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THE ACID–BASE BALANCE OF THE OUTER MANTLE EPITHELIUM OF *ANODONTA CYGNEA*

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Summary

1. Under short-circuit conditions the outer mantle epithelium of *Anodonta cygnea* is known to produce an acidification of the solution bathing the shell side and an alkalinization of the solution bathing the haemolymph side.

2. At steady state, the rates of secretion of acid and base were numerically equal to the simultaneously measured short-circuit current, expressed in the same units.

3. The rates of acid and base secretion, and the short-circuit current, showed close similarity in the reductions caused by anoxia, diamox, DIDS (from haemolymph side), DNP and iodoacetamide.

4. The short-circuit current (I_{sc}) was sensitive to the concentration of CO_2, bicarbonate or protons in the solution on the shell side.

5. The short-circuit current was insensitive to vanadate or oligomycin, was slowly inhibited by DCCD added under anoxia to the shell side, and was almost completely inhibited within seconds by TBTO (shell side) which also caused a 40% reduction in transepithelial conductance.

6. It is suggested that I_{sc} is due to a Cl^-/HCO_3^-exchange shunted by a Cl^- recirculation across the basolateral membrane and to the operation of an electrogenic proton pump located in the apical membrane.

Introduction

The outer mantle epithelium (OME) of *Anodonta cygnea* produces a spontaneous electrical potential *in vitro* (Istin and Kirschner, 1968). Under short-circuit conditions it generates a current (I_{sc}) which, when expressed as a molecular flux, has a magnitude the same as that of the simultaneously measured [^{14}C]bicarbonate net flux from shell to haemolymph side (Coimbra *et al.* 1988). Both the net flux of bicarbonate and I_{sc} are abolished by 4-acetamido-4′-isothiocyamatostilbene-2,2′-disulphonic acid (SITS) and 4,4-diisothiocyanostilbene 2,2-disulphonic acid (DIDS). The current is blocked by iodoacetamide, partially blocked by diamox

Key words: *Anodonta*, mantle, acid–base balance.

and is very sensitive to exogenous CO_2. To prevent the accumulation of protons (acid) within the cell (with potentially harmful effects), the net flux of bicarbonate must be accompanied by a net flux of protons to either the haemolymph or shell side. If protons are transported to the shell side, then the net flow of bicarbonate across the basolateral membrane, the net flow of protons across the apical membrane and I_{sc} should have the same magnitude, when expressed in the same units. The results presented here support this hypothesis. Within experimental error the three quantities are similar under a variety of conditions.

Materials and methods

Specimens of *Anodonta cygnea* were collected from the Lagoon of Mira in northern Portugal and kept in the laboratory in aerated dechlorinated water for up to 2 weeks. The OME was dissected out, mounted in an Ussing-type chamber and continuously short-circuited as previously described (Coimbra *et al.* 1988).

The usual solution bathing both sides of the preparation (control solution) had the following composition (in $mmol\,l^{-1}$): Na^+, 11; K^+, 7; Cl^-, 19; Mg^{2+}, 0.5; Ca^{2+}, 1; bicarbonate, 2. Both half-chambers were filled with 2 ml of this solution and gassed with a humidified mixture of CO_2 (5%) and oxygen (95%). In some experiments the haemolymph side of the preparation was bathed with control solution while ionic replacements were performed on the solution bathing the shell side (shell solution). When chloride was replaced by gluconate, isethionate, thiocyanate or sulphate, the osmolality of the solution was maintained by the addition of sucrose. When the effect of pH on the short-circuit current (I_{sc}) was studied, the shell solution contained $1\,mmol\,l^{-1}$ NaH_2PO_4 instead of $2\,mmol\,l^{-1}$ bicarbonate. Since most of the experiments were performed in the presence of exogenous CO_2 the pH-stat method was not used (Sanders *et al.* 1973). The amount of acid or base delivered into the baths over a period of 20–30 min was measured by titrating a known amount of fluid from each half-chamber, previously equilibrated with humidified nitrogen to remove all the dissolved CO_2. The titrant (HCl) was delivered from a precision syringe driven by a micrometer until all the bicarbonate had been removed from the solution. Each time a small amount of titrant was delivered there was an initial fall in pH followed by a slower rise (Fig. 1). This pattern results from the slowness of the hydration of CO_2 and of the dehydration of H_2CO_3. To avoid an overshoot, the amount of titrant delivered each time was progressively reduced. The titration was stopped when there was no rise in pH after the addition of acid (Fig. 1, second arrow). 50 samples of the bathing solution containing $2\,mmol\,l^{-1}$ bicarbonate yielded a value of $2.03\pm0.002\,mmol\,l^{-1}$ (mean±S.E.). When inhibitors were used the standards included the same amount of inhibitor as the samples. The inhibitors used were applied at the following final concentrations: amiloride (3,5-diamino-6-chloropyrazinoylguanidine), $1\,mmol\,l^{-1}$; diamox, $1\,mmol\,l^{-1}$; vanadate $1\,mmol\,l^{-1}$; dinitrophenol (DNP), $1\,mmol\,l^{-1}$; DIDS, $0.5\,mmol\,l^{-1}$; oligomycin, $200\,\mu g\,ml^{-1}$; dicyclohexylcarbodiimide (DCCD), $50\,\mu g\,ml^{-1}$.

