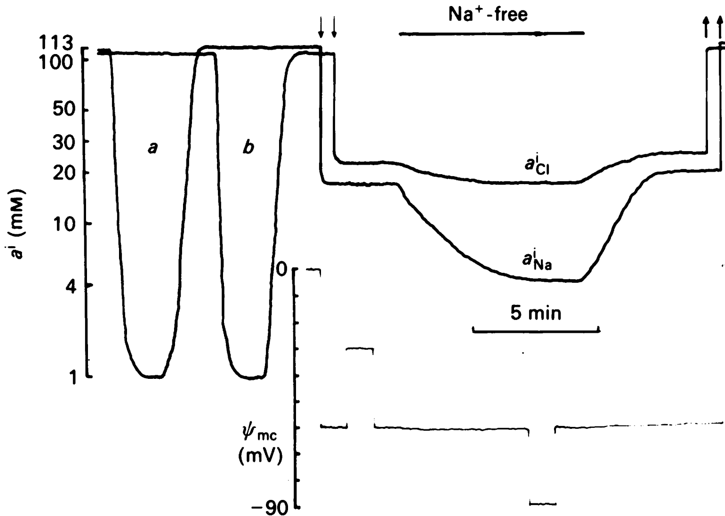

Fig. 3. Recordings of intracellular Na activity (a^i_{Na}) and intracellular Cl^- activity (a^i_{Cl}) by two separate double-barrel ion-sensitive micro-electrodes. Mucosal membrane potential (ψ_{mc}) was recorded from the reference side of the double-barrel Na-sensitive micro-electrode. A calibration of the electrodes in the mucosal bath is shown on the left-hand side of the trace giving in (*a*) the response of the double-barrel Na^+-sensitive micro-electrode on changing from normal Ringer to choline Ringer containing 1 mM-NaCl, and in (*b*) the response of the double-barrel Cl^--sensitive micro-electrode on changing from normal Ringer to SO_4^{2-} Ringer containing 1 mM-KCl. At the first arrow the double-barrel Na^+-sensitive micro-electrode penetrated a cell recording a^i_{Na} and ψ_{mc}, and the second arrow another cell (at 300 μm distance) was impaled by a double-barrel Cl^--sensitive micro-electrode. Neither a^i_{Na} nor a^i_{Cl} changed in response to a transepithelial current pulse of 75 $\mu A\ cm^{-2}$ in the mucosa–serosa direction which depolarized ψ_{mc} by 30 mV. Replacement on the mucosal side of normal Ringer with Na^+-free (choline) Ringer (during bar) caused a fall in a^i_{Na} and a^i_{Cl} with no change in ψ_{mc}. During this period hyperpolarization of ψ_{mc} by 30 mV caused by passing a transepithelial current pulse in the serosa–mucosa direction did not alter a^i_{Cl}. Both a^i_{Na} and a^i_{Cl} recovered to control values on return to normal Ringer. At third and fourth arrows the Na^+ and Cl^--sensitive micro-electrodes were withdrawn from the cells respectively.

In six trout bladders when the luminal perfusate was changed from normal Ringer to K^+-free Ringer the intracellular activities of Na and Cl ions remained unchanged although ψ_{mc} hyperpolarized by up to 15 mV. Furthermore, all of these bladders responded normally to amphotericin B in the absence of luminal K^+. When 5 mM-$BaCl_2$ was added to normal luminal Ringer no change in a^i_{Na} nor in a^i_{Cl} was recorded whereas ψ_{mc} depolarized by 5–10 mV. However, the addition of 5 mM-$BaCl_2$ decreased the amphotericin B induced V_t and I_{sc} by up to 50 % and caused a small increase in

R_t (12 %). These effects of Ba were also observed in luminal K^+-free Ringer. However, $BaCl_2$ added to the serosal Ringer had no effect on the ionophore induced V_t or I_{sc}. We also observed that when all Na^+ was replaced by K^+ in the mucosal Ringer the V_t and I_{sc} provoked by amphotericin B were reduced by 90 % and 72 % respectively when compared with values observed in normal Ringer.

