Catecholamine-induced Transport Systems in Trout Erythrocyte

Na^+/H^+ Countertransport or NaCl Cotransport?

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ABSTRACT It has previously been shown (Baroin, A., F. Garcia-Romeu, T. Lamarre, and R. Motais. 1984*a*, *b*. *Journal of Physiology*. 350:137, 356:21; Mahé, Y., F. Garcia-Romeu, and R. Motais. 1985. *European Journal of Pharmacology*. 116:199) that the addition of catecholamines to an isotonic suspension of nucleated red blood cells of the rainbow trout first stimulates a cAMP-dependent, amiloride-sensitive Na^+/H^+ exchange. This stimulation seems to be transient. It is followed by a more permanent activation of a coupled entry of Na^+ and Cl^-, which is inhibited by amiloride but also by inhibitors of band 3 protein (DIDS, furosemide, niflumic acid). The coupled entry of Na^+ and Cl^- could therefore result from the parallel and simultaneous exchange of Na^+_{out} for H^+_{in} (via the cAMP-dependent Na^+/H^+ antiporter) and Cl^-_{out} for HCO_{3in}^- (via the anion exchange system located in band 3 protein). However, in view of the following arguments, it had been proposed that NaCl uptake does not proceed by the double-exchanger system but via an NaCl cotransport: (*a*) Na^+ entry requires Cl^- as anion (in NO_3^- medium, the Na uptake is strongly inhibited, whereas NO_3^- is an extremely effective substitute for Cl^- in the anion exchange system); (*b*) Na uptake is not significantly affected by the presence of HCO_3^- in the suspension medium despite the fact that in red cells, Cl^-/HCO_3^- exchange occurs more readily than the exchanges of Cl^- for basic equivalents in a theoretically CO_2-free medium (the so-called Cl^-/OH^- exchanges). The purpose of the present paper was a reassessment of the two models by using monensin, an ionophore allowing Na^+/H^+ exchange. From this study, it appears that NaCl entry results from the simultaneous functioning of the Na^+/H^+ antiporter and the anion exchange system. The apparent Cl dependence is explained by the fact that, in these erythrocytes, NO_3^- clearly inhibits the turnover rate of the Na^+/H^+ antiporter. As Na^+/H^+ exchange is the driving component in the salt uptake process, this inhibition explains the Cl requirement for Na entry. The lack of stimulation of cell swelling by bicarbonate is explained by the fact that the rate of anion exchange in a CO_2-free medium (Cl^-/OH^- exchange) is roughly equivalent to that of Na^+/H^+ exchange and thus in practice is not limiting to the net influx of NaCl through the two exchangers. The acidification

observed at the onset of hormonal stimulation, although the turnover rate of Na^+/H^+ exchange does not exceed that of Cl^-/HCO_3^- exchange, results from the large buffer capacity of cytoplasm: it tends to make pH equilibration movements through band 3 lag behind the H^+ flux mediated by the Na^+/H^+ antiporter.

INTRODUCTION

The addition of catecholamines to an isotonic suspension of nucleated red blood cells of the rainbow trout, *Salmo gairdneri*, causes the cell volume to increase (Nikinmaa, 1982; Bourne and Cossins, 1982; Baroin et al., 1984*a*). This increase in volume, which is cAMP dependent (Mahé et al., 1985), is the result of a ouabain-insensitive uptake of NaCl and osmotically obligated water. This accumulation of salt and water is affected by different transport inhibitors: it is completely inhibited by amiloride, an inhibitor of Na permeability, and largely but not completely (~80%) inhibited by known inhibitors of anion exchanges in erythrocytes, such as DIDS, niflumic acid, and furosemide (Baroin et al., 1984*a*). The residual swelling observed in the presence of DIDS and similar compounds is due to a net Na^+ entry without a simultaneous net Cl^- uptake, the Na ions penetrating into the cell in exchange for H^+ from the intracellular buffers with a stoichiometry of 1:1 (Baroin et al., 1984*b*). This exchange of H^+ for Na^+ causes a strong acidification of the medium even when this medium is buffered with 15 mM HEPES. In the absence of an inhibitor, the addition of catecholamines also induces a strong but transient (of ~1 min) acidification of the medium that is amiloride sensitive, which suggests that, under uninhibited conditions, Na^+/H^+ exchange remains active (Baroin et al., 1984*b*). It must be pointed out that Cossins and Richardson (1985) described the existence in volume-static trout erythrocytes of a virtually quiescent Na^+/H^+ countertransport, which thus is greatly stimulated by catecholamines. Taken together, these data should indicate that the entry of NaCl into the red blood cells results from the parallel and simultaneous exchanges of Na^+_{out} for H^+_{in} (via the cAMP-dependent Na^+/H^+ antiporter) and Cl^-_{out} for HCO_{3in}^- (via the well-known anion exchanger of the red cell membrane, band 3). Furthermore, this model is consistent with several other observations (Baroin et al., 1984*a*): the simultaneous entry of Na^+ and Cl^- is not due to an electrical coupling of the fluxes occurring through conductive pathways; it is insensitive to external K; and it is inhibited by amiloride, which is considered an inhibitor of Na^+/H^+ exchange, and by furosemide, which inhibits the anion exchange system (Brazy and Gunn, 1976; Cousin et al., 1976), but not by furosemide analogues such as piretanide and bumetanide. Nevertheless, it seemed to us that the major part of the NaCl uptake could not proceed by a mechanism involving the anion exchange system, but must proceed via an NaCl cotransport because of various observations, e.g., that NO_3^- anion substitutes very poorly for Cl^- in salt uptake (Baroin et al., 1984*a*, *b*). Recently, however, it has been suggested that in the red blood cells of *Amphiuma* (Cala, 1983) and dog (Parker, 1983), NO_3^- could directly inhibit Na^+/H^+ exchange. This suggestion prompted us to reassess all the arguments we advanced to reject the double-exchanger model and then to examine further the mechanisms of NaCl entry induced by catecholamines in trout erythrocytes.

