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EVIDENCE THAT K-DEPENDENT TRANSPORT COMPONENTS IN ADDITION TO Na-K ATPase ARE INVOLVED IN Na AND Cl EXCRETION IN MARINE TELEOST GILLS

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SUMMARY

Although Tl^+ ions can substitute for K^+ with a ten-fold higher affinity in activating the sodium pump in mullet red cells and mullet gill microsomal ATPase, they cannot mimic the effects of K^+ in stimulating Na^+ and Cl^- efflux across the gill. This is interpreted in the light of an additional K^+-sensitive transport component being involved.

INTRODUCTION

In marine teleosts, the branchial ionocytes are probably responsible for Na^+ and Cl^- exchange fluxes. These cells are very rich in Na–K ATPase activity suggesting that this enzyme may be involved in Na^+ excretion (e.g. Maetz & Bornancin, 1975). Using autoradiography with [$_3$H]ouabain, this enzyme has been localized in the basolateral membrane of the cell and its numerous invaginations, the tubular reticulum (Karnaky *et al.* 1976). Ouabain inhibits the Na–K ATPase more effectively from the serosal side, supporting the idea that the enzyme is located on the serosal face of the cell (Karnaky *et al.* 1976; Silva *et al.* 1977). This situation, similar to that in fresh water fish, favours the transport of Na^+ into the blood instead of excretion to the external medium. It therefore raises the problem of the exact role of the Na–K ATPase in marine teleosts, particularly regarding the extrusion of NaCl into the external medium.

Since the inhibition of Na–K ATPase by ouabain parallels the inhibition of Na^+ and Cl^- efflux, the enzyme seems to be involved in ionic excretion (Silva *et al.* 1977). Evidence for coupling between Na^+ and Cl^- fluxes had previously been provided by the fact that both fluxes are simultaneously activated by external K^+ ions or inhibited by SCN^- injection, when a marine fish is rapidly transferred from salt to fresh water (Epstein, Maetz & de Renzis, 1973; Maetz & Pic, 1975). On this basis it was suggested that the Cl^- pump is associated with Na^+/K^+ exchange.

As the Na–K ATPase of the basolateral membrane faces the internal medium, plasma K^+ would be high enough to activate the enzyme irrespective of the K^+ level

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in the external medium. Therefore, the activation of ionic excretion by *external* K^+ suggests the involvement of other mechanisms, complementary to the sodium pump in ionic extrusion across the gill epithelium in sea water.

In the present work, we try to confirm the proposal that other systems are involved by comparing the effects of external Tl^+ with K^+. Thallium (Tl^+) can substitute for K^+ with a 10- to 100-fold greater affinity when activating a rabbit kidney Na–K ATPase preparation (Britten & Blank, 1968) or human red cell sodium pump activity (Cavieres & Ellory, 1974). In a recent study (Bell, Tondeur & Sargent, 1977), the seawater-adapted eel gill Na–K ATPase has been shown to be very similar in its ionic affinities to the kidney and brain Na–K ATPase preparations. It is therefore interesting to see whether the Tl^+ ion can mimic the K^+ effect on Na^+ and Cl^- fluxes in marine teleosts, to determine if activation of Na–K ATPase is directly involved in this stimulation.

MATERIAL AND METHODS

Fish. All experiments were carried out on juvenile mullet, *Mugil capito*, weighing 5–20 g. The fish were maintained in sea water for at least 3 weeks before use.

Blood. Blood was taken by cardiac puncture into a heparinized saline medium containing 5 mM Na EDTA. The saline was NaCl 140 mM, KCl 5 mM, Tris 20 mM, pH 7·5 at 20 °C. The cells were washed three times by centrifugation (2500 $g \times 5$ min) and loaded with ^{24}Na by incubation for 2 h in a medium containing NaCl 150 mM (+$^{24}NaCl$, 100 μCi/ml), glucose 10 mM, Tris 10 mM, pH 7·5 at 20 °C. The cells were washed five times in ice cold $MgCl_2$ 110 mM, Tris 10 mM and dispensed into a tube to measure ^{24}Na efflux. Cells were suspended at 3% hematocrit in a solution similar in composition to the loading medium, but containing either KCl 10 mM, or $TlNO_3$ 0·2 or 1 mM. Samples were run in triplicate, together with a series containing 1 mM-ouabain. At time intervals of 5, 25 and 45 min the samples were centrifuged and aliquots of the supernatant counted for ^{24}Na by Cerenkov radiation in a β-counter. Total ^{24}Na counts in a deproteinized cell preparation were also determined, and the efflux calculated as a rate constant.

