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A TRANSIENT SODIUM–HYDROGEN EXCHANGE SYSTEM INDUCED BY CATECHOLAMINES IN ERYTHROCYTES OF RAINBOW TROUT, *SALMO GAIRDNERI*

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SUMMARY

1. The addition of isoprenaline to an isotonic suspension of red blood cells of rainbow trout induces an amiloride-sensitive Na^+ transport which is independent of Cl^- and insensitive to 4,4′-diisothiocyano-2,2′-stilbene disulphonic acid (DIDS) and furosemide.

2. Na^+ uptake is accompanied by amiloride-sensitive H^+ release. The H^+ efflux is dependent upon the external Na^+ concentration, the $K_{0.5}$ value for Na^+ being 16 mM.

3. In the presence of DIDS, when the coupled NaCl entry (NaCl co-transport) induced by catecholamine is blocked, the results provide evidence for a linked movement of Na^+ and H^+, with a stoicheiometry of 1 : 1.

4. Exchange of H^+ for Na^+ induces osmotic swelling of the cells which is due to the replacement of a bound proton by an osmotically active Na^+ cation.

5. In the absence of DIDS when the bulk of the Na^+ uptake is the result of a coupled entry of Na^+ and Cl^-, H^+ extrusion still occurs and the magnitude of acid excretion is identical to that found in DIDS-treated cells. This suggests that Na^+–H^+ exchange remains active.

6. Addition of isoprenaline first stimulates the Na^+–H^+ exchange but only transiently. This is followed by a more permanent stimulation of the NaCl co-transport.

INTRODUCTION

Addition of catecholamines to an isotonic suspension of nucleated red blood cells of the rainbow trout, *Salmo gairdneri*, causes the cell volume to increase. This increase in volume is the result of net uptake of Na^+ and osmotically obligated water (Baroin, Garcia-Romeu, Lamarre & Motais, 1984). Two different pathways are involved in the salt uptake.

The bulk of the Na^+ uptake results from a one-for-one entry of Na^+ and Cl^-. We demonstrated that the mechanism responsible for this NaCl absorption is a neutral co-transport specifically requiring Cl^-. It is independent of extracellular K^+, sensitive to amiloride, 4,4′diisothiocyano-2,2′-stilbene disulphonic acid (DIDS), niflumic acid and furosemide, but insensitive to other loop diuretics such as piretanide and

bumetanide. Pharmacologically, this NaCl co-transport differs from the co-transport described in other cells, such as avian erythrocytes, where the co-transport is operationally defined as a neutral Na^+–K^+–$2Cl^-$ transport (Kregenow, 1977, 1978; Schmidt & McManus, 1977).

The minor component of Na^+ entry (about 20 %) results from Na^+ uptake which is independent of Cl^- and sensitive to amiloride, but insensitive to DIDS, furosemide and niflumic acid. A Na^+–H^+ counter-transport was proposed. This paper described the experiments performed to test this hypothesis.

METHODS

Preparation of cells. Rainbow trout, *Salmo gairdneri*, were anaesthetized by immersing in fluid containing crushed ice, and a small sample of blood was drawn from the caudal vessels using heparinized syringes. Afterwards, the fish were rewarmed, and survived with no adverse effect.

The blood of several animals was pooled. The cells were washed three times in fish Ringer solution to remove the buffy coat, and then suspended at a haematocrit of 15 % and incubated for 13 h at 5 °C in the fish Ringer solution to ensure that they reached steady state with respect to ion and H_2O content before experimental treatment. Fish Ringer solution (pH 7·9) contains (mM): NaCl, 145; $CaCl_2$, 5; $MgSO_4$, 1; KCl, 4; HEPES, 10; glucose, 5. When the external Na^+ concentration was varied, choline chloride was substituted for NaCl to maintain osmolarity.