MATERIALS AND METHODS

Fish Blood

Rainbow trout (*Salmo gairdneri*), ranging in weight from 200 to 300 g, were obtained from a commercial hatchery and kept for 1 wk in the laboratory in tanks provided with running tap water (temperature, 13°C). The fish were not fed. Blood was drawn from the caudal vessels using heparinized syringes. The blood of several fish was pooled. The cells were washed three times in fish-Ringer solution to remove the buffy coat. They were then suspended at a hematocrit of 15% and incubated overnight at 4°C in the fish-Ringer solution to ensure that they had reached a steady state with respect to ion and water content before experimental treatment.

Experimental Solutions

The fish-Ringer solution contained (mM): 145 NaCl, 5 $CaCl_2$, 1 $MgSO_4$, 4 KCl, 10 HEPES, 5 glucose, pH 7.6. The nitrate Ringer solution used in these experiments was obtained by substituting $NaNO_3$ for NaCl and $Ca(NO_3)_2$ for $CaCl_2$. As the rate of swelling and Na absorption is modulated by oxygenation of the medium (unpublished results), all the experiments were made in solution flushed with N_2.

In experiments performed to test the effect of different concentrations of HCO_3^-, the fish-Ringer solution was equilibrated at constant pH with different mixtures of N_2 and CO_2.

Cell Ion and Water Contents

After the incubation period, the red cells were washed four times in the experimental solution and the hormone or the ionophore was added to the suspension. At intervals, samples of the whole suspension were poured into nylon tubes that were centrifuged at 20,000 *g* for 10 min in a Sorvall RC 2B refrigerated centrifuge. These specially prepared tubes contain up to 0.7 ml.

For each time sample, at least four nylon tubes were filled with cell suspension. After centrifugation, the packed cell mass was separated from the supernatant by slicing the tube with a razor blade below the top of the red cell column. Cells were then prepared for analysis of cell water, ion, and hemoglobin contents.

Cell water content. The packed cell mass was expressed with a plastic rod fitted onto weighed aluminum foil. After weighing, the packed cells were dried to constant weight for 10 h at 90°C and reweighed. Cell water content is expressed as kilograms water per kilogram cell solid. Triplicate samples were used.

Ion content. The column of nylon tube containing the packed cell mass was put in 10 ml $MgSO_4$ solution (1 mM) and mixed to hemolyze the cells. 2 ml was reserved for hemoglobin analysis and 160 μl 80% perchloric acid was added to the remaining 8 ml hemolyzed suspension in order to precipitate cell proteins. After centrifugation at 15,000 rpm for 15 min, the clear supernatant was saved for analysis of cations and Cl^-. A flame photometer (Eppendorf GmbH, Hamburg, Federal Republic of Germany) was used for cation determination and Cl^- was analyzed by colorimetry on an automatic titrator. Fluid trapped between the packed cells after centrifugation in the nylon tube was estimated with [^{14}C]inulin. A trapping correction of 1.5% was routinely included in the final calculations. Ion contents are expressed as millimoles per liter of volume-static cells (i.e., before hormonal stimulation). The hemoglobin concentration was used as a reference to calculate cell volume. In our experimental conditions, a concentration of hemoglobin of 1 mg/liter was found to correspond to 5.424 ± 0.051 mg of wet cells under volume-static conditions.

Hemoglobin. The 2 ml of supernatants saved after hemolysis was centrifuged in an Eppendorf microfuge. Hemoglobin was determined as methemoglobin by spectrophotometry (Uvikon 820, Roche Kontron, Velizy, France) at a wavelength of 418.5 nm.

Protons Efflux Experiments

In the experiments performed to measure H^+ release by the cells, the procedure described in Baroin et al. (1984*b*) was used.

Rate of Hemolysis in Ammonium Salts

The procedure has been described previously (Aubert and Motais, 1975).

Materials

A fresh aqueous solution of 2.75×10^{-5} M isoproterenol (isoproterenol bitartrate; Sigma Chemical Co., St. Louis, MO) was prepared for each experiment. A sufficient amount was routinely added to experimental solutions to yield a final concentration of 5.5×10^{-7} M. Amiloride (Merck, Sharp & Dohme, West Point, PA), DIDS (Sigma Chemical Co.), and monensin (Sigma Chemical Co.) were prepared in dimethylsulfoxide such that the final concentration of dimethylsulfoxide was no more than 1%. The tripropyltin chloride (BDH Chemicals, Poole, England) was added to the external medium as an alcoholic solution.

RESULTS

Effects of NO_3^- on Na Permeability Induced by Isoproterenol

The problem was to determine whether the simultaneous entry of Na^+ and Cl^- into the red blood cells results either from the parallel exchange of Na^+_{out} for H^+_{in} (via the cAMP-dependent Na^+/H^+ antiporter) and Cl^-_{out} for HCO_{3in}^- (via the well-known anion exchanger of the red cell membrane) or from the functioning of a cotransport system. One of the criteria that can be used to discriminate between these two models is the effect of NO_3^-. Because NO_3^- and Cl^- are transported at about equal rates by the anion exchanger, the substitution of NO_3^- for Cl^- should not change the rate of salt uptake and swelling. On the contrary, the Cl^- requirement is one of the characteristics of the cotransport mechanism, NO_3^- among other anions being not accepted or badly accepted by this transport system (Ellory et al., 1982).