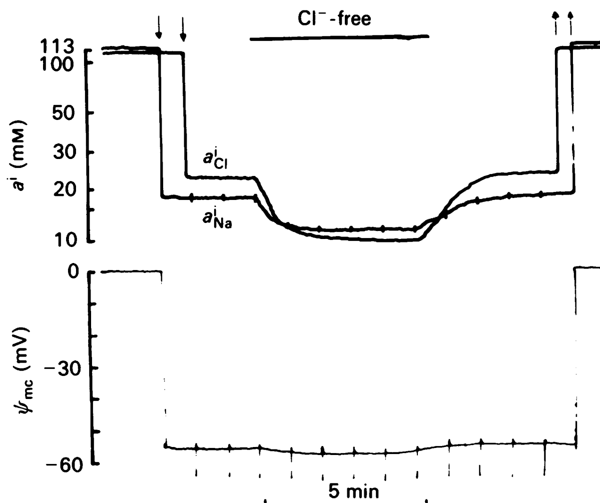

Fig. 4. Recording arrangement similar to Fig. 3. At first arrow, double-barrel Na^+-sensitive micro-electrode penetrated a cell to record a^i_{Na} and ψ_{mc}, at second arrow a double-barrel Cl^--sensitive micro-electrode impaled a separate cell to record a^i_{Cl}. During the period marked by a bar the normal mucosal Ringer was replaced by Cl^--free (SO_4^{2-}) Ringer causing a fall in both a^i_{Cl} and a^i_{Na} with little change in ψ_{mc} or membrane resistance as observed from the height of the potential spikes of ψ_{mc} trace in response to transepithelial constant current pulses (75 μA cm^{-2}).

It would appear from these results that transcellular NaCl co-transport is not K^+ dependent. However, the ionophore-induced Na transport and V_t are sensitive to Ba (whether K^+ is present in the Ringer or not), but are still generated in the absence of luminal K. This result may indicate that Ba ions block Na^+ movement through the membrane cation channels created by the ionophore.

The apical membrane shows good selectivity for K ions. Iso-osmotic substitution of KCl for NaCl in the mucosal Ringer (in the range 2·5–125 mM) produced a depolarization of ψ_{mc} of 40 mV per decade change in external K^+ activity (Fig. 8). The contribution of external K ions to the mucosal membrane e.m.f. was estimated from the K^+ dependent partial e.m.f. ratio (T_K) obtained from the equation:

$$T_K = \Delta E_a \bigg/ \frac{RI}{ZF} \ln a'_K/a_K, \tag{22}$$

where ΔE_a is the change in mucosal membrane e.m.f. following Na^+ replacement by K^+ in the mucosal Ringer, and a'_K and a_K are K ion activities in control Ringer and K^+ Ringer respectively. A mean T_K of 0·7 was found in seven bladders tested over the range of external K concentrations of 2·5–125 mM.

DISCUSSION

The urinary bladder is well developed in a number of teleosts and modifies the ureteral urine composition in such a way that it is involved in osmoregulation both in fresh water and in sea water (Lahlou, 1967). Bladder transport is under hormonal control and the osmolarity of the transported fluid decreases from fresh water to

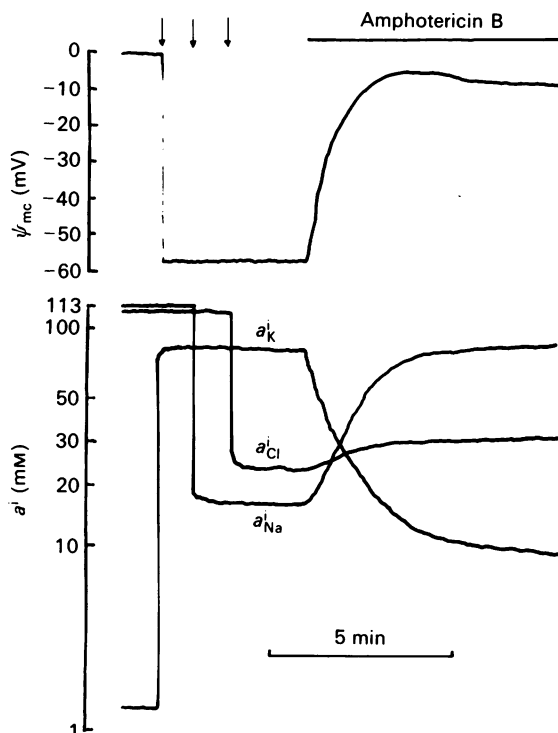

Fig. 5. Measurements of intracellular K activity (a^i_K), Na activity (a^i_{Na}) and Cl^- activity (a^i_{Cl}) by double-barrel ion-sensitive micro-electrodes. Mucosal membrane potential (ψ_{mc}) was measured by the reference side of the double-barrel K^+-sensitive micro-electrode. At first, second and third arrows the K^+, Na^+ and Cl^- electrodes penetrated separate cells respectively. During the period marked by a bar, amphotericin B (40 μg ml^{-1}) was added to the mucosal Ringer causing a rapid depolarization of ψ_{mc}, a large increase in a^i_{Na} and decrease in a^i_K with only a small increase in a^i_{Cl}.