Na-K ATPase. A microsomal ATPase preparation was made from *Mugil* gill scrapings by homogenization and differential centrifugation following the methods previously used for intestine (Ellory & Smith, 1969) and fish gill (Bornancin & de Renzis, 1972). The final pellet was treated with sodium deoxycholate to a concentration of 1·5 mM and ouabain-sensitive ATPase activity assayed at 25 °C for 15 min with incubation in a medium containing 150 mM-NaCl, 2·5 mM-Mg ATPase, 15 mM-Tris, pH 7·5, with various concentrations of KCl or $TlNO_3$ added. The reaction was stopped by the addition of TCA to a final concentration of 5% and the inorganic phosphate liberated assayed as previously described (Ellory & Maher, 1977).

Gill potential and flux measurements. Measurements of the transgill potential difference, and continuously monitored ^{24}Na and ^{36}Cl efflux were made following 'rapid transfer' of whole conscious fish from seawater to freshwater. The experimental techniques were as previously described (Maetz & Pic, 1975, 1977; Pic, 1978).

Table 1. *The effect of external K^+ and Tl^+ concentrations on the ouabain-sensitive Na^+ efflux from mullet red cells. Means* ± 1 S.E.M. ($n = 4$)

	Rate constant for Na efflux (h^{-1})		
	Control	Ouabain	Difference
150 mM-NaCl	0·135 ± 0·006	0·114 ± 0·004	0·021 ± 0·007
+10 mM-KCl	0·215 ± 0·004	0·128 ± 0·005	0·087 ± 0·006
+0·2 mM-$TlNO_3$	0·208 ± 0·001	0·132 ± 0·003	0·076 ± 0·011
+1 mM-$TlNO_3$	0·224 ± 0·008	0·121 ± 0·003	0·103 ± 0·009

Table 2. *The effect of K^+ and Tl^+ concentrations on the ouabain-sensitive ATPase activity of mullet gill microsomes: means* ± 1 S.E.M. ($n = 4$)

	ATPase activity (μmol P_i mg^{-1} protein.h^{-1} at 25 °C)		
	Control	Ouabain	Difference
150 mM-NaCl	5·8 ± 0·4	5·3 ± 0·3	0·5 ± 0·5
+10 mM-KCl	7·4 ± 0·3	4·9 ± 0·2	2·5 ± 0·4
+0·2 mM-$TlNO_3$	6·8 ± 0·2	4·6 ± 0·2	2·2 ± 0·3
+1 mM-$TlNO_3$	7·3 ± 0·3	4·7 ± 0·4	2·6 ± 0·5

RESULTS

The data presented in Table 1 indicate that Tl^+ can substitute equally effectively and at lower concentrations for K^+ in supporting ouabain-sensitive Na^+ efflux. These cells showed a ouabain-sensitive Na^+/Na^+ exchange in the absence of K^+ (e.g. Garrahan & Glynn, 1967) but K^+ 10 mM or Tl^+ 1 mM gave a significant activation. Similar results for gill ATPase activity (Table 2) indicate that, in line with other tissues so far studied, the sodium pump in mullet cells will accept Tl^+ as an effective substitute for potassium.

Table 3 presents the results for Na^+ and Cl^- effluxes and P.D. measurements. The absolute values are very similar to previous observations (Maetz & Pic, 1975, 1977). Addition of 10 mM-KCl in freshwater causes an increase in the Na^+ (200%) and Cl^- (100%) effluxes and a depolarization of the gill. In contrast the addition of 0·75 mM-$TlNO_3$ does not significantly alter the transgill P.D. or Na efflux. A small increase in Cl^- efflux is on the limit of significance ($P \sim 0{\cdot}05$). Thus there is no action *in vivo* of a Tl concentration sufficient to activate the sodium pump in erythrocytes and microsomal Na–K ATPase of the gill *in vitro*.

DISCUSSION

The difference between Tl^+ and K^+ in affecting efflux across the gills could be mediated in at least three possible ways. First, neither K^+ nor Tl^+ could be reaching the Na–K ATPase, but rather the external K^+ only acts on apical mechanisms independent of the Na–K ATPase; alternatively, only K^+ reaches the Na–K ATPase, Tl^+ being prevented from access to the basolateral membranes by diffusion through the cell. Finally, it is possible that both cations are activating the Na–K ATPase

Table 3. *Effects of K^+ and Tl^+ added to freshwater (FW) on Na^+ and Cl^- effluxes and transgill P.D., of* Mugil capito *adapted to seawater (SW)*