We previously showed (Baroin et al., 1984*a*) that when internal and external Cl^- were replaced by NO_3^-, the catecholamine-induced swelling was greatly reduced compared with the control (Fig. 1). This inhibition of swelling resulted from a reduced uptake of Na as reported in Table I for three sets of experiments. In the presence of NO_3^-, the Na uptake represents only 27–36% of the Na entry measured in the presence of Cl. Since we verified that in trout erythrocytes as well the anion exchange system cannot distinguish between Cl^- and NO_3^-, the fact that NO_3^- could not replace Cl^- in the swelling process had been interpreted by us as indicating that the NaCl uptake does not result from the parallel and simultaneous exchanges of Na^+ for H^+ and Cl^- for HCO_3^- but from an NaCl cotransport mechanism (Baroin et al., 1984*a*). A direct proof that the operation of a simultaneous double exchanger must normally induce swelling in trout erythrocytes in the presence of NO_3^- is given by the following experiment using monensin, an Na^+/H^+ exchange ionophore. The ionophore was added (5×10^{-6}

M final) to red blood cells suspended in Cl^- or in NO_3^- medium in the absence of catecholamines. The ionophore promotes an electrically silent Na^+_{out}/H^+_{in} countertransport, thereby inducing a pH shift that is compensated by an extrusion of a basic equivalent (OH^- or HCO_3^-) in exchange for the external anion (NO_3^- or Cl^-) through the natural membrane anion exchanger (band 3). The result is an uptake of NaCl or $NaNO_3$. From this experiment (Fig. 2), it is clear that the rate of swelling is not Cl dependent when Na^+/H^+ exchange is mediated by monensin.

It is evident, however, that the salt uptake could result from the operation of a simultaneous double exchanger and nevertheless be apparently Cl dependent if, in trout erythrocytes, NO_3^- is inhibitory to the cAMP-dependent Na^+/H^+ exchange, as suggested by Cala (1983) and Parker (1983) for the red blood cells of *Amphiuma* and dog, respectively. To test this possibility, we measured the entry of Na^+ in the presence of DIDS, i.e., in a condition allowing for a quantitative determination of Na^+/H^+ exchange alone. From the experiments reported in Table I, it appears that the amount of Na penetrating into the cell

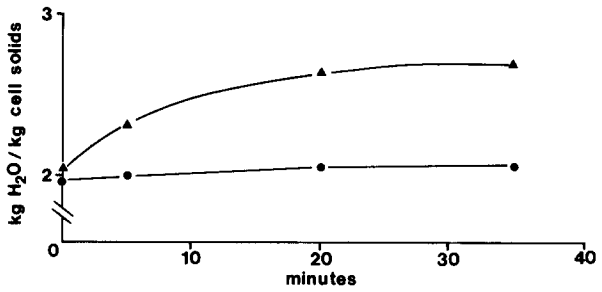

FIGURE 1. Effect of replacement of Cl^- by NO_3^- on cell swelling induced by the addition of isoproterenol (5×10^{-7} M) at time 0. ▲, Cl; ●, NO_3.

by the Na^+/H^+ antiporter in NO_3^- medium (the group called NO_3^- + DIDS) represents only 51–65% of the Na^+ uptake in Cl medium (the group called Cl^- + DIDS). Thus, it is clear that Na^+/H^+ countertransport is significantly inhibited in the presence of NO_3^-.

As the simulation of Na^+/H^+ is cAMP dependent, this inhibition could result from some effect on the adenylate cyclase system. From the following results, this possibility can be discarded: (*a*) isoproterenol induces the same accumulation of cAMP in red cells suspended either in Cl^- or in NO_3^- medium; (*b*) swelling and salt uptake, which are normally induced by catecholamines, can also be promoted by adding 8 Br cAMP (10^{-3} M) and theophylline (10^{-3} M) to the external medium (Mahé et al., 1985). When 8 Br cAMP was added in NO_3^- medium, swelling was inhibited and the magnitude of inhibition was similar to that observed with isoproterenol (data not shown).

Effect of Trialkyltin Derivatives

In the model of double exchangers operating in parallel, Na^+/H^+ countertransport induces a pH shift that is compensated by an extrusion of a basic equivalent

TABLE I

Comparison of Net Na Entry in a Cl^- and in an NO_3^- Medium for 30 Min after the Addition of Isoproterenol

	Total Na uptake			Na uptake in exchange with H^+		
	Cl medium	NO_3 medium	Percent of inhibition	Cl medium + DIDS (10^{-4} M)	NO_3 medium + DIDS (10^{-4} M)	Percent of inhibition
	mM·liter^{-1} × 30 min^{-1}			*mM·liter^{-1} × 30 min^{-1}*		
First experiment (n = 3)	36.98±0.52	9.98±0.27	73.0	10.79±0.21	5.53±0.18	48.8
Second experiment (n = 6)	49.51±1.08	18.17±0.39	63.3	12.06±0.20	7.82±0.35	35.2
Third experiment (n = 6)	52.35±0.42	14.57±0.27	72.2	13.23±0.11	8.06±0.16	39.1

The Na content expresses a concentration in a constant volume of cells (millimolar per liter cells), the cell volume before hormonal stimulation being used as reference (see Materials and Methods).