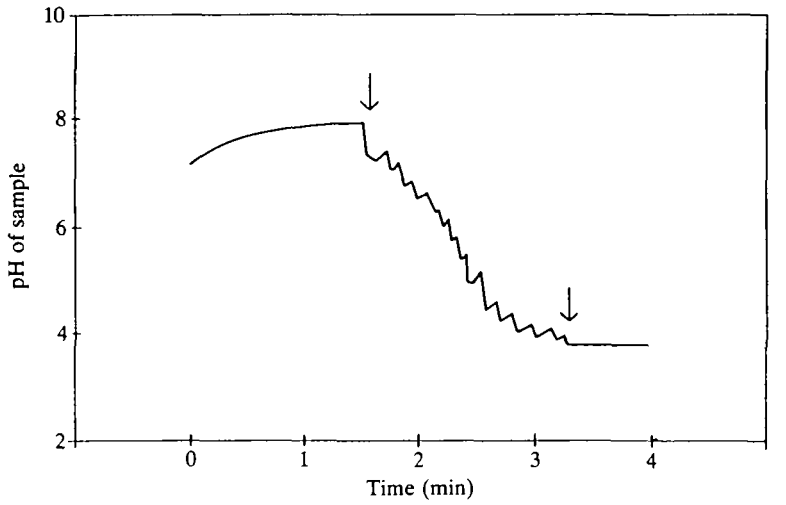

Fig. 1. Titration of a sample. First arrow, start of titration; second arrow, end of titration. The sample, under continuous agitation, was gassed from time zero onwards with nitrogen saturated with water vapour.

To compare the rate of alkalinization of the haemolymph compartment, or rate of acidification of the shell compartment, with I_{sc}, the amount of base, or acid, delivered by the epithelium, expressed in $\mu mol\,cm^{-2}\,s^{-1}$ was multiplied by the Faraday (96 500 C $equiv^{-1}$).

Results

The I_{sc} across the OME, which is known to be equal to the rate of bicarbonate transport to the haemolymph side when expressed in the same units (Coimbra *et al.* 1988), was also found to be equal to the rate of transport of acid to the shell side (Fig. 2). Similar observations have been reported for the amphibian gastric mucosa (Teorell, 1951) and for the turtle urinary bladder, in which the transepithelial sodium transport had been blocked (Husted *et al.* 1979).

I_{sc} in the OME is inhibited by diamox, DIDS, DNP, amiloride and iodoacetamide (Coimbra *et al.* 1988). Diamox, DIDS and DNP induced changes in the rates of secretion of base (haemolymph side) and acid (shell side) which were very similar to the concurrent changes in I_{sc} (Fig. 3). Amiloride induced a decrease in the amounts of acid and base secreted and had almost no effect on I_{sc} during the same period. This agrees with the observation (Coimbra *et al.* 1988) that the inhibition of I_{sc} by amiloride is very slow.

These results show that over a wide range of transport rates and within experimental error there is good agreement between I_{sc} and the net transport of acid and base, both under control conditions and when the preparation is treated with diamox, DIDS or DNP. Thus, provided the current is stable, it can be used to

monitor the transport of acid (towards the extrapallial compartment) and base (towards the haemolymph compartment).