sea water. *In vitro* studies showed that the isolated trout bladder when bathed with Ringer on both sides transports Na^+ and Cl^- from lumen to serosa in the form of a solution up to 10 times more hypertonic to the mucosal Ringer. The apical entry of Na and Cl proceeds via an obligatory coupled electroneutral process and is the limiting step in transepithelial NaCl absorption. The trout bladder presents some unusual properties such as higher permeability to Cl^- and non-cation-selective paracellular pathways. The electroneutral mode of NaCl co-transport is associated with a null transepithelial potential, however, in the presence of amphotericin B a transient V_t and short-circuit current are produced (cf. Fossat & Lahlou, 1977, 1979*a*,

TABLE 4A. Intracellular ionic activities of Na^+, K^+ and Cl^- in isolated trout urinary bladder bathed in Forster's Ringer before and after the addition of amphotericin B (40 μg ml^{-1}). ($n = 12$ bladders)

a^i_{Na} (mM)	a^i_K (mM)	a^i_{Cl} (mM)
	Control	
15.7 ± 1.9	75.5 ± 6.3	21.2 ± 3.4
	Amphotericin B	
79.4 ± 9.9	6.7 ± 0.8	28.4 ± 4.0

TABLE 4B. Electrochemical driving forces ($\Delta\bar{\mu}$ in mV) for transfer of K^+, Na^+ and Cl^- across the cell membranes of trout urinary bladder in control Normal Ringer and in the presence of amphotericin B (40 μg ml^{-1}) under open-circuit and short-circuit conditions. A positive sign denotes that $\Delta\bar{\mu}$ favours uptake of the ion whereas a negative sign signifies an outward flux is favoured. ($n = 12$ bladders)

	Mucosal membrane open-circuit	Serosal membrane open-circuit	Mucosal and serosal membranes short-circuit
$\Delta\bar{\mu}_K$			
Control	−37	−37	−37
Amphotericin B	−26	−16	−22
$\Delta\bar{\mu}_{Na}$			
Control	+106	+106	+106
Amphotericin B	+16	+26	+20
$\Delta\bar{\mu}_{Cl}$			
Control	−14	−14	−14
Amphotericin B	+26	+17	+23

1982). For a recent review dealing with the osmoregulatory role of fish urinary bladder see Lahlou & Fossat (1984).

Resistances and electrochemical potentials in control conditions

Our values of membrane resistances and electrochemical potentials are the first to be reported for fish urinary bladder. It can be calculated from the resistance data in Table 2 that 96% of transepithelial current bypasses the cells, thus the shunt pathway is intercellular and the trout urinary bladder may be classified as a 'leaky' epithelium as defined by Frömter (1972).

Leaky epithelia characteristically produce a low transepithelial potential which may reflect the electroneutral mode of NaCl transport and the shunting effect of the paracellular pathway (Frizzell & Duffey, 1980).

As a rule the urinary bladder of fish produces a low transepithelial potential which may be positive or negative even within a given species and may be dependent on the adaptive medium. For example, fresh water adapted flounder urinary bladder produces a V_t of approximately 2 mV serosa negative when mounted in an Ussing chamber and bathed in Forster's saline. This V_t gradually declines to zero over the space of one hour. The negative V_t and I_{sc} in this tissue may be accounted for by K transport (Dawson & Andrew, 1980, 1981). In the fresh water adapted trout bladder, however, the V_t is zero and from circuit analysis the membrane e.m.f.s are