(OH^- or HCO_3^-) in exchange for external Cl^-. One of the predictions of the model is that when the anionic exchanger is blocked by an inhibitor such as DIDS, the NaCl uptake can be restored by adding an artificial ionophore that permits Cl^-/OH^- exchanges. Monovalent trialkyltin derivatives are ionophores that allow such exchanges across the membranes of mitochondria, erythrocytes,

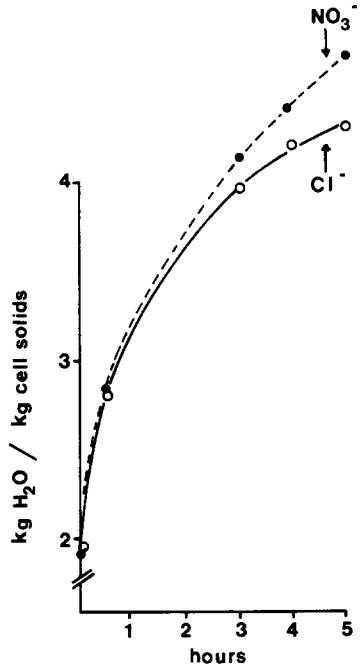

FIGURE 2. Effect of replacement of Cl^- by NO_3^- on cell swelling induced by the addition of monensin (5×10^{-6} M) at time 0.

and lipid bilayers (Selwyn et al., 1970; Aubert and Motais, 1975; Motais et al., 1977; Wieth and Tosteson, 1979; Tosteson and Wieth, 1979).

A direct proof of the accuracy of this prediction is given by an experiment using monensin (Fig. 3). The addition of monensin to red cells not stimulated by catecholamines allows Na^+/H^+ exchange, which induces a pH shift compensated by Cl^-/HCO_3^- exchange via band 3, and thus swelling occurs. Inhibition of anion exchange by DIDS blocks swelling. The addition of tin derivatives to DIDS-inhibited cells again allows a normal swelling.

We previously showed that, on the contrary, when Na^+/H^+ exchange was induced by catecholamines and the anion exchange system was inhibited by DIDS, we could no longer restore NaCl uptake and swelling by adding tin derivatives. We considered this negative result evidence that the double-exchanger model could be eliminated (Baroin et al., 1984*a*).

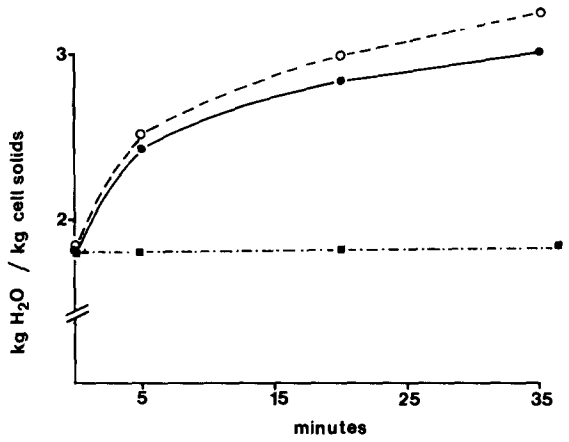

FIGURE 3. Effect of tripropyltin chloride (5×10^{-6} M) on cell swelling induced by monensin (5×10^{-6} M) when the Cl^-/OH^- exchanges are inhibited by DIDS (5×10^{-4} M). O, monensin; ●, monensin plus DIDS and tripropyltin chloride; ■, monensin plus DIDS.

However, recently, in parallel experiments, we observed that swelling and NaCl uptake were greatly inhibited when the tin derivative alone was added to the suspension (i.e., without DIDS), which indicates that this compound impairs the functioning of the catecholamine-induced transport system. The reason for this inhibiting effect is unknown but it is not via cAMP, since inhibition was also observed when swelling was induced by adding 8 Br cAMP in the solution. Whatever the explanation for the inhibition, which could result from some cytotoxic effect, it is evident that the use of tin derivatives cannot give any information concerning the mode of transport involved in NaCl uptake induced by catecholamines.

Effect of HCO_3^-

In the double-exchanger model, if the rate of anion exchange is sufficiently slow, the movement of anions can become a rate-limiting step for the overall reaction,

i.e., the NaCl uptake. In trout red cell, if NaCl entry proceeds according to this mode of transport, the pH shift induced by Na^+/H^+ exchange is compensated by the exchange of Cl^- for a basic equivalent. The latter process is mediated by the anion exchange system (band 3). It is possible to modulate the rate of this anion countertransport by modifying the concentration of HCO_3^-. It is known that in human red cells, the exchange of Cl^- for HCO_3^- occurs very rapidly because of the high affinity of HCO_3^- for the exchanger. The rate at which OH^- per se can exchange with Cl^- is not known, but because of the very small concentration of this anion, and possibly also its low affinity, the amount of OH^- transferred is in any case much smaller than that of HCO_3^- (for review see Hladky and Rink, 1977). It is difficult to obtain a real HCO_3^--free medium (Cousin et al., 1975; Jennings, 1976), especially at alkaline pH. Nevertheless, the amount of basic equivalents that are transferred in exchange for Cl^- in a theoretically

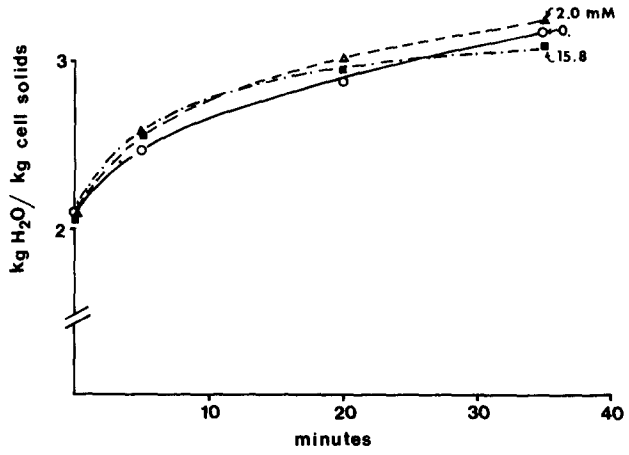

FIGURE 4. Effect of different concentrations of HCO_3^- on the rate of cell swelling induced by isoproterenol. The saline solutions were equilibrated at constant pH with convenient mixtures of N_2 and CO_2.