The dependence of I_{sc} on O_2 was assessed. Six preparations were gassed with O_2

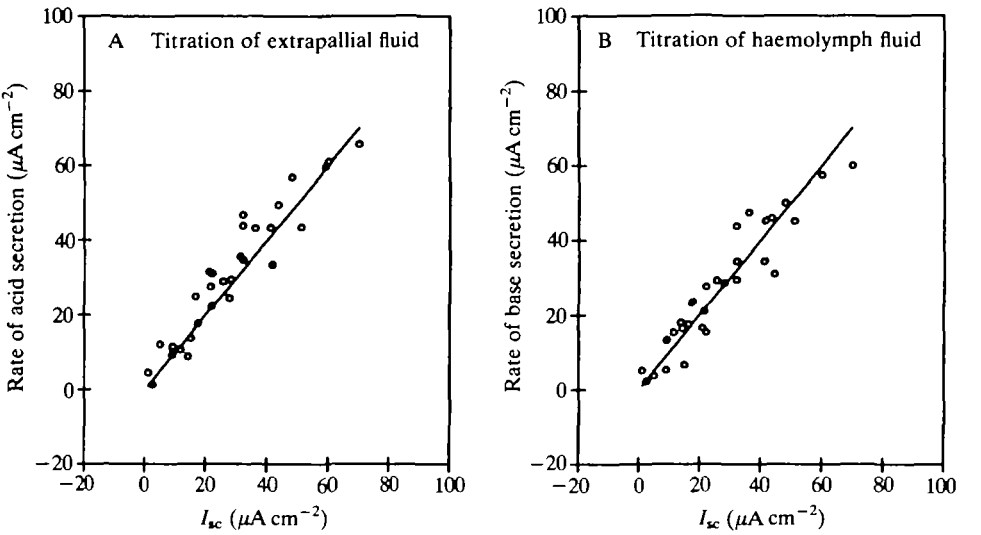

Fig. 2. Relationship between the short-circuit current (I_{sc}) and the rates of secretion of acid (A) and base (B). The continuous lines represent a slope of titration rate *vs* I_{sc} of 1.

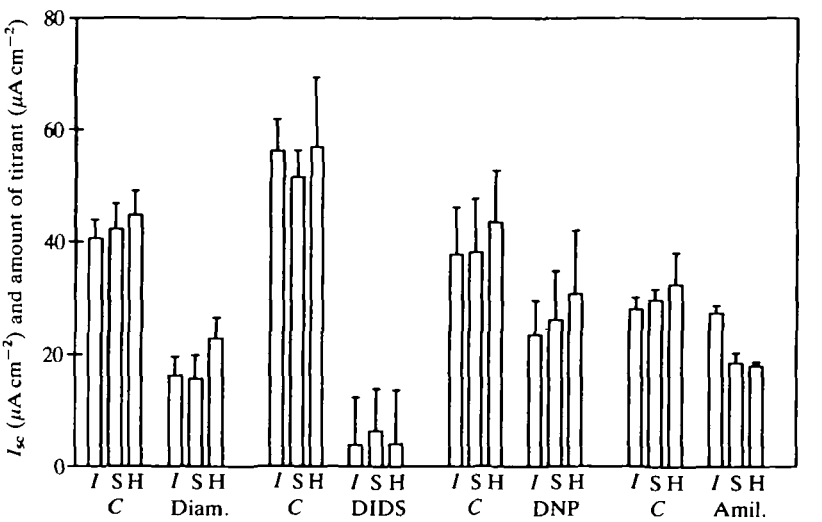

Fig. 3. Effect of diamox 1 mmol l^{-1} (Diam.), DIDS 0.5 mmol l^{-1}, DNP 1 mmol l^{-1} and amiloride 1 mmol l^{-1} (Amil.) on the short-circuit current (I), on the rate of secretion of acid towards the shell side (S) and on the rate of secretion of base towards the haemolymph side (H). *C*, control period. Six experiments for each condition. Bars show S.E.