	SW	FW	FWK	(FWK–FW)
$J^{Na+}_{out\,(15)}$	6763 ± 719	1155 ± 209	3839 ± 441	+2684 ± 288*
$J^{Cl-}_{out\,(15)}$	3292 ± 337	691 ± 131	1290 ± 182	+599 ± 143*
$V_{ext} - V_{int}$ (25)	−22·0 ± 1·62	+41·1 ± 2·92	+17·5 ± 1·24	−23·6 ± 2·05*
	SW	FW	FWTl	(FWTl–FW)
$J^{Na+}_{out\,(8)}$	6753 ± 801	1667 ± 263	2023 ± 1020	+356 ± 165**
$J^{Cl-}_{out\,(5)}$	3031 ± 980	1045 ± 352	1208 ± 321	+163 ± 59
$V_{ext} - V_{int}$ (7)	−15·4 ± 1·91	+23·0 ± 5·21	+19·9 ± 4·69	−3·1 ± 2·19**

J_{out}: Na^+ or Cl^- effluxes in μequiv h^{-1}. 100 g^{-1}; $V_{ext} - V_{int}$: transgill P.D. in mV. All data are expressed as mean ± S.E.; number of experiments in parentheses. Statistics; paired differences: * $P < 0{\cdot}001$; comparison of Tl^+ and K^+ effects: ** $P < 0{\cdot}001$. Since Tl^+ is around ten times more efficient than K^+ to stimulate gill Na^+ ATPase or erythrocyte Na^+ pump of *Mugil*, we used as final concentration for Tl^+: 0·75 m-equiv l^{-1} and for K^+: 10 m-equiv l^{-1}.

and increasing Na^+ (and perhaps indirectly, Cl^-) concentration in the tubular reticulum, however there is a K^+-dependent step subsequent to this, at which Tl^+ cannot substitute effectively. Both the first and second hypotheses seem unlikely, since lanthanum added to the external medium penetrates quickly through short leaky junctions between the ionocytes (Sardet, Pisam & Maetz, 1978). This indicates a possible route, communicating with the tubular reticulum, for the entry of Tl^+ and K^+ ions. Moreover, the first hypothesis is inconsistent with the effect of ouabain on ionic fluxes (Silva *et al.* 1977). In the second hypothesis it is difficult to reconcile the location of Na–K ATPase on the basolateral membrane with sodium extrusion since the pumps are wrongly directed. In the third hypothesis, activation of Na–K ATPase is necessary for Na^+ and Cl^- excretion, which is compatible with the inhibition of effluxes by ouabain, but this activation is not enough for ionic extrusion.

For Na^+ extrusion, several authors have suggested that the transgill electrical potential effectively compensates the chemical gradient in seawater and that the increased Na^+ efflux after K^+ addition results from a further gill depolarization (Potts & Eddy, 1973; Greenwald, Kirschner & Sanders, 1974; Kirschner, Greenwald & Sanders, 1974). However, activation of Na^+ efflux by K^+ is respectively two and three times greater than one would expect from depolarization in *Dormitator maculatus* (Evans, Carrier & Bogan, 1974) and *Mugil* (Maetz & Pic, 1975, 1977). Further the Cl^- secretion in seawater occurs against an electrochemical gradient, and the Cl^- efflux is in an opposite direction from that predicted from the potential change. Thus the alteration in transgill electrical potential induced by K^+ will only partly explain an increased Na^+ efflux and cannot be responsible for the Cl^- efflux component. It is therefore impossible that the difference between K^+ and Tl^+ effects can be only attributed to their different effects on transgill P.D.

The magnitude of Na^+ and Cl^- gill exchanges in seawater, and the K^+-activation mechanism depends on the presence of Ca^{2+} (and Mg^{2+}) in the external solution (Bornancin, Cuthbert & Maetz, 1972; Isaia & Masoni, 1976). Since Ca^{2+} is an inhibitor of Na–K ATPase (Fahn, Koval & Albers, 1966; Tobin *et al.* 1973) this involvement of Ca^{2+} is a further pointer to the involvement of other mechanisms in addition to the Na^+ pump in this effect. Finally, the inhibition of ionic excretion and

K^+ activated mechanisms by colchicine (Maetz & Pic, 1977) suggests that microtubules, cell polarity and motility may play a role in Na^+ and Cl^- excretion in seawater.

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REFERENCES

Bell, M. V., Tondeur, F. & Sargent, J. R. (1977). The activation of sodium plus potassium ion-dependent adenosine triphosphatase from marine teleost gills by univalent cations. *Biochem. J.* **163**, 185–187.

Bornancin, M., Cuthbert, A. W. & Maetz, J. (1972). The effects of calcium on branchial sodium fluxes in the sea water adapted eel, *Anguilla anguilla*. *J. Physiol.* **222**, 487–496.

