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THE EFFECTS OF CALCIUM ON BRANCHIAL SODIUM FLUXES IN THE SEA-WATER ADAPTED EEL, *ANGUILLA ANGUILLA*, L.

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SUMMARY

1. The sodium influx through the gills of eels placed in calcium-free sea water for 15 hr was double that of controls. The effect was reversed in 1 hr by addition of calcium.

2. The total sodium outflux through the gills of fish placed in calcium-free sea water for 15 hr was double that of controls. The effect was only partially reversed in 15 hr by addition of calcium.

3. The passive outflux component of the total outflux was increased fourfold when calcium was removed and was restored to normal in 15 hr by addition of calcium. The active (exchange) outflux component of the total outflux was halved by calcium removal and increased above normal following calcium addition.

4. The inability of calcium to restore the total outflux to normal within 15 hr in calcium-depleted fish, together with the raised plasma sodium concentration at this time, suggests that the raised outflux is caused by homoeostatic mechanisms, rather than permeability changes in the gill epithelium.

INTRODUCTION

In a recent paper we have described the effects of calcium removal on the branchial fluxes in a fresh-water teleost, *Carassius auratus*, L. Calcium removal was found to cause a reversible increase in sodium influx in this species (Cuthbert & Maetz, 1972). We have now turned our attention to the effects of calcium on sodium fluxes through the gills of the sea-water-adapted eel, *Anguilla anguilla*, L. to see if a similar situation obtains. In

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sea water there is 10 mM calcium compared to only 1·5 mM in fresh water; however, the sodium/calcium ratio is much greater in sea water since there is 500 m-equiv sodium/l. in the latter compared to only 100 μ equiv/l. in fresh water. Thus if a sodium-calcium interaction occurs the removal of calcium may have more dramatic effects on sodium balance in sea-water animals. Although no detailed studies of the effect of calcium on electrolyte balance in the eel have been reported it is known that eels die within a few days if calcium is removed from the sea water (Sharratt, Bellamy & Chester Jones, 1964). Potts & Fleming (1971) have recently shown in another teleostean, *Fundulus kansae*, that removal of calcium from sea water produces an increase in sodium outflux of 130%.

The problems of measuring sodium fluxes in gills in sea water are more difficult than in fresh water, since it is not possible to measure accurately the disappearance of sodium from the external milieu when this is sea water containing 500 mM sodium. Consequently influx and outflux through gills must be measured in separate experiments in sea water. In addition, sodium outflux through the gills proceeds along several pathways and by different mechanisms (Maetz, 1971) and these pathways may be affected differently by calcium removal.

The sea-water animal is hyposmotic to the environment and so water is lost to the environment through the gills. This loss in volume is compensated by drinking. The absorption of salt from the gut provides the driving force for water absorption and the extra salt imbibed by the fish is eliminated by active extrusion through the gills. Sodium losses through the kidneys and gut are negligible (Maetz, 1971).

In this paper we have studied the effect of calcium removal and addition to the external medium on both sodium influx and outflux. Both influx and outflux were increased in the absence of external calcium.

METHODS

Eels were caught in fresh water in the Rhône valley and shipped to the laboratory. They were placed initially in fresh water and then adapted to sea water. All the animals used had been in sea water for at least 3 weeks at 16–18° C before use. The fish used for flux experiments weighed between 100–200 g.

Measurement of sodium outflux

Sodium outflux through the gills was measured following the intraperitoneal injection of ^{24}Na. ^{24}Na (as sulphate) 200 μc/100 g fish was given and the animal placed in a plastic mesh tube in an aquarium containing running sea water. Thirty to 45 min were allowed for distribution of the isotope. At the end of this time the animals plus mesh were placed, in quick succession in four aquaria containing known volumes of fluid. The bathing media and times of exposure were as follows: (i) sea water (SW 1) 10 min (ii) fresh water (FW) 6 min (iii) fresh water with 10 mM potassium chloride

(FWK) 6 min and (iv) sea water (SW 2) 10 min. The animals were given a quick rinse (for 2 min) in fresh water between each exposure.