H^+ release measurement. In all the experiments performed to measure H^+ release by the cells a CO_2-free medium was used (obtained by bubbling fish Ringer solution with N_2) in order to avoid the buffering effect of HCO_3^- which, because it is a volatile buffer, renders difficult a quantitative expression of the results. A 10 ml portion of cell suspension (haematocrit 20 %) was stirred at 15 °C and the time course of the extracellular pH was followed after addition of isoprenaline. A similar amount of the same suspension (haematocrit 20 %) was used to establish the 'titration' curve: pulses of 5 μl 0·1 N-HCl were added step by step and the changes in the extracellular pH were monitored. To determine the amount of H^+ release during a defined period of time, ΔpH was measured on the experimental pH trace; the changes obtained on the titration curve by addition of known amounts of acid were used as a reference. This procedure takes into account intra- and extracellular buffering capacities. For the experiments with DIDS, which essentially isolate intracellular from extracellular buffer, the calculations were performed with titration curves obtained with DIDS-treated cells.

Cell ion and water contents. These were determined as described previously (Baroin *et al.* 1984).

The variation of intracellular Na^+ in 1 min measures net Na^+ uptake (J_nNa^+) and is expressed in mmol l cells^{-1} min^{-1}

Materials. Sigma was the source of ouabain, isoprenaline bitartrate and DIDS. Amiloride was generally provided by Chibret (63203 Riom France).

RESULTS

The dependence of H^+ excretion on the external Na^+ concentration

We have shown previously that the increase in cell volume induced by isoprenaline was drastically, but not completely, inhibited by DIDS and furosemide (Fig. 1*A*). The slight swelling in the presence of DIDS was due to net Na^+ entry (Fig. 1*C*) without a simultaneous net Cl^- uptake (Fig. 1*B*). This amiloride-sensitive component of Na^+ uptake was thought to be due to the exchange of intracellular H^+ for extracellular Na^+. If such a Na^+–H^+ exchange takes place, the external Na^+ concentration would be expected to modulate H^+ excretion.

As illustrated in Fig. 2, no modification of the extracellular pH was observed when 5×10^{-7} M-isoprenaline was added to an Na^+-free suspension of erythrocytes (choline chloride Ringer solution). However, acidification of the extracellular medium occurred in the presence of external Na^+; acidification increased with increasing

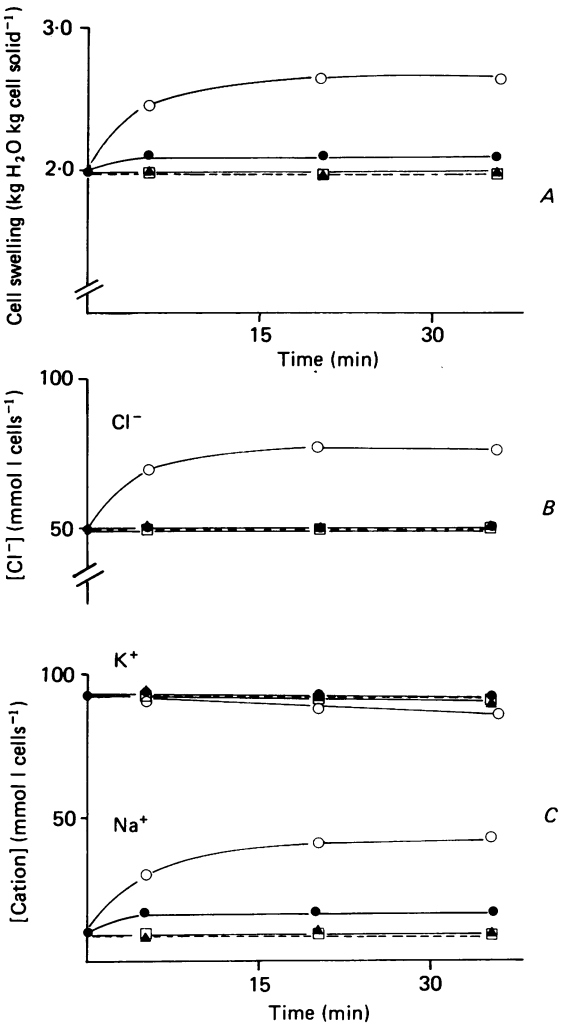

Fig. 1. Effect of inhibitors on cell swelling (*A*), chloride content (*B*) and cation content (*C*). 5×10^{-7} M-isoprenaline was added at time zero. □, control; ○, isoprenaline; ▲, isoprenaline + 2×10^{-4} M-amiloride; ●, isoprenaline + 5×10^{-4} M-DIDS.