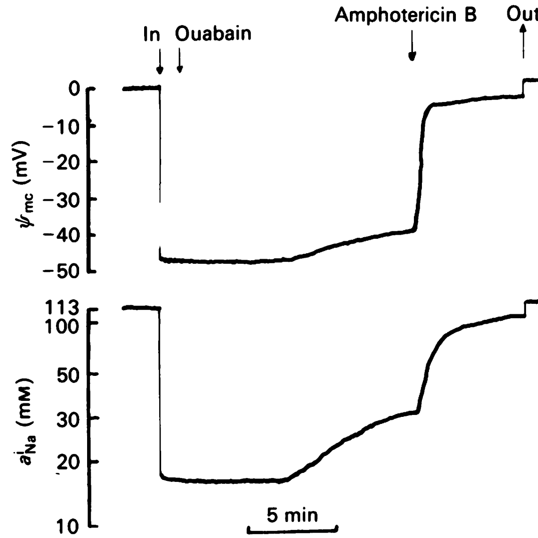

Fig. 6. Recording of the effect of prior treatment of trout urinary bladder with serosal applied ouabain (10^{-4} M) on the amphotericin B induced changes in mucosal membrane potential (ψ_{mc}) and a^i_{Na} measured with a double-barrel Na^+-sensitive micro-electrode. At the first arrow the Na^+ electrode penetrated a cell and recorded ψ_{mc} and a^i_{Na} and at the second arrow ouabain (10^{-4} M) was added to the serosal Ringer causing a depolarization of ψ_{mc} and an increase in a^i_{Na}. At the third arrow amphotericin B (40 μg ml^{-1}) was added to the mucosal Ringer producing a rapid depolarization of ψ_{mc} and a further increase in a^i_{Na}. At the fourth arrow the electrode was withdrawn from the cell.

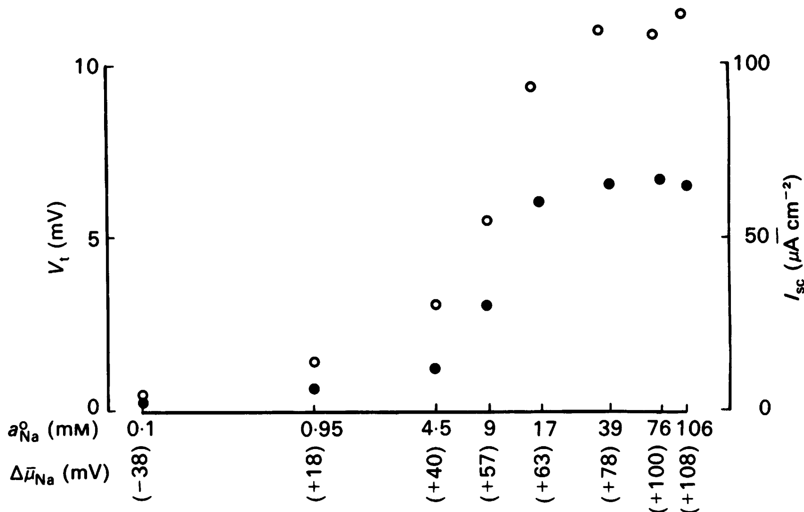

Fig. 7. The relationship between mucosal Na^+ activity (a^o_{Na}) and the amphotericin B induced transepithelial potential (V_t) and short-circuit (I_{sc}) in trout urinary bladders selected for similar initial transepithelial resistances. The Na^+ electrochemical gradient ($\Delta\bar{\mu}_{Na}$ in mV) calculated from measurements of mucosal membrane potential and intracellular Na^+ activity is given for each a^o_{Na} value in parentheses on the abscissa. Half-maximal V_t and I_{sc} were provoked by amphotericin B at an a^o_{Na} of 9·5 mM and $\Delta\bar{\mu}_{Na}$ of +58 mV (n = 8 bladders).

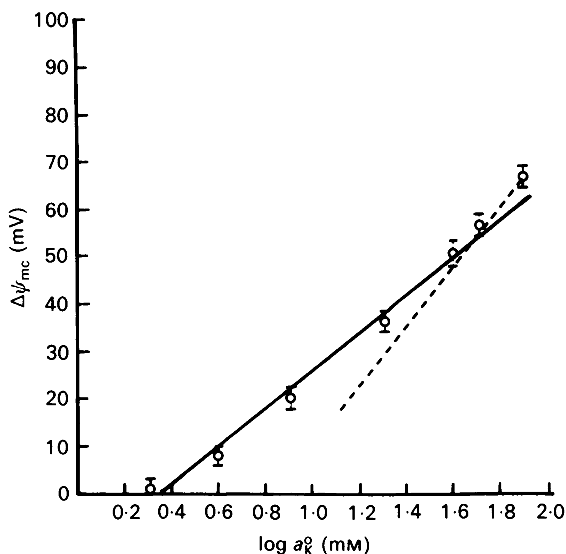

Fig. 8. Changes in mucosal membrane potential provoked by increasing mucosal K^+ activity (given on log scale). The continuous line was obtained from linear regression analysis of the data and has a slope of 39·5 mV. The dashed line gives the slope expected for a pure K electrode. Note that at higher values of a^{o}_{K} the response of ψ_{mc} becomes more nernstian. Each point is the mean ± S.E. of mean of eight impalements in the same bladder for each a^{o}_{K} change.

equal to the membrane potential. If both cell membranes are permselective to K ions we would expect E_a and E_b to be close to the E_K where in fact they are 40 mV less negative.