The order was varied between (ii) and (iii) in alternate experiments. Outflux of the isotope from the fish proceeds exponentially with a half-time of 2·5–3·0 hr; however, since the experimental periods were short the radioactive outflux remained constant during the measurements. The outflux during each exposure was monitored on a β or γ counter which was in closed circuit with the aquarium. A pump and a cryostat were used to control the temperature at 17° C and to circulate the fluid. The amount of radioactivity circulating through the counter each minute was shown on a digital print out (Tanguy, 1970). At the end of the experiment a blood sample was removed from the caudal artery for counting and flame photometry of the plasma. At the end of a series of experiments a solution of ^{24}Na was pumped through all the counters so that their relative efficiency could be measured. The radiosodium outfluxes from a

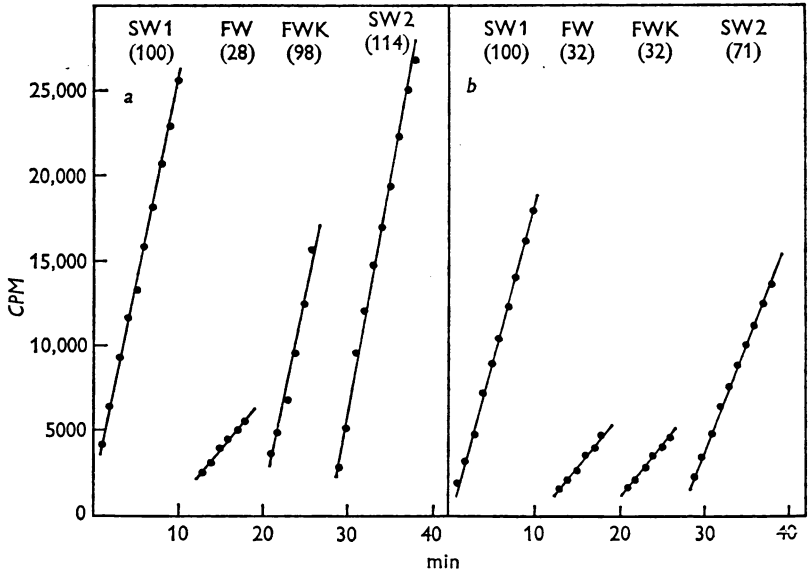

Fig. 1. Outflux of ^{24}Na (*CPM*) versus time in different media. The fish was exposed to SW for 10 min (SW 1), FWK for 6 min, FW for 6 min and finally sea water for 10 min (SW 2). The fish was washed for 2 min in fresh water between each flux determination. The results shown in *a* refer to fish in ASW, while *b* shows fluxes for the same fish after exposure to calcium-free ASW. The figures in parentheses indicate the flux rates expressed with respect to the flux in SW 1. All fluxes have been corrected for the different counting efficiency of the various flow counters used. Note that in *a* the fish showed no reduction of outflux in the SW 2 period following two exposures to hyposaline media. This behaviour was seen in three out of fourteen fish studied. After exposure to calcium-free ASW (*b*), the fish showed the more usual behaviour pattern with a reduction in outflux in the SW 2 period compared to SW 1. It is important to realize that the results illustrated show only relative and not actual fluxes. The actual flux during the SW 1 period in *a* was 1018 μ equiv/100 g.hr whereas in *b* the flux during the SW 1 period was 3830 μ equiv/100 g.hr.

typical fish are shown in Fig. 1. The activities have been corrected to take account of the relative efficiency of the counters.