concentrations of Na^+. The relationship between Na^+ and H^+ fluxes indicates that on hormonal stimulation the red cells excrete H^+ and that the excretion is dependent on the presence of external Na^+. Fig. 3 shows the dependence of the H^+ excretion on the external Na^+ concentration. The half-maximum effect for Na^+ activation was observed at 16 mM. As previously demonstrated, entry of Na^+ was inhibited by amiloride (Fig. 1*C*); 2×10^{-4} M-amiloride also inhibited the excretion of protons, even when the extracellular medium contained 145 mM-Na^+ (Fig. 4). In both cases the inhibition was instantaneous. Over-all, these results indicate a coupled movement of Na^+ and H^+. What is more, since these variations of pH are measured in a strongly buffered suspension (10 mM-HEPES), they must be quantitatively very large.

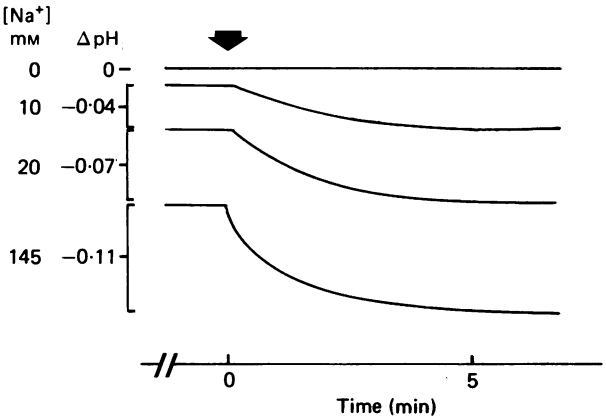

Fig. 2. The effect of Na^+ on proton excretion after hormonal stimulation in trout erythrocytes. The external medium contained increasing quantities of Na^+ (0, 10, 20, 145 mM); choline chloride was substituted for NaCl to maintain osmolarity. Isoprenaline was added at time zero. The evolution of extracellular pH was measured. Haematocrit: 20%.

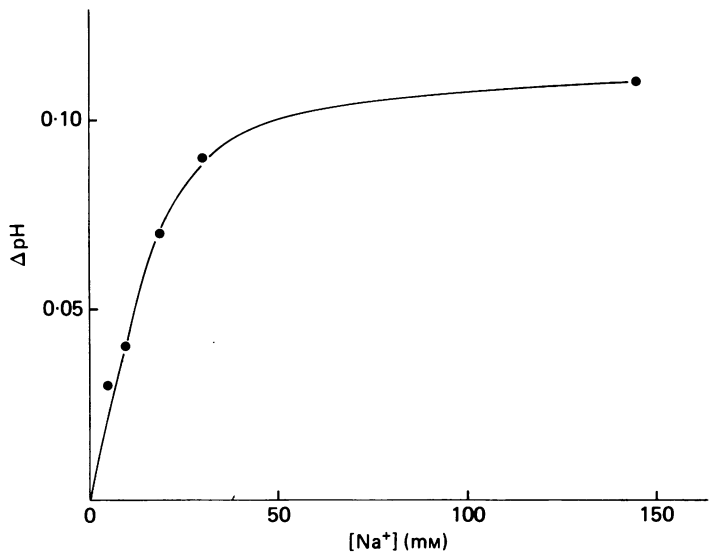

Fig. 3. Dependence on Na^+ of the amiloride-sensitive rate of H^+ release by trout erythrocyte.