Bornancin, M. & de Renzis, G. (1972). Evolution of the branchial sodium outflux and its component, especially the Na/K exchange and the Na–K dependent ATPase activity during adaptation to sea water in *Anguilla anguilla*. *Comp. Biochem. Physiol.* **43**A, 577–591.

Britten, J. S. & Blank, M. (1968). Thallium activation of the (Na^+–K^+) activated ATPase of rabbit kidney. *Biochim. biophys. Acta* **159**, 160–166.

Cavieres, J. D. & Ellory, J. C. (1974). Thallium and the sodium pump in human red cells. *J. Physiol.* **243**, 243–266.

Ellory, J. C. & Maher, P. (1977). A change in the internal affinity of LK goat red-cell sodium pumps induced by high pH. *Biochem. biophys. Acta* **471**, 111–117.

Ellory, J. C. & Smith, M. W. (1969). Deoxycholate stimulation of goldfish intestinal (Na^+–K^+)-ATPase and its relation to digoxin binding. *Biochim. biophys. Acta* **193**, 137–145.

Epstein, F. H., Maetz, J. & de Renzis, G. (1973). On the active transport of chloride by the teleost gill. Inhibition by thiocyanate. *Am. J. Physiol.* **224**, 1195–1199.

Evans, D. H., Carrier, J. C. & Bogan, M. B. (1974). The effect of external potassium ions on the electrical potential measured across the gills of the teleost *Dormitator maculatus*. *J. biol. Chem.* **61**, 277–283.

Fahn, S., Koval, G. J. & Albers, R. W. (1966). Sodium–potassium-activated adenosine triphosphatase of *Electrophorus* electric organ. *J. biol. Chem.* **241**, 1882–1889.

Garrahan, P. J. & Glynn, I. M. (1967). Factors affecting the relative magnitudes of the sodium–potassium and sodium–sodium exchanges catalysed by the sodium pump. *J. Physiol.* **192**, 189–216.

Greenwald, L., Kirschner, L. B. & Sanders, M. (1974). Sodium efflux and potential difference across the irrigated gill of sea water adapted rainbow trout *Salmo gairdneri*. *J. gen. Physiol.* **64**, 135–147.

Isaia, J. & Masoni, A. (1976). The effects of calcium and magnesium on water and ionic permeabilities in the sea water adapted eel, *Anguilla anguilla* L. *J. comp. Physiol.* **109**, 221–223.

Karnaky, K. J., Kinter, L. B., Kinter, W. B. & Stirling, C. E. (1976). Teleost chloride cell. II. Autoradiographic localization of gill Na, K-ATPase in killifish *Fundulus heteroclitus* adapted to low and high salinity environments. *J. cell Biol.* **70**, 157–177.

Kirschner, L. B., Greenwald, L. & Sanders, M. (1974). On the mechanism of sodium extrusion across the irrigated gill of sea water adapted rainbow trout (*Salmo gairdneri*). *J. gen. Physiol.* **64**, 148–165.

Maetz, J. & Bornancin, M. (1975). Biochemical and biophysical aspects of salt excretion by chloride cells in teleosts. *Fortschr. Zool.* **23**, 322–362.

Maetz, J. & Pic, P. (1975). New evidence for a Na/K and Na/K exchange carrier linked with the Cl^- pump in the gill of *Mugil capito* in sea water. *J. comp. Physiol.* **102**, 85–100.

Maetz, J. & Pic, P. (1977). Microtubules in the 'chloride cell' of the gill and disruptive effects of colchicine on the salt balance of the sea water adapted *Mugil capito*. *J. exp. Zool.* **199**, 325–337.

Pic, P. (1978). A comparative study of the mechanism of Na^+ and Cl^- excretion by the gill of *Mugil capito* and *Fundulus heteroclitus:* effects of stress. *J. comp. Physiol.* (In the Press.)

Potts, W. T. W. & Eddy, F. B. (1973). Gill potentials and sodium fluxes in the flounder *Platichthys flesus*. *J. comp. Physiol.* **87**, 29–48.

SARDET, C., PISAM, M. & MAETZ, J. (1978). The surface epithelium of teleostean fish gills. Cellular and junctional adaptations of the chloride cell in relation to salt adaptation. *J. cell Biol.* (In the Press.)

SILVA, P., SOLOMON, R., SPOKES, K. & EPSTEIN, F. H. (1977). Ouabain inhibition of gill Na–K–ATPase: relationship to active chloride transport. *J. exp. Zool.* **199**, 419–426.

TOBIN, T., AKERA, T., BASKIN, S. I. & BRODY, T. M. (1973). Calcium ion and sodium- and potassium-dependent adenosine triphosphatase: its mechanism of inhibition and identification of the $E_1 - P$ intermediate. *Molec. Pharmacol.* **9**, 336–349.