Outfluxes in sea water represent sodium loss by either exchange diffusion, passive flow or by active exchange for potassium (Maetz, 1969, 1971). The outflux in the second sea water period (SW 2) was usually less than that in the first sea water period (Table 1, Fig. 1*b*). It must be emphasized that the outflux during SW 1 is in a fully sea-water-adapted fish, while in the SW 2 period the fish may have begun to show adaptation to hyposaline media following the two fresh-water exposures. This adaptation is characterized by a reversible reduction of the sodium outflux upon return to sea water. The shorter the exposure to hyposaline media, the smaller the reduction and the faster the recovery (Motais, 1967). In hypophysectomized fish the adaptive changes do not occur (Maetz, 1970) indicating involvement of the hypophysis in the adaptive changes. The relative magnitudes of the fluxes in FW and FWK have been calculated assuming that adaptation proceeds linearly between SW 1 and SW 2 and the fluxes have been expressed with respect to $\frac{1}{2}$(SW 1 + SW 2).

The flux occurring after transfer to fresh water is a measure of passive outflux since very little sodium is available for exchange diffusion, and no potassium is available for active exchange. Similarly, the outflux in FWK is a measure of the active extrusion of sodium in exchange for potassium plus passive outflux. The value of the exchange flux is obtained by subtracting the FW flux from the FWK flux. It is not claimed that these fluxes are equal to the passive and active exchanges occurring in sea water, but rather an indication of such exchanges and a means of testing the relative importance of the separate components of the total outflux following various manipulations such as calcium removal.

The outflux during the SW 1 and SW 2 periods was calculated from

$$f_{out} = \frac{CPM_{aq} \times 6 \times V}{RAS_c \times W/100}\ \mu\text{-equiv Na/100 g.hr},$$

where CPM_{aq} is the activity in 1 ml. of aquarium fluid at the end of the 10 min outflux period and V is volume of the aquarium fluid (usually 1 l. per 150 g fish). RAS_c is the mean specific activity of the plasma which was obtained from the specific activity of the plasma at the end of the experiment plus a correction for the activity lost between the end of the experiment and the middle of either the SW 1 or SW 2 flux period.

Measurement of sodium influx and sodium space

Sodium influx was measured simultaneously in batches of six eels. To do this the eels were weighed and placed in 4 l. sea water at 17° C, bubbled with air. At the time zero (t_0) ^{24}Na (as sulphate), 5 mc was added to the aquarium. Exactly 1 hr later (t_1) the eels were transferred to fresh water containing MS 222 (1·5 g/l.). As soon as the fish were anaesthetized (2 min) samples of blood were taken from the candal artery of each fish into heparinized syringes. The fish were then placed in individual aquaria containing exactly 1 l. sea water, bubbled with air and left for 20 hr.

Samples of blood plasma were separated by centrifugation and used for ^{24}Na counting and flame photometry against external sodium standards. Samples of aquarium fluid were also taken during the initial exposure to the isotope and used for counting and flame photometry. In addition samples of aquarium fluid were taken for counting after 20 hr, at which time the label was uniformly distributed and the internal and external specific activities were identical.

The sodium influx through the gills and the sodium space were calculated as follows.

The total activity, T_1, lost during 20 hr plus an allowance for that remaining in the fish was calculated from

$$T_1 = CPM_{aq} \times (1000 + \tfrac{1}{9}W)$$

(where CPM_{aq} is the activity in 1 ml. aquarium fluid after 20 hr and W is the weight of the fish in grams). The allowance for the activity remaining in the fish ($W/9$) assumes a sodium space of 33 % and a plasma sodium concentration of 1/3 SW. Also T_2, the radiosodium lost by backflux during the 1 hr exposure to the isotope, was calculated from

$$T_2 = f_{out} \times W/100 \times RAS_{int}/2,$$

where f_{out} is the mean outflux in sea water in identical conditions (determined in previous outflux experiments) and RAS_{int} is the internal specific activity defined as [activity in 1 ml. plasma]/[Na] plasma at the end of the influx experiment.

The sodium influx, f_{in}, was found from

$$f_{in} = \frac{[T_1 + T_2]}{RAS_{ext} \times W/100} (\mu\text{-equiv/100 g.hr})$$

where RAS_{ext} is the external specific activity defined as [activity in 1 ml. external fluid during exposure to isotope]/[Na] external. The sodium space was found from

$$\frac{[T_1 + T_2]100}{CPM_{plasma} \times W} (\%),$$

where CPM_{plasma} was the activity in 1 ml. plasma.