The quantitative relation between Na^+ uptake and H^+ release in cells treated with DIDS

Uptake of Na^+ due to NaCl co-transport is inhibited by DIDS. The inhibition allows a quantitative determination of the coupled movement of Na^+ and H^+. Simultaneous measurement of the rate of acid extrusion and the net uptake of Na^+ in cells treated with DIDS allowed determination of the stoicheiometry of the exchange. The experiments were conducted in the presence of ouabain to prevent Na^+ efflux through the Na^+ pump, and the net uptake of Na^+ was blocked at the end of the experimental period by the addition of amiloride (5×10^{-4} M).

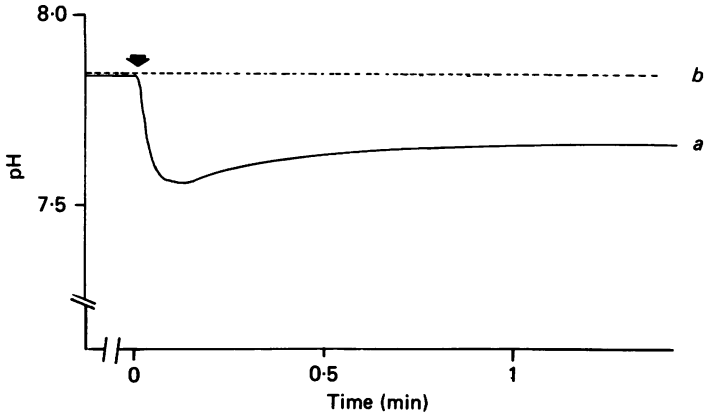

Fig. 4. Inhibition by amiloride of the Na^+-induced excretion of proton. *a*, modification of the extracellular pH for control cell suspended in normal Ringer solution containing 145 mM-Na^+. *b*, no modification of the extracellular pH in presence of 2×10^{-4} M-amiloride.

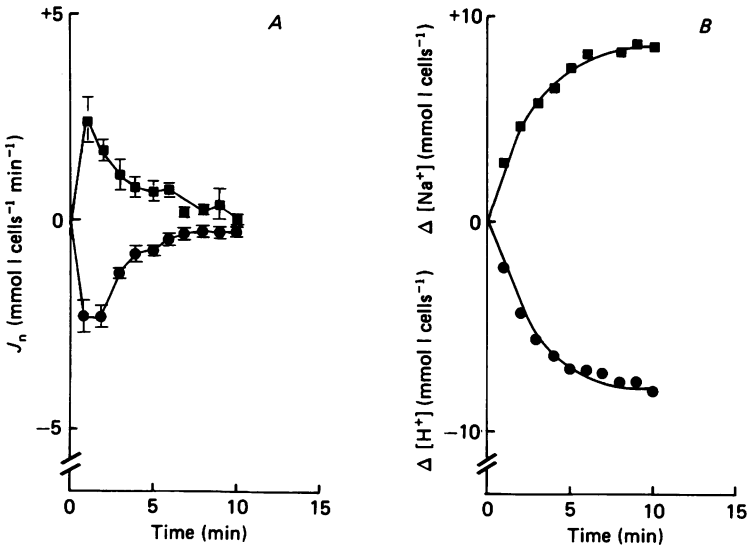

Fig. 5. The relationship between absorption of Na^+ and proton excretion in trout erythrocytes treated with DIDS. *A*, evolution of Na^+ (■) and H^+ (●) fluxes during the first 10 min after hormonal stimulation. *B*, evolution of the intracellular concentrations of Na^+ (■) and H^+ (●).