The apical membrane does show good K^+ selectivity with a 40 mV response per decade change in mucosal K^+ activity. The difference between E_a and E_K may be accounted for by the existence of an inward current carried by net cation entry or anion exit across the apical membrane. The ion species which may carry such a current has not been identified and deserves further investigation. Na and Cl transport is strictly specific in trout bladder and is not affected by pH changes in the bathing solutions and is therefore unlikely to be linked to Cl–HCO_3^- exchange (B. Fossat & B. Lahlou, unpublished results). Furthermore amiloride which acts on Cl^--independent Na^+ sites in epithelia reduces mucosa to serosa Na flux in flounder but not in trout bladder (Renfro, 1977). Thus, the entry of Na^+ by electrodiffusion may not be significant in trout bladder and may not be implicated in depolarizing ψ_{mc} below E_K.

Intracellular ionic activities

Our main findings are that intracellular Cl^- accumulation is dependent on mucosal Na^+ and that mucosal Na uptake requires the presence of Cl ions in the luminal solution. The co-transport of NaCl is electroneutral since voltage clamping ψ_{mc} by ± 30 mV did not change a^{i}_{Na} nor a^{i}_{Cl}. The NaCl co-transport mechanism of trout bladder is comparable to linked NaCl uptake by rabbit ileum (Nellans, Frizzel &

Schultz, 1973, 1974) and rabbit gall-bladder (Diamond, 1962; Cremaschi & Henin, 1975).

The absorption of Cl ions is a secondary active process which is dependent on a favourable transmembrane chemical gradient for Na entry (μ_{Na}). Since the measured a^i_{Na} is about 60 times less than that predicted for passive diffusion, the maintenance of μ_{Na} is an active process. The basolateral membrane of trout bladder possesses Na–K-ATPase activity associated with an active Na–K exchange pump sensitive to ouabain (Fossat, Lahlou & Bornancin, 1974; Renfro, Miller, Karnaky & Kinter, 1976). In the present study ouabain was found to increase a^i_{Na} approximately twofold and thus decrease the transapical membrane μ_{Na}. Associated with the increase in a^i_{Na}, the a^i_{Cl} decreased by 20% providing further evidence that Cl^- uptake is dependent on μ_{Na}. Substitution of K^+ by Na^+ or addition of Ba^{2+} to the mucosal Ringer did not affect the intracellular accumulation of Cl ions. The mechanism of NaCl co-transport in trout bladder differs therefore, from the triple association of Na^+, 2 Cl^-, K^+ found in the kidney cortical thick ascending limb (Greger & Schlatter, 1981).

The apical membrane of trout bladder exhibits a high transference number (T_K) for K ions of 0·7 and this K^+ selectivity is also a feature of *Necturus* gall-bladder (Reuss & Finn, 1975). Since a^i_K is almost 4·5 times that predicted for passive distribution, K ions are actively accumulated by the cell. Given the presence of a basolateral Na–K exchange pump and high T_K for the luminal membrane we would expect K^+ secretion to occur and this is a feature of fish urinary bladders (Dawson & Andrew, 1980, 1981; Lahlou & Fossat, 1984).

Effects of amphotericin B on electrical parameters

We used flat cable analysis to determine the effects of amphotericin B on resistances and e.m.f.s, we also employed the ionophore itself as a tool to perform a circuit analysis.

Flat cable analysis indicated that the main effect of the ionophore was to reduce the electrical resistance of the apical membrane with little change occurring in the resistance of the paracellular pathway. Following prolonged action of the antibiotic (30 min) the basolateral membrane resistance was approximately halved. The fall in R_a was due to the increase in transapical Na^+ and K^+ diffusion down their respective electrochemical gradients. The reduction in R_b could have been caused by a mechanical effect of cell swelling or as a result of a direct action of amphotericin B on the basolateral membrane following cell penetration.