CO_2-free medium, even if a trace of HCO_3^- is present, is expected to be drastically reduced in comparison with that observed in an HCO_3^--containing medium. This effect of bicarbonate has been effectively shown in different experimental conditions: pH re-equilibration (Deuticke, 1972; Jacobs and Stewart, 1942; Crandall et al., 1971; Jennings, 1976) and NH_4Cl-induced cell lysis (Cousin et al., 1975).

Thus, if the coupled entry of NaCl induced by catecholamines in trout red cell results from the simultaneous functioning of the Na^+/H^+ antiporter and the anion exchange system, the rate of Na uptake and swelling should be different in an (HCO_3^-) CO_2-free medium and in an HCO_3^--containing medium. This reasoning has been used with *Amphiuma* red blood cells, and HCO_3^- was shown to have a strong stimulatory effect (Cala, 1983). However, as cited above in trout red blood cells, we observed that swelling and Na uptake are practically insensitive to HCO_3^- (Baroin et al., 1984*a*). This is illustrated in Fig. 4, which shows the evolution of swelling when the media HCO_3^- is increased from 0 to 16 mM at

fixed pH (7.42). We previously thought that this result indicated that the double-exchanger model could be ruled out. This interpretation must be re-examined by taking in account the relative rates of turnover of Na^+/H^+, Cl^-/OH^-, and Cl^-/HCO_3^- exchanges.

The fact that the rate of NaCl uptake and swelling after hormonal stimulation is practically insensitive to HCO_3^- could be explained by assuming that, in trout erythrocytes, the exchange of Cl^- for a basic equivalent in a theoretically CO_2-free medium could occur as readily as Cl^-/HCO_3^- exchange in HCO_3^--containing medium. This possibility seems unlikely but can easily be checked by measuring the rate of hemolysis in ammonium salt (Aubert and Motais, 1975; Motais, 1977). When red cells are suspended in an isotonic solution of NH_4Cl, osmotic hemolysis occurs by the mechanism represented in Fig. 5, according to which NH_3, which

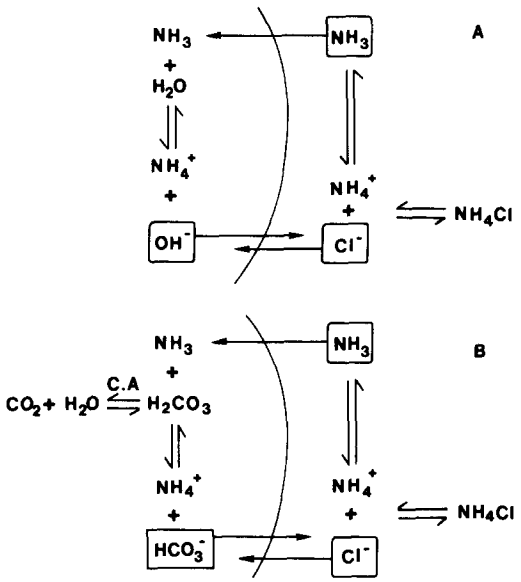

FIGURE 5. Transfer processes involved in the net movement of NH_4Cl into red blood cells in CO_2-free medium (*A*) and in the presence of CO_2 (*B*).

is highly permeable, first enters the cell and there becomes converted into NH_4 ions; a subsequent shift of anions through the anion exchanger completes the process. In CO_2-free medium (Fig. 5*A*), the overall rate of transfer of the salt is slow, chiefly because of the extremely low concentration of OH ions in the cell at any given time ($\sim 2.10^{-7}$ M). If HCO_3^- is then added to the external solution, we have the conditions presented in Fig. 5*B*, in which the concentration of internal anions able to be exchanged with external Cl^- is relatively high (in the millimolar range) and this concentration is kept constant by the cycling effect of CO_2 (a molecule with a very great penetrating power) and carbonic anhydrase. HCO_3^- therefore serves as a catalyst to get the $NH_4^+Cl^-$ into the cell more rapidly than in a CO_2-free solution. The time course of hemolysis can be estimated by measuring the decrease in the optical density of a suspension of red cells in

isotonic NH_4Cl solution (Aubert and Motais, 1975; Motais, 1977). It can be seen in Fig. 6 that hemolysis occurs very slowly when trout red cells are suspended in a theoretically CO_2-free medium and extremely rapidly in a medium in which the amount of HCO_3^- is 5 mM.

From this experiment, it is evident that in trout erythrocytes, as in human erythrocytes, the exchange of Cl^- for a basic equivalent in a theoretically CO_2-free medium (defined as Cl^-/OH^- exchange) occurs much more slowly than Cl^-/HCO_3^- exchange in an HCO_3^--containing medium. Thus, the fact that the rate of NaCl uptake after hormonal stimulation seems practically insensitive to HCO_3^- cannot be explained by assuming that Cl^-/OH^- exchange occurs at the same rate as Cl^-/HCO_3^- exchange.