Fig. 5*A* shows the average of four experiments. In these experiments the Na^+ and H^+ fluxes were measured every minute for the first 10 min following hormonal stimulation. During the first minute, the addition of isoprenaline induced a large uptake of Na^+ ($2{\cdot}41 \pm 0{\cdot}55$ mmol l cells^{-1}) and a simultaneous excretion of H^+ of similar magnitude ($2{\cdot}28 \pm 0{\cdot}36$ mmol l cells^{-1}), after which the quantity of Na^+ which was absorbed and the quantity of H^+ which was excreted decreased rapidly at first

(1–5 min), then more moderately, and nearly reached zero at 10 min. The movements of Na^+ and H^+ were synchronized, producing symmetric variations of the concentration of intracellular Na^+ and the concentration of extracellular H^+ (Fig. 5*B*). These synchronized movements strongly suggest an Na^+–H^+ exchange. Fig. 6 compares the

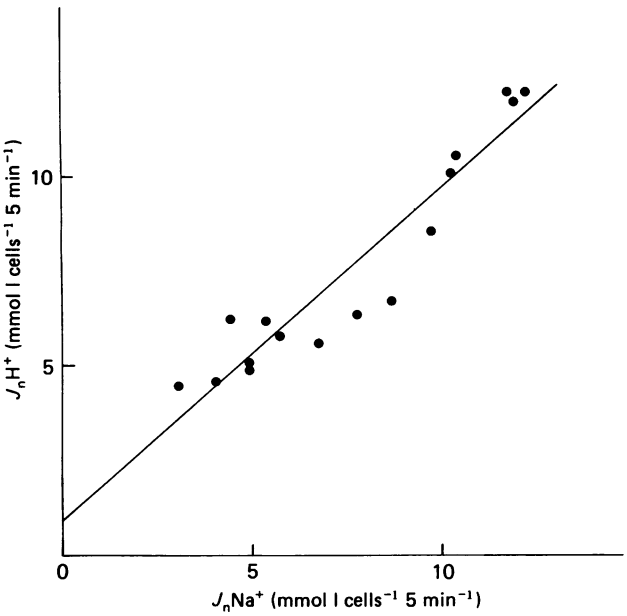

Fig. 6. Relationship between the net Na^+ influx and net H^+ efflux during the 5 min after hormonal stimulation in trout erythrocytes treated with DIDS.

net H^+ excretion for the first 5 min after hormonal stimulation with the net Na^+ absorption for the same period. The relation is expressed by the following equation:

$$J_nH^+ = (0{\cdot}88 \pm 0{\cdot}08)\, J_nNa^+ + (0{\cdot}92 \pm 0{\cdot}63),$$

where $r = 0{\cdot}95$ and $n = 16$.

The slope suggests that each Na^+ ion is exchanged for an H^+ ion.

The quantitative relation between Na^+ uptake and H^+ release in the absence of DIDS

Fig. 7 shows the results obtained with cells which were stimulated in the absence of DIDS, that is, when the NaCl co-transport facilitated simultaneous penetration of large quantities of Na^+ and Cl^-.

As was the case for preceding experiments, the external pH was monitored throughout. From the pH trace, the excretion of H^+ was calculated every minute and compared with Na^+ uptake for the same period of time. As illustrated in Fig. 7*A*, which is representative of seven similar experiments, the excretion of H^+ was very large during the first minute (6·22 mmol l cells^{-1}); 1 min later it was zero or very close to zero.

The kinetics of this proton excretion differ from those observed in the presence of DIDS, but the flux of H^+ in the absence of DIDS was 1·07 times the flux of H^+ in its presence.