Since flat cable analysis required at least 30 min to perform we were unable to determine the short-term effects of amphotericin B on membrane resistances using this method. The ionophore itself, in altering the voltage-divider ratio α and R_t may be used as a tool to perform a circuit analysis of the Thévenin equivalents of the epithelium. The technique is similar to that used in the amphotericin B treated rabbit gall-bladder (Cremaschi, Henin, Meyer & Bacciola, 1977; Henin & Cremaschi, 1978), toad urinary bladder (Reuss, Gatzy & Finn, 1978) and in the nystatin treated rabbit urinary bladder (Lewis, Eaton, Clausen & Diamond, 1977; Wills, Lewis & Eaton, 1979). The validity of the absolute values of R_a, R_b and R_j for control conditions calculated by this method depend on the assumption that amphotericin B does not have an immediate effect on R_b nor R_j. We know from cable analysis that in the long

term following 30 min exposure to the drug that R_j was only marginally affected whereas R_b was decreased by 57 %. We have reason to believe, however, as discussed below that amphotericin B does not affect R_b in the first 10 min of its action.

The advantage of using amphotericin B to perform equivalent circuit analysis is that quasi-continuous measurements can be made of the transepithelial conductance G_t and of the voltage divider ratio $\alpha = \Delta\psi_{mc}/\Delta\psi_{cs} = G_b/G_a$.

The relationship G_t *versus* $(1+\alpha)^{-1}$ calculated before and during the first 10 min of amphotericin B exposure is linear with a unique slope = G_b and y-intercept = G_j. Following 20 min exposure the function $[G_t, (1+\alpha)^{-1}]$ has a steeper slope (increase in G_b) with a significant change in the y-intercept (G_j increased).

For periods longer than 20 min both the slope and y-intercept have changed indicating an increase in G_j and a further decrease in G_b.

Using the values of resistances, calculated from the reciprocal of these conductances, we found from eqns. 5 and 6 that the apical membrane e.m.f. reversed polarity and approached E_{Na} whereas the E_b hyperpolarized immediately following amphotericin B exposure. After 30 min the E_b had depolarized and the values of R_a, R_b, R_j, E_a and E_b were in close agreement with those calculated by flat cable analysis.

Since during amphotericin B action the E_b is more negative than E_a, current flow through the paracellular pathway is in the serosa to mucosa direction and as a result the apical membrane is hyperpolarized with respect to E_a and the basolateral membrane is depolarized in relation to E_b. The presence of the low resistance shunt also explains why after exposure to amphotericin B the difference between ψ_{mc} and ψ_{cs} is only 9 mV whereas the difference between E_a and E_b is 42 mV.

Amphotericin B produces a serosa positive V_t in *Necturus* gall-bladder (Reuss, 1978), rabbit gall-bladder (Cremaschi *et al.* 1977) and trout urinary bladder (Fossat & Lahlou, 1982) and several different hypotheses have been advanced to explain the origin of this V_t. In trout bladder the V_t induced by the ionophore may have resulted from (i) a fall in ψ_{mc} as a result of a decrease in R_a, (ii) a fall in E_a and/or a rise in E_b such that the net result is an increase in $(E_a - E_b)$, (iii) a serosa positive change in E_j, (iv) electrogenic extrusion of Na across the basolateral membrane.

The initial rapid fall in R_a by 70 % could account for a 16 mV change in ψ_{mc} but the actual depolarization is 49 mV. Therefore, E_a must also decrease and this is expected from the increase in a^i_{Na} and the decrease in E_K (since ψ_{mc} is in part determined by E_K). A change in the polarity of E_a from negative to positive values has been proposed by Cremaschi *et al.* (1977) to account for the effect of amphotericin B on the V_t of the rabbit gall-bladder. If we assume E_j is zero immediately following amphotericin B, then E_b is calculated to increase by 13 mV. This change results in a further increase in $(E_a - E_b)$, making V_t serosa positive. The initial net change in $(E_a - E_b)$ was 86 mV; however, the corresponding change in V_t was 13 mV, thus a sizeable paracellular leak exists to shunt the membrane battery.