In some experiments the animals failed to recover from the anaesthetic and the measurement of ^{24}Na loss over 20 hr could not be made. In these instances influx was calculated making use of the mean sodium space for the survivors of the group. T_1 was then calculated from

$$CPM_{plasma} \times \text{mean sodium space} \times W/100.$$

The calculation of influx was then made as described above.

To examine the effects of calcium addition or removal on sodium influx the fish were treated in the appropriate way before influx measurements commenced.

The solutions used in the experiments were as follows

(i) Sea water (SW).

(ii) Artificial sea water (ASW) containing NaCl, 500 mM; $MgSO_4$, 30 mM; $MgCl_2$, 20 mM; KCl, 10 mM; Na_2HPO_4, 1 mM and $CaCl_2$, 10 mM.

(iii) Calcium-free artificial sea water (ASW − Ca). As above without added calcium.

(iv) Fresh water (FW) containing approximately 100 μM-Na, 1·5 mM-Ca, 1 mM-Mg and 100 μM-Cl.

(v) Fresh water with potassium (FWK). Fresh water plus 10 mM-KCl.

(vi) Deionized water (DW).

(vii) Deionized water with potassium (DWK). Deionized water with 10 mM-KCl.

DW and DWK were used in calcium-free experiments instead of FW and FWK.

RESULTS

The effects of calcium on sodium outflux and its components

The characteristics of the sodium outflux in sea-water eels was determined in fourteen separate experiments. A summary of the findings is given in Table 1. The outflux in the first experimental period (SW 1) was 984 ± 77 μ-equiv/100 g.hr but fell to 694 ± 81 μ-equiv/100 g.hr during the second sea-water exposure (SW 2) due to adaptive changes caused by intervening exposure to fresh water. The average plasma sodium concentration in these animals was $155{\cdot}2 \pm 2{\cdot}3$ m-equiv/l. This mean value is similar to the average plasma sodium concentration of all the control fish used in this work (see Table 4).

TABLE 1. Detailed summary of sodium outflux in control fish

Experimental fluxes				Derived values		
SW 1	FWK	FW	SW 2	$\frac{1}{2}$(SW 1 + SW 2)	FWK–FW	SW 1/SW 2
984 ± 77	661 ± 54	194 ± 17	694 ± 81	839 ± 71	467 ± 50	$1{\cdot}43 \pm 0{\cdot}18$

Fluxes are given as μ-equiv/100 g.hr. The results are for fourteen fish, means $\pm$ S.E. are given. The values for the SW 1 and SW 2 outflux are significantly different ($P < 0{\cdot}02$).

TABLE 2. The effect of calcium on sodium outflux and its components

	Outflux μ-equiv/100 g.hr			
Medium	Total outflux	P	FWK outflux	P
SW	839 ± 71 (14)	—	661 ± 54 (14)	—
ASW–Ca	1967 ± 437 (5)	$< 0{\cdot}001$	1019 ± 154 (5)	$< 0{\cdot}02$
(ASW–Ca) + Ca	1479 ± 323 (5)	$< 0{\cdot}01$	965 ± 269 (5)	N.S.
	FW outflux	P	FWK–FW	P
SW	194 ± 17 (14)	—	467 ± 50 (14)	—
ASW–Ca	765 ± 178 (5)	$< 0{\cdot}001$	254 ± 64 (5)	$< 0{\cdot}01$
(ASW–Ca) + Ca	213 ± 116 (5)	N.S.	752 ± 243 (5)	N.S.

The P values refer to comparisons with fish kept in sea water. Fish were depleted of calcium by placing in ASW without calcium for 15 hr. The values for the calcium repleted fish were determined 15 hr after addition of calcium. The numbers in parentheses indicate the number of fish used.