Without DIDS, the evolution of the net Na^+ influx was also different. The large

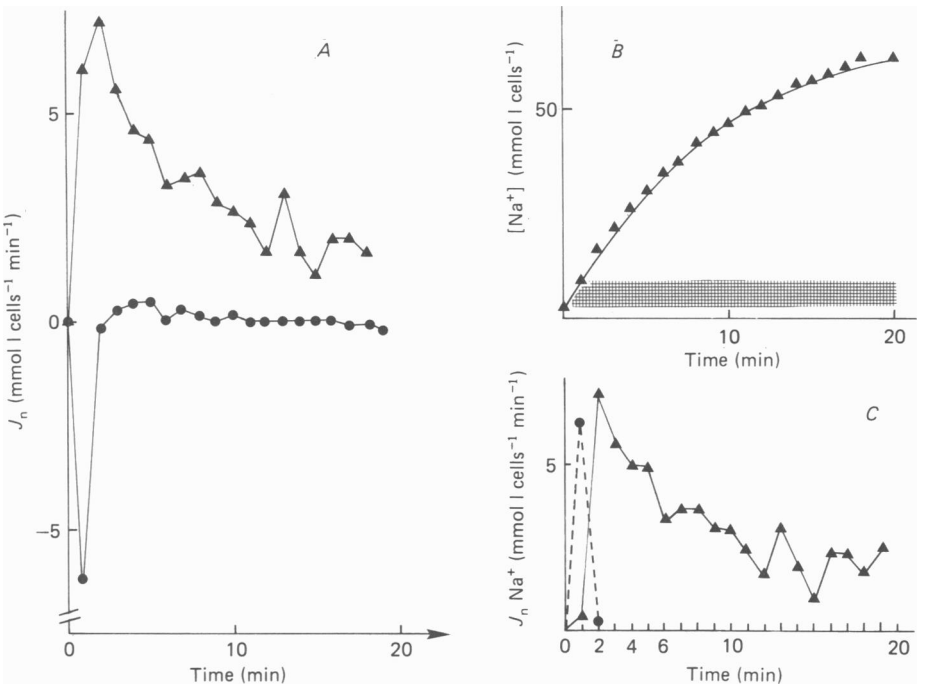

Fig. 7. The relationship between absorption of Na^+ and proton excretion in trout erythrocytes not treated with DIDS. *A*, evolution of Na^+ (▲) and H^+ (●) fluxes during the first 20 min after hormonal stimulation. *B*, evolution of the intracellular Na^+ concentration (▲). The shaded portion indicates the increase in Na^+ due to Na^+–H^+ exchange. *C*, two components responsible for the net Na^+ influx: ●, net Na^+ influx due to Na^+–H^+; ▲, net Na^+ influx due to NaCl co-transport (see text).

net influx seen in the first minutes (6·24 mmol l cells^{-1} at 1 min, with a peak of 7·28 mmol l cells^{-1} at 2 min) was followed by a relatively slow, though steady reduction of the net Na^+ influx, which none the less remained elevated even at the end of the experiments (20 min).

Without DIDS, there was no longer a symmetrical evolution of the Na^+ and H^+ fluxes. It is reasonable to consider that, in the absence of DIDS, two Na^+ transport processes function (i.e. Na^+–H^+ exchange and the NaCl co-transport). If each H^+ ion excreted was actually exchanged for an Na^+ ion (as in the case of cells treated with DIDS), then the net Na^+ influx can be divided into two components, Na^+–H^+ exchange and NaCl co-transport. These two components are depicted in Fig. 7*C*. The two transport mechanisms did not seem to operate simultaneously: Na^+–H^+ exchange functioned during the first 2 min after hormonal stimulation while NaCl co-transport began during the second minute and continued throughout the experiment (20 min).

The quantity of Na^+ which entered via Na^+–H^+ exchange is minor compared to

the total quantity of Na^+ which penetrated into the cell. During the first minute it accounted for 100 % of the Na^+ intake, but by 20 min it was only 10–15 % of the total (Fig. 7*B*).