A serosa positive change in E_j may occur following amphotericin B given the hypertonic nature of NaCl transport (Fossat & Lahlou, 1977) and the apparent anion selectivity of the shunt pathway (Fossat & Lahlou, 1979*a*). However, we calculate that little change in R_j occurs and furthermore transepithelial Cl^- fluxes are not modified by amphotericin B (Fossat & Lahlou, 1982). Cl^- does, however, appear to exert a shunting effect on the V_t since the replacement of mucosal Cl^- by SO_4^{2-} or

gluconate enhances the ionophore induced V_t and converts the response from that of a transient to a sustained V_t (Fossat & Lahlou, 1982).

If the basolateral Na pump becomes electrogenic under an increased intracellular Na load during amphotericin B action, then an increase in E_b would be expected. An increase in E_b in the amphotericin treated gall-bladder was reported by Rose & Nahrwold (1976) and by Graf & Biebisch (1979), but a decrease in E_b was reported by Reuss (1981). The latter author proposed that the result obtained by the former workers differed as they had assumed that R_b remained constant in the presence of the ionophore. In the present study, however, we have calculated an increase in E_b to be present even 10 min following the addition of the antibiotic, taking into consideration the calculated fall in R_b within this time.

In contrast, we were unable to detect any effect of ouabain on ψ_{cs} in control Ringer prior to changes in a^i_{Na}. Also, Reuss reported that ouabain had little effect on ψ_{cs} in *Necturus* gall-bladder (Reuss, 1979). It must be borne in mind, however, that the Na–K pump may become electrogenic only under the influence of an increased a^i_{Na} and that the electrogenic contribution to ψ_{cs} may not be measurable in the presence of low R_b and R_j. During amphotericin B action it may be difficult to determine the electrogenicity of the pump from changes in E_b since the diffusion potential may decrease with increased a^i_{Na} and decreased a^i_K and this effect may offset an increase in the current (pump) generated potential. For a detailed discussion of this point see Zeuthen (1981).

It was not possible to study ouabain effects on the electrogenic component of ψ_{cs} under amphotericin B conditions because of the transient nature of ψ_{cs} in this situation. However, the calculated E_b in the continued presence of ouabain and amphotericin B was less than that calculated in the presence of the ionophore alone, indicating the removal of an electrogenic Na–K pump contribution to E_b.

We conclude from our experiments that the amphotericin B induced V_t results from a decrease in E_a due to an increase in luminal membrane Na permeability and an increase in E_b resulting from electrogenic Na extrusion across the basolateral membrane. The transient nature and decline in V_t is related to the decrease in the difference $E_a - E_b$ which may be due to a direct effect of the antibiotic on the basolateral membrane. Transepithelial Cl^- movement has a shunting effect on the V_t and most likely occurs across a paracellular route since a^i_{Cl} is little affected by the ionophore.

Effect of amphotericin B on intracellular ionic activities

From the changes observed in a^i_{Na} and a^i_K in the amphotericin treated trout bladder we may conclude that the ionophore creates pores in the mucosal membrane through which Na and K ions flow down their respective electrochemical gradients.

The amphotericin B induced V_t was found to be dependent on the electrochemical gradient existing for passive Na entry across the apical membrane, with the maximum response occurring at external Na concentration ($[Na]_o$) = 50 mM and $\Delta\bar{\mu}_{Na} = +100$ mV. The ionophore produced a V_t of only 0·2 mV when all mucosal Na was replaced by K, thus the hypothesis presented by Reuss, Weinmann & Grady (1980) for *Necturus* gall-bladder, where K^+ accumulated in unstirred layers near the luminal membrane generates a paracellular diffusion potential contributing to V_t,

cannot be applied to trout bladder. Our flux studies in open-circuit conditions (Fossat & Lahlou, 1982) and in short-circuit conditions (Harvey, 1982) also rule out the possibility proposed by Rose & Nahrwold (1976) for rabbit gall-bladder according to which amphotericin B diminishes Na backflux from intercellular spaces to the mucosal medium, since this backflux was found to be increased in the ionophore treated trout bladder.

In conclusion we report the first intracellular electrophysiological study of the urinary bladder of an osmoregulating teleost. The study has served to characterize the trout urinary bladder as a leaky epithelium and to describe the cellular mechanism of electroneutral NaCl co-transport and the origin of the amphotericin B induced transepithelial potential difference.

The adaptation of euryhaline fishes such as trout to various external salinities is reflected in functional (histological and transport) changes in their ion transporting epithelia including urinary bladder. The present study may serve as a reference basis for further electrophysiological investigations of the ionic transport mechanisms involved in the adaptive osmoregulation in these animals.

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