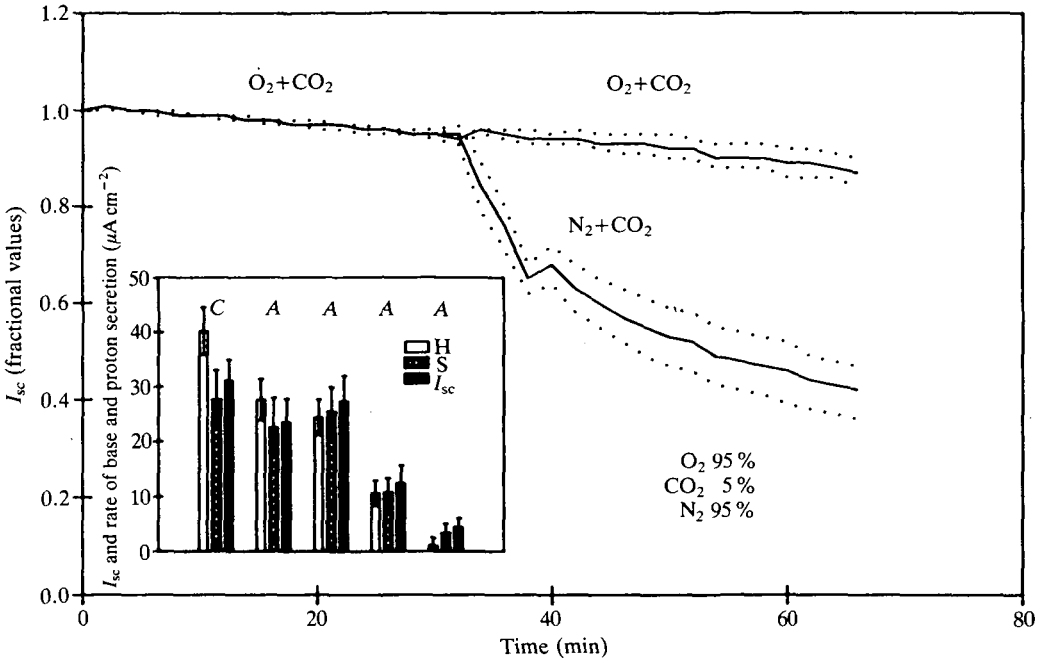

Fig. 4. Oxygen dependence of I_{sc}. Six pieces of OME isolated from six mantles were prepared and gassed with O_2+CO_2 (carbogen). After a control period, three of the preparations were gassed with a mixture of N_2 and CO_2. Continuous lines correspond to the averages of the instantaneous currents divided by the corresponding currents at zero time. Dotted lines correspond to ± one standard error of the mean (S.E.). Inset, I_{sc} and rates of secretion of acid and base measured in another group of six preparations. *C*, average ±S.E. of the three initial 30-min periods for the six preparations; *A*, average ±S.E. of 30-min periods under anoxia.

and CO_2 until a steady state was attained. Three of the preparations were then gassed with a mixture of N_2 and CO_2; the current fell continuously (albeit slowly) reaching a relative value of 0.5 after 30 min (Fig. 4). The inset shows the results of similar experiments (N=6) in which I_{sc} and the rates of acidification and alkalinization of the shell and haemolymph compartments, respectively, were measured simultaneously. Both under control conditions (*C*, three 30-min periods for each preparation) and under anoxia (*A*, four consecutive 30-min periods) there was good agreement between the three quantities.

When CO_2 was removed from the mixture gassing the shell side of the preparation, the I_{sc} fell to 40 % of the control value (Fig. 5). Under these conditions I_{sc} was almost insensitive to the concentration of bicarbonate on the same side in the range 10–1.25 mmol l^{-1}. When the shell solution was nominally bicarbonate-free, I_{sc} fell to a relative value of less than 0.1. However, an effect of pH on I_{sc} cannot be ruled out in these experiments.

In another series of experiments the effect on I_{sc} of the pH of the solution on the shell side was studied (Fig. 6). When the shell solution was nominally bicarbonate-