Dependence of cell swelling on Na^+–H^+ exchange

Upon hormonal stimulation of cells not treated with DIDS, swelling resulted from the simultaneous penetration of an anion, Cl^-, and a cation, Na^+, i.e. of two osmotically active particles. If the absorption of NaCl is accompanied by an isosmotic

TABLE 1. The influence of DIDS on net Na^+ influx (J_nNa^+) and net H_2O influx (J_nH_2O) after hormonal stimulation

	J_nNa^+ (mmol l cells^{-1} 5 min^{-1})	J_nH_2O (ml l cells^{-1} 5 min^{-1})	$\frac{J_nH_2O}{J_nNa^+}$
Control cells ($n = 17$)	$+19{\cdot}49 \pm 1{\cdot}70$	$+108{\cdot}98 \pm 9{\cdot}41$	$5{\cdot}59 \pm 1{\cdot}11$
Cells treated with DIDS ($n = 12$)	$+7{\cdot}19 \pm 1{\cdot}04$	$+19{\cdot}38 \pm 2{\cdot}62$	$2{\cdot}70 \pm 0{\cdot}53$

entry of water, and if the cells act as perfect osmometers, then, for each millimole of Na^+ entering, the intracellular tonicity increases by 2 mosM, and 6·25 ml H_2O enter (the Ringer solution being 320 mosM). Measuring the net influx of Na^+ and H_2O shows that $5{\cdot}59 \pm 1{\cdot}11$ ml H_2O enter per millimole Na^+ (Table 1).

As already indicated, in the presence of DIDS the hormone still induced a slight cellular swelling which resulted from an Na^+ influx without an accompanying Cl^- influx. Under these conditions, $2{\cdot}70 \pm 0{\cdot}53$ ml H_2O entered per millimole of Na^+ (Table 1), i.e. half the quantity which entered in cells not treated with DIDS. This ratio agrees with the fact that the Na^+ enters without Cl^-, meaning that only one osmotically active particle enters. As previously demonstrated, the Na^+ which entered in the presence of DIDS was always exchanged with an H^+, with a stoicheiometry of 1. Such an exchange is, in theory, without an osmotic effect, and yet the cellular swelling indicated that the excreted protons were not osmotically active. In other words, the protons must be released by the intracellular buffers, such as haemoglobin. The fundamental mechanism underlying cellular swelling in cells treated with DIDS involves the replacement of a bound proton by an osmotically active cation, Na^+.

DISCUSSION

The work reported in this paper indicates the presence of an amiloride-sensitive Na^+ transport which is induced by catecholamines in trout erythrocytes and has the following characteristics.

(1) The amiloride-sensitive, chloride-independent Na^+ uptake is accompanied by an amiloride-sensitive H^+ release. The H^+ efflux is dependent upon the external Na^+ concentration, and the $K_{0{\cdot}5}$ value for Na^+ is 16 mM.

(2) In the presence of DIDS, i.e. when the coupled NaCl entry (NaCl co-transport) is blocked, the results provide evidence for a linked movement of Na^+ and H^+, the stoicheiometry being one.

(3) This exchange of identical numbers of H^+ for Na^+, which would ordinarily be osmotically ineffectual, induces osmotic swelling in this case. The swelling is due to the buffering capacity of the cell content, which allows the replacement of a bound proton by an osmotically active Na^+ cation.

(4) In normal cells (not treated with DIDS), in which the bulk of the Na^+ uptake is the result of a coupled entry of Na^+ and Cl^-, H^+ extrusion still occurs, and the magnitude of acid excretion is identical to that found in DIDS-treated cells. This correspondence indicates that Na^+–H^+ exchanges always occur in this condition.

(5) There is a temporal sequence to the hormonal stimulation of the Na^+ transport mechanisms: first the Na^+–H^+ exchanger functions briefly, then the NaCl co-transport is more permanently stimulated.

Since the external acidification occurs very rapidly after the addition of isoprenaline, the hormone most likely activates a normally latent exchanger in the cell membrane, rather than inducing a new mechanism. This raises the question of the physiological significance of an Na^+–H^+ exchanger in trout erythrocytes. It is well established that H^+ is not at equilibrium across the plasma membrane of animal cells. A number of recent papers (see review by Boron, 1983) have indicated that the presence of a membrane transport system involving a coupled exchange of Na^+ and H^+ could be of major importance in the regulation of intracellular pH; in this system the extrusion of H^+ would be energized by Na^+ moving down its electrochemical gradient. However, in red blood cells the situation is quite different: due to the high efficiency of the anion transport system, the distribution of the H^+ ion is at steady state across the cell membrane, in accordance with the Donnan equilibrium. In erythrocytes, an Na^+–H^+ exchanger to equilibrate pH would obviously be redundant; the physiological significance is questionable.