The mean flux of 839 μ-equiv/100 g.hr reported here is close to the value given by Rankin (in Mayer & Nibelle, 1970) and intermediate to the values found by Mayer and Nibelle (1970). The value of the leak observed after transfer to fresh water (194 μ-equiv/l.100 g.hr) is about 23% of the total outflux and similar to that reported by Motais (1967). The addition of

10 mM potassium to fresh water induces a 467 μ-equiv increase of the sodium outflux indicating that in the absence of external sodium, Na/K exchange may account for as much as 55 % of the total outflux, a value similar to that reported by Maetz (1969) for the flounder.

When fish were placed in ASW without calcium for 15 hr there was a significant increase in the total outflux to 1967 μ-equiv/100 g.hr (Table 2). However, not all components of the outflux were increased, in fact the active extrusion of sodium was depressed, while the leak observed after transfer to fresh water was increased (Table 2).

When fish that had been exposed to calcium-free conditions for 15 hr were re-exposed to calcium (10 mM) for a further 15 hr the normal state was not completely restored. The total outflux remained elevated and significantly different from the control value. At this time the leak was normal, while the outflux due to active exchange was increased. However, the increase in active exchange did not reach significance due to the wide scatter of values.

The effects of calcium on sodium influx and sodium space

The values for sodium influx in twenty-four fish under various conditions are shown in Table 3. The values obtained were 614 ± 90 μ-equiv/100 g.hr in sea water and 765 ± 139 μ-equiv/100 g.hr in artificial sea water. These values are not significantly different. The values of the influx in SW or ASW are lower than those for outflux. This is to be expected since there is a net sodium loss through the gills in sea-water fish, equivalent to the sodium gained by the animal drinking the medium.

When eels were placed in ASW without calcium for 15 hr sodium influx increased twofold. At the same time the sodium space increased from 22·3 % to 29·5 %. It must be emphasized that the determination of sodium space and influx was made after the fish had been exposed to ^{24}Na for exactly 1 hr, while all the available sodium space may not be filled at this time. The increase in sodium space after removal of calcium may therefore represent a real increase in space or an increase in the rate at which the space is filled. These two possibilities were explored by exposing fish to ASW without calcium for 15 hr and then adding calcium (10 mM) 1 hr before the determination of influx. The procedure reduced the influx and sodium space to normal. Since it was known from outflux experiments that the total outflux remained high even 15 hr after re-exposure to calcium, indicating the load on the outflux mechanisms remained high at this time, the ability of calcium to reduce influx and space to normal within 1 hour shows that calcium increases the rate at which the space is filled rather than causing an increase in real space.

Plasma sodium levels

Values of plasma sodium concentrations in the fish used for influx and outflux measurements are given in Table 4. Fish exposed to calcium-free solutions for 15 hr showed an 8 % increase in plasma sodium concentration. The increase in sodium concentration in individual fish was very variable, as indicated by the large standard error and range compared to the controls. Addition of calcium (10 mM) to calcium-deprived fish for 15 hr (five fish) or 1 hr (six fish) produced no significant change in the plasma sodium concentration, although the plasma sodium concentration was now significantly different from the control level, since the scatter was less.

TABLE 3. The effect of calcium on sodium influx and sodium space

Medium	Influx (μ-equiv/100 g.hr)	P	Sodium space %	P
SW	614 ± 90 (6)	N.S.	21·2 ± 2·2 (5)	N.S.
ASW	765 ± 139 (6)	—	22·3 ± 1·4 (3)	—
ASW–Ca	1454 ± 203 (6)	< 0·02	29·5 ± 1·8 (4)	< 0·02
(ASW–Ca) + Ca	771 ± 129 (6)	N.S.	21·8 ± 1·2 (5)	N.S.

P values refer to comparisons with fish in ASW; N.S., not significant. Fish were deprived of calcium for 15 hr before influx was measured. Calcium repleted fish were exposed to calcium for only 1 hr before influx was measured. Values in parentheses refer to the number of fish used.