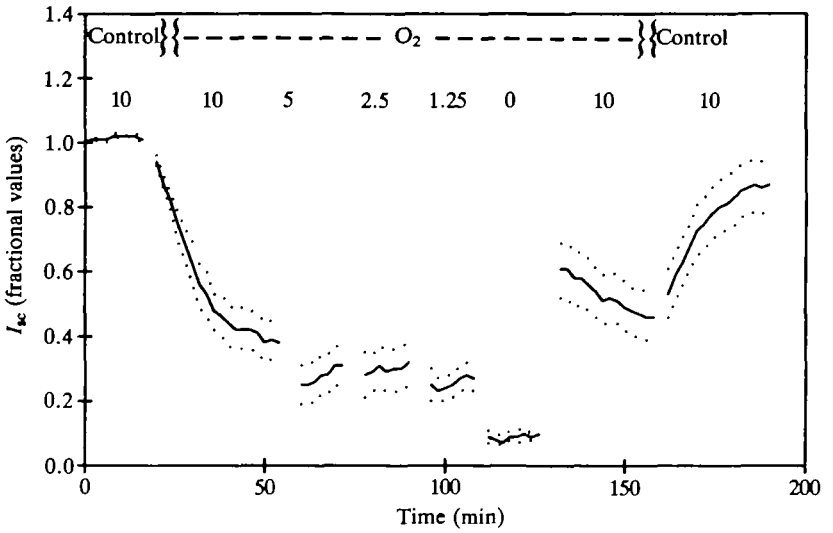

Fig. 5. Bicarbonate dependence of I_{sc}. Six preparations were studied. During the initial and final periods the preparations were bathed in control solution gassed with 95% O_2+5% CO_2 on both sides. In the intermediate periods the shell solution contained 10, 5, 2.5, 1.25 or (nominally) 0 mmol l^{-1} bicarbonate and was gassed with pure oxygen (dashed line). Bicarbonate was replaced by chloride. For an explanation of the dots and continuous lines, see legend to Fig. 4.

free, the preparation was gassed with pure oxygen. I_{sc} was measured with the shell solution pH adjusted (with HCl) to different values (9, 8, 7, 6 or 5). For comparison, a phosphate-free, nominally bicarbonate-free shell solution was also used. I_{sc} was pH-sensitive, in particular below pH 7 (Fig. 6).

I_{sc} was insensitive to vanadate (1 mmol l^{-1}) added to both sides (control 55.6±4.37 μA cm^{-2}; +vanadate 62.0±1.84 μA cm^{-2}, N=6) and to oligomycin (200 μg ml^{-1}) also added to both sides (control 42.0±12.4 μA cm^{-2}; + oligomycin 39.3±12.6 μA cm^{-2}, N=6). DCCD (50 μg ml^{-1}) caused a slow and moderate reduction of I_{sc} under anoxia when added to the shell side and was ineffective from the haemolymph side (Fig. 7). Tributyltin oxide (TBTO) was ineffective when added on the haemolymph side, but caused an almost complete inhibition of I_{sc} within less then 10 s when added to the shell solution to give final concentrations in the range 10–100 nmol l^{-1}. Such an effect could be observed when the main anion in the solution was isethionate, gluconate, sulphate, thiocyanate or chloride (Fig. 8). TBTO reduces transepithelial conductance by 40% (Machado *et al.* 1989).

Discussion

Our earlier work suggested that the short-circuit current of the OME under our experimental conditions cannot be due to a net flux of chloride, potassium or calcium and that the small net flux of sodium measured was in the opposite

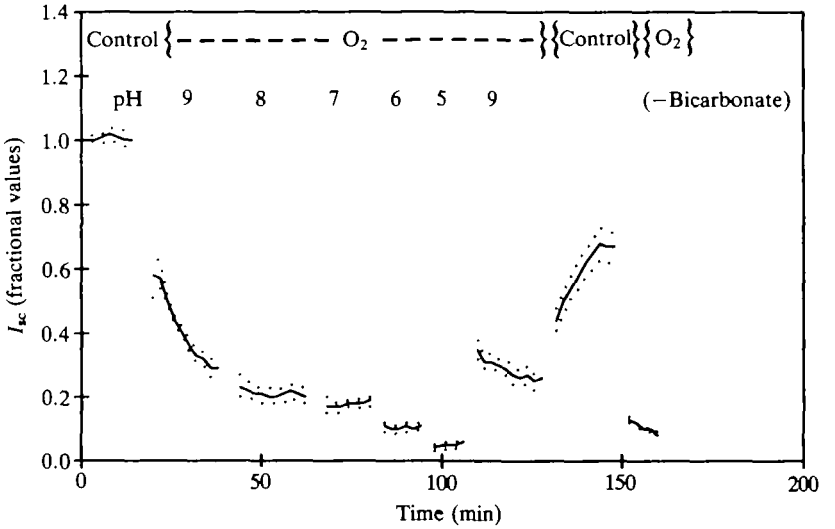

Fig. 6. pH dependence of I_{sc}. Six preparations were studied. After an initial control period the chamber on the shell side was emptied, refilled in succession with nominally bicarbonate-free solution containing 1 mmol l^{-1} NaH_2PO_4 titrated to pH 9, 8, 7, 6, 5 or 9 and gassed with 100 % O_2. The chamber was then refilled with control solution gassed with 95 % O_2+5 % CO_2. In the final period the chamber was filled with nominally bicarbonate-free solution gassed with pure oxygen. For an explanation of continuous lines and dots, see legend to Fig. 4.