Therefore, the only reasonable explanation that can be proposed is that under our experimental conditions the turnover rate of Na^+/H^+ exchange did not exceed that of the so-called Cl^-/OH^- exchange. This possibility can be tested by

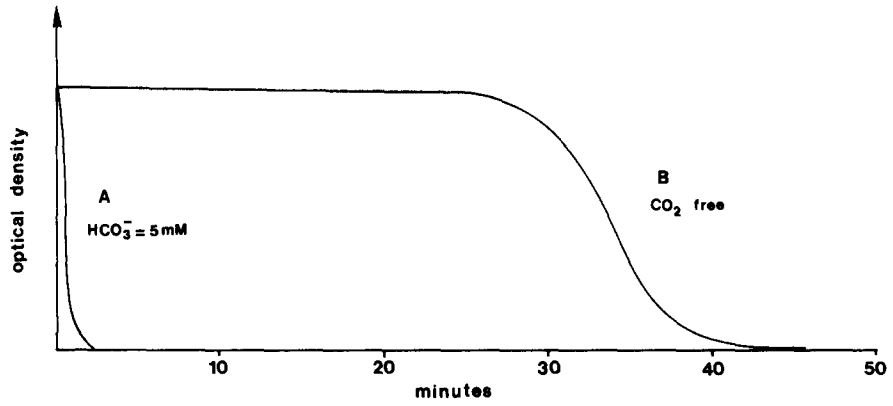

FIGURE 6. The time course of hemolysis, measured by the decrease in optical density of a suspension of trout red cells in an isotonic NH_4Cl solution. (*A*) NH_4Cl medium in equilibrium with 0.55% CO_2 (pH 7.9). (*B*) CO_2-free NH_4Cl medium. Temperature, 15°C.

using specific inhibitors. Fig. 7 illustrates the evolution of external pH in experiments performed in a CO_2-free medium. When we blocked Na^+/H^+ exchange by amiloride at the time of maximum acidification (~1 min after the addition of isoproterenol), we observed a rapid pH readjustment, and the new value of the pH steady state was close to the value of the initial pH. There is evidence that this pH readjustment occurs by exchange of an internal basic equivalent for external Cl^- since it is inhibited by DIDS (Fig. 7*A*). This result indicates that the turnover rate of Na^+/H^+ exceeds that of Cl^-/OH^-. Fig. 7*B* shows that 12 min after stimulation of red cells by isoproterenol, the Cl^-/OH^- exchange is still lower than that of Na^+/H^+, and this difference disappears at 15–20 min (Fig. 7*C*). If this interpretation is correct, one would expect that when the turnover rate of the anionic exchange is stimulated by HCO_3^-, the apparent delay in pH equilibration would disappear. When a similar experiment was

performed in a medium containing 2 mM HCO_3^-, the addition of isoproterenol induced some acidification ($\Delta pH = 0.04–0.07$), but when we blocked Na^+/H^+ exchange by amiloride 1 min later, no significant pH readjustment occurred. This clearly indicates that in the theoretically CO_2-free medium the turnover rate of the Na^+/H^+ exchanger initially exceeded that of the Cl^-/OH^- exchanger, and, owing to the decrease of the Na gradient across the membrane with time, the rate of Na^+/H^+ exchange progressively decreased until the rate of the so-called Cl^-/OH^- was no longer limiting. On the contrary, in HCO_3^--containing medium, Cl^-/HCO_3^- exchange was fast and did not represent a rate-limiting step for the overall reaction. Thus, we are faced with two different problems.

(*a*) An acidification of the extracellular medium occurs rapidly after the addition of isoproterenol to the cell suspension. If the anion exchange remains functional, the medium pH will decrease only to the extent that the turnover

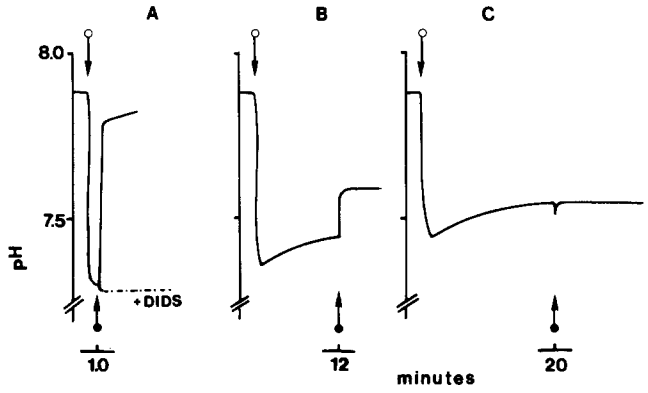

FIGURE 7. The evolution of extracellular pH after the addition of isoproterenol (5×10^{-7} M) to a red cell suspension (○) at time 0, followed by the addition of amiloride (4.5×10^{-4} M) (●) at 1, 12, and 20 min. The pH readjustment observed after the addition of amiloride can be blocked by the addition of DIDS as illustrated at 1 min. Hematocrit, 15%. 0.5 mM HEPES.

rate of the Na^+/H^+ exchanger exceeds that of the anion exchanger. Then how do we explain acidification in a medium containing HCO_3^-, since we concluded above that Cl^-/HCO_3^- exchange is potentially faster than Na^+/H^+ exchange?

The acidification observed at the onset of hormonal stimulation does not necessarily mean that the turnover rate of Na^+/H^+ exchange exceeds the carrying capacity of the anion exchanger, i.e., that it occurs because the anion exchange is kinetically late relative to the cation exchange. It could be true if the intracellular medium were unbuffered, with the excreted H^+ coming from dissociation of water. However, the experimental conditions were different. The red blood cell was strongly buffered. As soon as an H ion is excreted, the internal buffers tend to replenish the pool of intracellular exchangeable H^+ and the Na ion that has penetrated is electrically balanced by the ionization of buffer. In other words, at the onset of hormonal stimulation, only the Na^+/H^+ antiporter functions and

external acidification occurs, which results essentially from the extrusion of H^+ obtained by ionization of the internal buffers. As the protein buffer approaches saturation, then every H^+ moving via Na^+/H^+ is buffered by Cl^-/HCO_3^- exchange (simultaneous entry of NaCl) and no more acidification occurs.