TABLE 4. Plasma sodium concentrations in relation to various treatments

Plasma sodium m-equiv/l.		
Control	Ca-free	Ca-return
152·9 ± 1·6 (26)	164·3 ± 8·2 (11)	166·6 ± 6·3 (11)
Range 131·3 ± 167·3	Range 142·5 ± 241·8	Range 153–224·5
	P < 0·2	P < 0·05

Calcium-free fish has been deprived of calcium for 15 hr. The calcium return group were fish that had been deprived of calcium for 15 hr and then returned to calcium containing solutions for 15 hr (five fish) or 1 hr (six fish).

The figures in parentheses indicate the number of animals used. P values refer to comparisons with control fish.

DISCUSSION

It is convenient to discuss separately the several types of fluxes which have been measured in this work in relation to the effects of calcium.

Influx was increased twofold by placing fish in calcium-free solutions for 15 hr. The increase in influx was readily reversed by adding calcium, the reversal being complete within one hour. These results are similar to those found for the fresh-water teleost, *Carassius* (Cuthbert & Maetz, 1972), where a readily reversible increase in influx also followed on calcium removal. The present findings strengthen the view that calcium

is important in controlling the permeability of gill membranes to monovalent cations, as indeed it does in many other varieties of cell membrane (for references see Cuthbert & Wong, 1971). Sodium influx through the gills of sea-water fishes is thought to be a passive process, the sodium moving down its concentration gradient. What is not known is whether influx occurs exclusively through the salt-secreting cells (chloride-cells), or the respiratory epithelium, or even between the cells. Since calcium is important for establishing and maintaining intercellular contacts (Loewenstein, 1966) part of the extra-influx may be between the cells.

Sea-water fish excrete sodium to the environment through the gills to maintain ionic balance. Outflux is thus partly an active process since it is necessary to move sodium against a steep chemical gradient. Removal of calcium from the external milieu produced a twofold increase in outflux. Thus our results confirm the findings of Potts & Fleming (1971) on *Fundulus kansae*. However, the outflux in eels remained elevated 15 hr after calcium had been re-admitted to the aquaria. The failure to reverse probably results from the increase in plasma sodium which in consequence places a load on the homoeostatic mechanisms. In some fish the outflux increased following calcium removal even though no detectable changes in plasma sodium were present. This may indicate very complex homoeostatic mechanisms. For example, Potts & Fleming (1970) have shown that calcium removal increases branchial water permeability in the gills of *F. kansae*. If the same is true for the eel then excessive water loss through the gills will force the animal to drink sea water, and the increased salt load may be monitored by visceral receptors, as suggested by Kirsch (1971). Mayer & Nibelle (1970) have shown that infusion of hypersaline into eels causes an increase in total outflux. Some clarification of this problem is possibly by considering the various components of the total outflux.

The sodium flux after transfer to fresh water is an indication of a passive leak down a concentration gradient. This flux was increased fourfold by removal of calcium, the effect being reversible. This result is probably a direct effect of calcium on permeability as discussed for influx. The outflux of sodium represented by the (FWK-FW) flux is believed to be due to an outwardly directed sodium pump located in the outer surface of the gill epithelium (Maetz, 1969). Arguments have been made to exclude the possibility that the extra-flux obtained in changing from FW to FWK results from potential changes (Maetz, 1971). In addition the (FWK–FW) flux has a Q_{10} of 6 compared with only 2 for the passive flux, a strong indication that the (FWK–FW) flux is an active process (Maetz & Evans, 1972). Furthermore, R. Motais & X. Isaia (in preparation) have demonstrated that addition of ouabain to the external medium blocks this flux in the eel and the flounder. The (FWK–FW) flux was halved after 15 hr.

in calcium-free conditions, but then increased above control values when calcium was readmitted. Although the increase was not statistically significant due to the wide scatter of values, it was reflected in a significant elevation of the total outflux above control values. The increase in the (FWK–FW) flux following repletion of external calcium is probably an expression of the sodium load on the system. It is not known why calcium removal reduces this flux.

This work has shown that removal of calcium upsets the delicate balance between influx and outflux in eel gills in such a way that the animal gains sodium. The results provide an explanation for the inability of sea-water eels to survive in calcium-free sea water for more than a day or so (Sharratt *et al.* 1964).

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