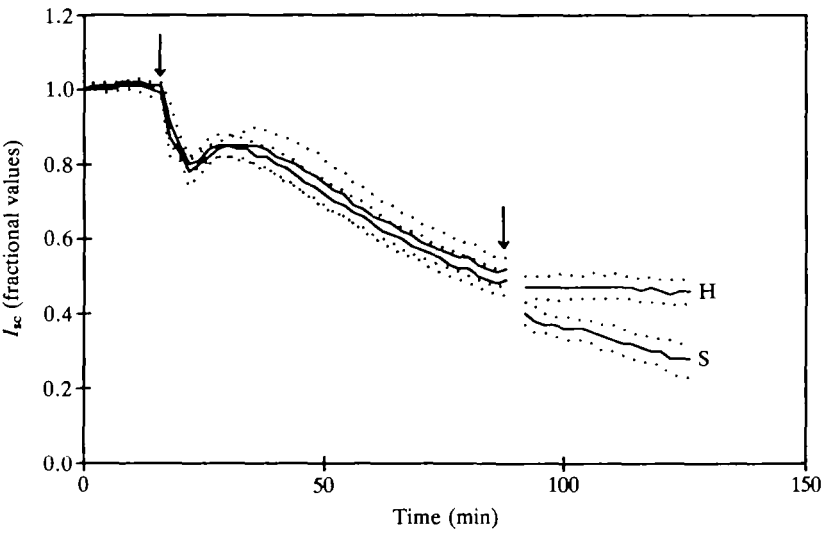

Fig. 7. Effect of DCCD on I_{sc}. Twelve preparations were studied. From the first arrow onwards the solutions were gassed with 95 % N_2+5 % CO_2. At the time indicated by the second arrow DCCD (50 $\mu g\,ml^{-1}$) was added to haemolymph (H) side of six preparations and the shell (S) side of other six. For an explanation of continuous lines and dots, see legend to Fig. 4.

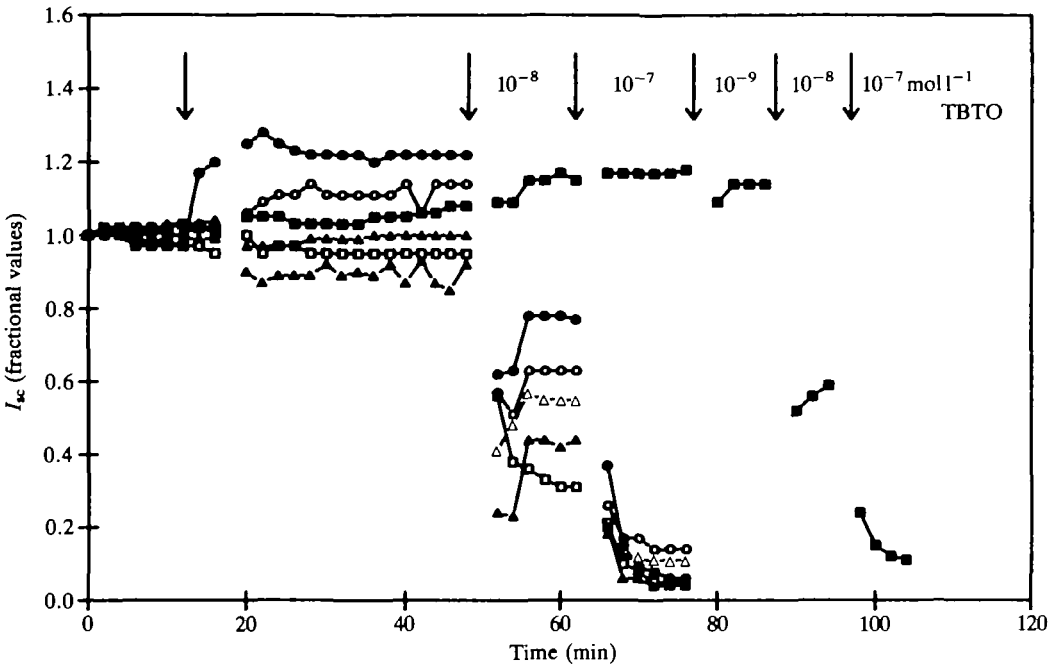

Fig. 8. Anion dependence of the effect of TBTO on I_{sc}. Six preparations were studied (one for each condition). At the time indicated by the first arrow, chloride (□) was replaced by isethionate (○), gluconate (●), sulphate (△) or thiocyanate (▲). After 30 min, five of the preparations were treated in succession (shell side) with 10 and 100 nmol l^{-1} TBTO. The sixth preparation (■), which had been kept as a control, was then treated with 1 nmol l^{-1}, 10 nmol l^{-1} and 100 nmol l^{-1} TBTA (also on the shell side).