In salamander (*Amphiuma*) erythrocytes, the presence of an Na^+–H^+ exchanger has already been described and the physiological role has been considered (Cala, 1980; Siebens & Kregenow, 1978, 1980). In *Amphiuma* this exchanger becomes apparent after osmotic perturbation: when erythrocytes are placed in a hypertonic medium, their initial shrinkage is followed by a gradual re-expansion over a period of several hours. Re-expansion is brought about by a net NaCl uptake. The authors proposed that an Na^+–H^+ exchanger mediates the Na^+ uptake while a Cl^-–HCO_3^- exchanger mediates the Cl^- uptake. Cala (1980) suggests that changes in internal pH associated with osmotic perturbation (shrinkage would cause a fall in intracellular pH) serve as a signal for the Na^+–H^+ exchanger which, in this case, would be considered as a volume-regulating mechanism. Incidentally, the response to osmotic perturbation in salamander erythrocytes is very different from the response to isoprenaline described above; in *Amphiuma*, Na^+–H^+ exchanges are not transient, but continue over a period of 3 h, and the transport rate is very slow.

In trout erythrocytes the Na^+–H^+ exchanger cannot be considered as a volume-regulating mechanism, since after hormonal stimulation about 80 % of swelling is due to NaCl co-transport, and the Na^+–H^+ exchanges account for only 20 %. Upon first consideration, the induction of the NaCl co-transport might be attributed to the Na^+–H^+ exchange: the quantity of Na^+ moving down its electrochemical gradient through the Na^+–H^+ exchanger might promote a rapid intracellular alkalinization despite the high buffering capacity of cell content. This increase in intracellular pH,

being the primary event after hormonal stimulation, could be the controlling factor (perhaps through the phosphorylation of a specific protein) in inducing the NaCl co-transport which appears later. Such a sequence of events has already been suggested for the mode of action of growth factors on proliferating cells (Smith & Rozengurt, 1978; Koch & Leffert, 1979; Moolenaar, Mummery, van der Saag & de Laat, 1981; Paris & Pouyssegur, 1983) and for that of insulin on frog skeletal muscle (Moore, 1981). None the less, in physiological conditions, i.e. in presence of HCO_3^-, it seems quite doubtful that the Na^+–H^+ exchanger could play such a role in an erythrocyte. It must be noted that under our experimental conditions, i.e. in the nominal absence of HCO_3^-, the anion exchange system cannot significantly or rapidly dissipate the pH gradient created across the cell membrane by the Na^+–H^+ exchanges: normally, pH equilibrium occurs via Cl^-–HCO_3^- exchanges and not via Cl^-–OH^- exchanges, OH^- ions being transported poorly or not at all by the anion transport system (Cousin, Motais & Sola, 1975; Jennings, 1978; Wieth, Brahm & Funder, 1980). Theoretically, in an HCO_3^--free medium, equilibration of pH could still proceed through a co-transport of Cl^- and H^+ which, according to the findings of Jennings (1978), requires an operating anion transport system. However, the rate of HCl co-transport is extremely slow above pH 7, which explains why H^+ excretion can be measured in cells not treated with DIDS (Fig. 7*A*). Conversely, in the presence of HCO_3^- it is likely that the increase in intracellular pH would be partially or totally abolished by the Cl^-–HCO_3^- exchanges catalysed by the anion transport system. Since NaCl co-transport has been found and described in the presence of CO_2 (Baroin *et al.* 1984), an increase in intracellular pH cannot be requisite for the induction of the NaCl co-transport. Experiments determining the relationship between Na^+–H^+ exchanger and NaCl co-transport are in progress.

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