(*b*) It was shown above that the rate of the Na^+/H^+ exchange induced by isoproterenol exceeds that of the anion exchanges in a CO_2-free medium but not in an HCO_3-containing medium. In other words, if NaCl entry results from the parallel functioning of the two exchangers, then in a CO_2-free medium the limiting step in the process of salt uptake is anion exchange, and in an HCO_3-containing medium it is Na/H exchange. Thus, swelling, which reflects NaCl uptake, should be stimulated by HCO_3^-. In fact, however, Fig. 4 shows that it is practically insensitive to HCO_3^-.

Using ouabain, it is possible to compare the time-dependent accumulation of Na^+ in cells suspended in a theoretically CO_2-free medium and cells suspended in a medium containing HCO_3^- (Table II). It can be seen that 6 min after hormonal stimulation, the amount of Na that penetrated into the cells was greater in the presence of HCO_3^- (83.43 $mM \cdot kg^{-1}$ cell solid) than in a CO_2-free medium (77.21 $mM \cdot kg^{-1}$ cell solid), which indicates, as expected, that anion exchange is a limiting step in the CO_2-free medium. However, the difference (6.22 mM) is small, which suggests that the turnover rate of the so-called Cl^-/OH^- exchange is only slightly slower than that of the Na^+/H^+ exchange. As a comparison, in the presence of DIDS, i.e., when the anion exchange is really limiting, the total amount of Na^+ entering during the same period of time is only 25.53 $mM \cdot kg^{-1}$ cell solid; these Na ions penetrate in exchange for H^+ and are electrically balanced by ionization of internal buffers (Baroin et al., 1984*b*). The increase in water content that is expected from the increase in Na content can be calculated as explained in Table II. From the comparison of the experimental and calculated values, it is clear that the variation in cell volume can be reasonably explained by the variation in Na content. 6 min after hormonal stimulation, the excess Na content of cells suspended in an HCO_3^--containing medium as compared with that of cells suspended in a CO_2-free medium was 6.22 mM kg^{-1} cell solid; this amount of Na osmotically forces 38.8 ml of water to penetrate into cells containing 2.360 ml of water. This means that the difference in swelling between the two batches of cells represents only 1.6% of the total intracellular water and is hardly detectable, as, for instance, in Fig. 4.

Five identical experiments were performed. Three gave similar results. In two, however, it was impossible to detect any difference in the evolution of the Na contents of cells suspended in media with or without HCO_3^-.

In brief, the turnover rate of the anion exchange is drastically reduced by removing HCO_3^- and CO_2 from the suspension medium (see Fig. 6). By following external pH after a sudden interruption of Na/H countertransport by amiloride (Fig. 7), it was shown that in a CO_2-free medium, the so-called Cl^-/OH^- exchange is kinetically late compared with the Na^+/H^+ exchange. Nevertheless, the comparison of salt uptake in different experimental conditions shows that the speeds of Cl^-/OH^- and Na^+/H^+ exchanges are very similar, which explains why a stimulatory effect of HCO_3^- on swelling is practically nonexistent.

TABLE II

Comparison of the Effect of Isoproterenol on Na and Water Contents of Trout Red Blood Cells Suspended in a Medium Containing 2 mM HCO_3^-, in a CO_2-free Medium, and in a CO_2-free Medium + DIDS 10^{-3} M

Time after hormonal stimulation	HCO_3^--containing medium			HCO_3^--free medium			HCO_3^--free medium + DIDS			
		Water content			Water content		Ion content		Water content	
	Na content	Experimental	Calculated	Na content	Experimental	Calculated	Na	Cl	Experimental	Calculated
min	*mM·kg⁻¹·cell solid*	*kg water·kg⁻¹ cell solid*		*mM·kg⁻¹·cell solid*	*kg water·kg⁻¹ cell solid*		*mM·kg⁻¹·cell solid*		*kg water·kg⁻¹ cell solid*	
0	39.07±0.42 (n = 3)	1.95	—	41.52±1.85 (n = 3)	1.96	—	49.28±0.83 (n = 3)	204.32±0.64 (n = 3)	1.97	—
6	122±1.08 (n = 3)	2.35	2.39	118.73±1.07 (n = 3)	2.33	2.36	74.81±0.81 (n = 3)	202.58±3.56 (n = 3)	2.04	2.05
21	175.76±1.47 (n = 3)	2.66	2.70	173.45±1.36 (n = 3)	2.61	2.68	82.43±0.65 (n = 3)	201.79±0.77 (n = 3)	2.05	2.07

The calculated values for water content are obtained as follows. (*a*) In the presence of DIDS, the hormone induces an Na^+ influx without an accompanying Cl^- influx. For each millimole of Na^+ entering, the intracellular tonicity increases by 1 mosmol (H^+, the counterion released by the internal buffers, is not osmotically active) and 3.125 ml H_2O enters (the saline being 320 mosmol). In 6 min, 25.53 mM Na and thus 79.78 ml H_2O enter into the cells. The water content was initially 1.97 kg water·kg^{-1} cell solid; after 6 min, it was 2.049 (*b*). In the absence of DIDS, a fraction of Na entering into the cell is not accompanied by Cl^- (the Na/H exchanges promote acidification of saline) and another fraction enters as $Na^+ + Cl^-$, two osmotically active particles. Assuming that the amount of Na^+ penetrating alone is the same as in the presence of DIDS (the magnitude of acid excretion is identical [Baroin et al., 1984*b*]), the net Na influx can be divided in two components. For example, in CO_2-free medium, in 6 min, the total net influx of Na is 77.21 mM kg^{-1} cell solid; 25.53 mM is assumed to drive 79.98 ml H_2O and 51.68 mM to drive 323.0 ml H_2O. The water content was initially 1.96 kg water·kg^{-1} cell solid; after 6 min, it was 2.36.