direction and corresponded, at most, to less then 15 % of I_{sc} (Coimbra *et al.* 1988). We have now shown that, under short-circuit conditions, the rates of accumulation of acid in the shell solution and of base in the haemolymph solution are numerically equal to I_{sc}, when expressed in the same units. These observations are also consistent with the observation that the transepithelial net flux of [^{14}C]bicarbonate (towards the haemolymph compartment) is also equal to I_{sc} (Coimbra *et al.* 1988).

I_{sc} and the rates of delivery of acid and base by the epithelium have a similar oxygen dependence, and drugs such as iodoacetamide and DNP, which inhibit the rate of ATP production, are potent inhibitors of I_{sc} (Coimbra *et al.* 1988 and present work).

Transmembrane hydrogen ion movements are difficult to characterize, both because proton secretion is often indistinguishable from uptake of hydroxyl or bicarbonate ions and because the proton concentration can, by affecting the degree of ionization of proteins, modify the membrane proton permeability and some transport systems, in particular those responsible for the regulation upward

of intracellular pH (Vaughan-Jones, 1988). A further complication is that proton movements may be modulated by intracellular pH (Boron, 1986), membrane potential, hormones, growth factors and osmotic challenges (Grinstein and Rothstein, 1986).

In the OME the tight links between I_{sc} and the acidification and alkalinization of the bathing fluids, together with the sensitivity of these processes to diamox, suggest the involvement of bicarbonate at some stage. Three alternative mechanisms may explain our observations: (1) a transepithelial transport of acid or simply of protons towards the shell side; (2) a transepithelial transport of base (perhaps bicarbonate) in the opposite direction; (3) or the simultaneous extrusion of base across the basolateral membrane and of protons across the apical membrane. Since the flux of [^{14}C]bicarbonate, I_{sc} and the rates of alkalinization and acidification of the bathing fluids are sensitive to SITS and DIDS acting from the haemolymph side, an extrusion of bicarbonate towards the haemolymph in exchange for chloride (Boron, 1983) seems to be present. Such a process explains why intracellular chloride concentration rises when the basolateral side is bathed by a nominally bicarbonate-free solution (Coimbra *et al.* 1988) and entails a recirculation of chloride across the basolateral membranes. The low sensitivity of I_{sc} to the replacement of chloride on the haemolymph side may be due to a high affinity of the anion exchanger for chloride, as was observed in the turtle urinary bladder (Fischer *et al.* 1983).

The sensitivity of I_{sc} to bicarbonate and CO_2 on the shell side suggests that the absorption of bicarbonate across the apical membranes may be part of the process responsible for the I_{sc}. However, when the shell side is bathed by a solution nominally free of CO_2 and bicarbonate and adjusted to a pH of 7.2, currents of the order of 6 μA cm^{-2} can still be measured.

Alternatively, protons may be transported across the apical barrier towards the shell solution. Protons can be translocated across cell membranes by a variety of mechanisms, such as a Na^+/H^+ antiport, a K^+/H^+ pump or an electrogenic pump (Steinmetz and Andersen, 1982). The Na^+/H^+ antiport has been identified in a large number of cell systems from vertebrates and invertebrates and in particular in epithelia (Grinstein and Rothstein, 1986). The OME I_{sc} was sensitive to amiloride acting from the basolateral side, but, as the inhibition was very slow and as the replacement of sodium by choline in the bath of the same side has a small effect on I_{sc} (Coimbra *et al.* 1988), the contribution of this antiport (if it exists in this preparation) to the proton exchanges across the basolateral barrier is probably small. I_{sc} was insensitive to SITS, DIDS or amiloride added to the shell side or to the replacement of sodium or potassium on this side (Coimbra *et al.* 1988). It is thus unlikely that the acid secretion to the shell side was due to a Na^+/H^+ antiport, to a K^+/H^+ pump or to the uptake of bicarbonate across the apical membrane involving Na^+/HCO_3^- cotransport (Boron, 1986) and a recirculation of bicarbonate across the same barrier. Furthermore, since the resistance of the apical barrier is 4–10 times that of the basolateral barrier and is insensitive to the replacement of sodium, potassium or chloride by impermeant ions on the shell