DISCUSSION

We previously proposed (Baroin et al., 1984*a*, *b*) that the addition of isoproterenol to an isotonic suspension of nucleated red blood cells of the rainbow trout first stimulates a transient Na^+_{out}/H^+_{in} countertransport and then a neutral entry of NaCl. The nature of the mechanism underlying this NaCl uptake was considered. Several experimental results, mainly the pharmacological properties of the system, were consistent with a model assuming an electrically silent double antiport (Na^+/H^+ and Cl^-/HCO_3^-). Some others, however, were inconsistent with this model. (*a*) The NaCl entry could not be restored when, the anion exchange process being inhibited by DIDS, a lipid-soluble artificial ion carrier allowing electrosilent hydroxyl/chloride exchange (trialkyltin derivative) was introduced in the red cell membrane. (*b*) The Na uptake was apparently Cl dependent: NO_3^-, which is an extremely effective substitute for Cl^- in the anion exchange system, was found to be a very poor substitute for Cl in the NaCl uptake process. (*c*) The net Na uptake was practically insensitive to HCO_3^-. Thus, we suggested that an NaCl cotransport process was the underlying mechanism. Additional information has now been obtained that allows an assessment of the cotransport hypothesis, as opposed to that of the double-exchanger hypothesis, to be made.

Concerning the first argument, we have experimentally confirmed, using monensin, our proposition that NaCl entry must proceed when we replace the inhibited anion exchange system of the red cell membrane (band 3) by an artificial ionophore (tributyltin) that can mediate an electrosilent Cl^-/OH^- exchange (Fig. 3). Then we also confirmed our previous results showing that when the Na^+/H^+ exchange is mediated not by monensin but by the cAMP-dependent antiporter, such a substitution is ineffective. However, as pointed out above, the trialkyltin derivatives, when used alone, have an inhibitory effect on NaCl uptake induced by catecholamine. Whatever the reason for this inhibiting effect (cytotoxic effect?), the first argument can no longer be used to discriminate between the two models.

Concerning the second argument, we have experimentally verified, using monensin, our assumption that the double-exchanger system must allow a normal swelling in NO_3^- as well as in Cl^- solution (Fig. 2). We have also confirmed our previous results showing that when Na^+/H^+ exchange is mediated not by monensin but by the cAMP-dependent antiporter, the net Na^+ uptake is drastically reduced in the NO_3^- medium, to ~70% inhibition when compared with that in the Cl^- medium (see Table I). However, we have also demonstrated in the present paper that in trout erythrocytes, NO_3^- clearly reduces the turnover rate of the Na^+/H^+ antiporter itself. This inhibition does not involve the adenylate cyclase system, but, as suggested by Parker (1984) in elegant experiments performed with dog red cells, it could have to do with the activation of the antiporter. In the double-exchanger model, Na^+/H^+ exchange is the driving component. Thus, since NO_3^- inhibits Na^+/H^+ exchange, it follows that Na uptake is inhibited. Consequently, the apparent Cl dependence of Na uptake cannot be retained as an argument to refute the double-exchanger model, even if the degree of inhibition is less for the Na^+/H^+ exchange than for the total Na uptake (Table I). In brief, the above two arguments, which we previously considered to support the cotransport model, can no longer be retained.

The third argument we considered to refute the double-exchanger model was that swelling induced by hormone shows practically no HCO_3^- dependence (Fig. 4). In trout erythrocytes, the rate of anion exchanges is very much slower in CO_2-free medium than in HCO_3^--containing medium (Fig. 6). Taken together, these data mean—if NaCl uptake and thus swelling result from a dual-exchange process—that the anion exchanges (including the so-called Cl^-/OH^- occurring in a theoretically CO_2-free medium) are not rate-limiting to the net influx of NaCl. On the other hand, the delay in equilibration of the proton gradient established by the Na^+/H^+ countertransport (Fig. 7) suggests that the activity of the anion exchanger may be slower than that of the Na^+/H^+ pathway and thus rate-limiting to net NaCl uptake when the experiment is performed in CO_2-free medium, but not in HCO_3^--containing medium. This apparent paradox is explained by the fact that in a CO_2-free medium, the rate of anion exchanges is only slightly slower than that of cation exchanges and therefore the expected stimulatory effect of HCO_3^- on salt uptake and swelling is hardly significant (Table II). The observation of Parker (1983) that, in dog red blood cells, reciprocal Na and proton movements were not stimulated by bicarbonate could probably be explained in the same way. On the other hand, the stimulatory effect on salt uptake reported by Cala (1983) in *Amphiuma* red cells is difficult to interpret: indeed, the isoproterenol-stimulated Na^+/H^+ exchanges reported here occur at rates far exceeding the movements of Na recorded in *Amphiuma* in response to shrinkage of the cells.

In conclusion, it seems reasonable to propose that the coupled entry of Na^+ and Cl^- induced by isoproterenol in trout red blood cells results from the parallel and simultaneous exchange of Na^+_{out} for H^+_{in} (via an Na^+/H^+ antiporter) and Cl^-_{out} for HCO_{3in}^- (via the anion exchanger located in band 3 protein). First, this proposition is consistent with several experimental findings, mainly the effect of various drugs on salt uptake and the stimulation of a cAMP-dependent Na^+/H^+ antiporter by isoproterenol (Baroin et al., 1984*a*, *b*; Mahé et al., 1985). Second, the present study shows that the three arguments that we previously considered to be inconsistent with the double-exchanger model can no longer be considered to be so.

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