side, it is unlikely that there is a direct link, or an electrical coupling, between the fluxes of protons and the fluxes of sodium, potassium or chloride ions across the apical barrier (Coimbra *et al.* 1988). In addition, I_{sc} is quickly and dramatically inhibited by DNP and by iodoacetamide, drugs that affect the rate of ATP production. We suggest that the acid secretion towards the extrapallial compartment is, at least in part, due to the operation of an electrogenic proton pump. Its location at the apical barrier is identical to that of turtle urinary bladder (Steinmetz and Andersen, 1982).

There is no specific inhibitor of the electrogenic proton pump found in epithelia. However, the inhibition of I_{sc} by DCCD (in anoxic conditions) and by TBTO from the shell side suggests the presence of a proton pump (Goffeau and Boutry, 1986) and the speed and nature of the TBTO effect indicate that it inhibits an electrogenic process by blocking a conductive channel rather than by creating an electrically silent anion exchange path (Linnet and Beechey, 1979). The observed sensitivity of I_{sc} to the pH of the shell solution is also compatible with the behaviour expected from an apical proton pump. Further characterization of this system requires measurements of intracellular pH under a variety of conditions.

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References

BORON, W. F. (1983). Transport of H^+ and of ionic weak acids and bases. *J. Membr. Biol.* **72**, 1–16.

BORON, W. F. (1986). Intracellular pH regulation in epithelial cells. *A. Rev. Physiol.* **48**, 377–388.

COIMBRA, J., MACHADO, J., FERNANDES, P. L., FERREIRA, H. G. AND FERREIRA, K. G. (1988). Electrophysiology of the mantle of *Anodonta cygnea*. *J. exp. Biol.* **140**, 65–88.

FISCHER, J. I., HUSTED, R. F. AND STEINMETZ, P. R. (1983). Chloride dependence of the HCO_3 exit step in urinary acidification by the turtle bladder. *Am. J. Physiol.* **245**, F564–F568.

GOFFEAU, A. AND BOUTRY, M. (1986). Three proton-pumping ATPases in yeast. *Microbiol. Sci.* **3**, 164–168.

GRINSTEIN, S. AND ROTHSTEIN, A. (1986). Mechanisms of regulation of the Na^+/H^+ exchanger. *J. Membr. Biol.* **90**, 1–12.

HUSTED, R. F., COHEN, L. H. AND STEINMETZ, P. R. (1979). Pathways for bicarbonate transfer across the serosal membrane of turtle urinary bladder: studies with a disulfonic stilbene. *J. Membr. Biol.* **47**, 27–37.

ISTIN, M. AND KIRSCHNER, L. B. (1968). On the origin of the bioelectrical potential generated by the freshwater clam mantle. *J. gen. Physiol.* **51**, 478–496.

LINNET, P. E. AND BEECHEY, R. B. (1979). Inhibitors of the ATP synthetase system. In *Methods in Enzymology*, vol. LV (ed. S. Fleischer and L. Packer), pp. 172–511. New York: Academic Press.

MACHADO, J., COIMBRA, J. AND SÁ, C. (1989). Shell thickening in *Anodonta cygnea* by TBTO treatments. *Comp. Biochem. Physiol.* **92C**, 77–80.

SANDERS, S. S., HAYNE, V. B., JR AND REHM, W. S. (1973). Normal H^+ rates in frog stomach in absence of exogenous CO_2 and a note on pH stat method. *Am. J. Physiol.* **225**, 1311–1321.

STEINMETZ, P. R. AND ANDERSEN, O. S. (1982). Electrogenic proton transport in epithelial membranes. *J. Membr. Biol.* **65**, 155–174.

TEORELL, T. (1951). The acid–base balance of the secreting isolated gastric mucosa. *J. Physiol., Lond.* **114**, 267–276.

VAUGHAN-JONES, R. D. (1988). Regulation of intracellular pH in cardiac muscle. In *Proton Passage Across Cell Membranes* (ed. G. Bock and J. Marsh), pp. 23–35. Chichester: John Wiley & Sons.