

Desensitization by External Na of the Cyclic AMP-dependent Na⁺/H⁺ Antiporter in Trout Red Blood Cells

F. GARCIA-ROMEU, R. MOTAIS, and F. BORGESÉ

From the Laboratoire Jean Maetz, Département de Biologie du Commissariat à l'Energie Atomique, 06230 Villefranche-sur-Mer, France

ABSTRACT The erythrocytes of the trout, *Salmo gairdneri*, react to β -adrenergic stimulation by activating a cyclic AMP-dependent and amiloride-sensitive Na⁺/H⁺ antiporter (see Borgese, F., F. Garcia-Romeu, and R. Motais, *Journal of General Physiology*, 1986, 87:551–566). The present study traces the kinetic behavior of the unidirectional Na fluxes after stimulation by isoproterenol. A very considerable increase (100-fold) of the unidirectional Na influx (J_{in}^{Na}) follows the addition of isoproterenol to the erythrocyte suspension. After 1.5 min, J_{in}^{Na} falls suddenly, and asymptotically diminishes toward the nonstimulated flux level. The unidirectional Na efflux (J_{out}^{Na}) proceeds according to similar kinetics. The decrease of J_{in}^{Na} and J_{out}^{Na} is not linked to either a change in the driving forces of the transported ions or a decrease of the cyclic AMP concentration but to a desensitization of the Na⁺/H⁺ antiporter. This desensitization is dependent on the external Na concentration and is not controlled by internal Na, cell swelling, or external Ca.

INTRODUCTION

In the presence of catecholamines, the permeability of trout erythrocytes to Na is greatly increased by stimulation of an Na⁺/H⁺ antiporter, facilitating a downhill entry of Na ions. This Na entry is accompanied by an uptake of Cl in exchange for HCO₃ or OH ions (Baroin et al., 1984; Cossins and Richardson, 1985; Borgese et al., 1986); the consequent osmotic entry of water causes the erythrocytes to swell. Stimulation of the cation antiporter is cAMP dependent (Mahé et al., 1985) and amiloride sensitive (Baroin et al., 1984; Cossins and Richardson, 1985; Mahé et al., 1985). The time curve of the catecholamine-stimulated net Na flux goes through a maximum in the first few minutes and then it decreases asymptotically toward the nonstimulated flux value (Baroin et al., 1984; Borgese et al., 1987a).

Various hypotheses could explain the reduction of the net Na absorption with

Address reprint requests to Dr. F. Garcia-Romeu, Laboratoire Jean Maetz, Département de Biologie du Commissariat à l'Energie Atomique, B. P. 68, 06230 Villefranche-sur-Mer, France.

time. The present study concerns the kinetics of the unidirectional Na fluxes: it shows that the diminution of the net Na absorption is not a consequence of reduced driving forces acting on the Na^+/H^+ antiporter or of a fall in the cAMP concentration; rather, it is the result of desensitization of the exchanger after a transitory phase of activation. The mechanism of antiport desensitization does not involve either cell swelling or the intracellular Na concentration but is dependent on the Na concentration of the external medium.

MATERIALS AND METHODS

Fish Blood and Experimental Solutions

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 saline buffered with HEPPS (15 mM) at the physiological pH (pH 8.0), and the buffy coat was removed. The red blood cells were then suspended at a hematocrit of 20% and incubated overnight at 4°C in the saline (pH 8.0) to ensure that they had reached a steady state with respect to ion and water contents before experimental treatment. After the incubation period, red cells were washed four times in the experimental solution; the hematocrit was maintained at 20%.

The washing saline contained (millimolar): 145 NaCl, 5 CaCl_2 , 1 MgSO_4 , 4 KCl, 5 glucose. The experiments were made at 15°C with the saline buffered with HEPES (15 mM; pH 7.50). In some experiments, salines of different pH's were used (see Results), in which cases buffers (15 mM) with pK_a 's in the region of the desired pH were utilized. KCl or choline Cl replaced the Na in experiments in which the Na concentration of the Ringer solution was reduced. Since the rates of swelling and Na absorption are influenced by the oxygen concentration of the medium (Motais et al., 1987), all experiments were made in solutions flushed with N_2 .

Preparation of Samples and Cell Water Content

At intervals after the addition of isoproterenol or forskolin, samples of the whole suspension were poured into nylon tubes and centrifuged at 30,000 g for 10 min in a refrigerated centrifuge. These specially prepared tubes contain up to 170 μl .

For each time sample, at least three 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. The cells were then expressed with a close-fitting plastic rod onto weighed aluminium foil. After weighing, the packed cells were dried to constant weight for at least 10 h at 90°C and reweighed. Cell water content is expressed as grams of water per gram of cell solid.

Na Content

The packed dry cells with their aluminium foil were put in 5 ml distilled water and mixed carefully for 4 h. 100 μl of 70% perchloric acid was then added to the suspension. After centrifugation at 50,000 g for 15 min, the clear supernatant was saved for analysis of sodium by means of an Eppendorf (Hamburg, Federal Republic of Germany) flame photometer. A trapping correction of 1.5% was routinely applied to the final calculation (see below). Na content is expressed in micromoles per gram of cell solid.

Unidirectional Na Influx

Unidirectional Na influxes were measured with tracer quantities of ²²Na (final concentration, 0.4 μCi/ml). To measure the initial unidirectional flux immediately after stimulation ($t_0 - t_{3\text{min}}$), ²²Na solution with isoproterenol or forskolin was added to the cell suspension (20% hematocrit) and mixed thoroughly. The suspension was then divided between at least three nylon tubes and the reaction was terminated 3 min later by centrifugation at 30,000 *g* in the refrigerated centrifuge. Flux measurements were made after longer time intervals in hormone or forskolin by adding suspensions of stimulated cells to the ²²Na solution and the reaction was terminated, as before, after 3 min. Extracellular trapping was estimated by adding the isotope to a suspension of non-stimulated cells immediately before centrifugation. An extracellular space of 1.5% was found.

After centrifugation, the nylon tubes were treated as described above. Triplicate aliquots (30 μl) of the supernatant were counted. The packed cell mass was weighed immediately and dried for at least 10 h at 90°C. After weighing, the dry pellets were counted in a well-type counter. The unidirectional Na influx was expressed in micromoles per gram of dry cells per minute. The data obtained were taken to represent the flux values at the middle of the measurement period; e.g., the flux measured for the 0–3-min period after addition of hormone represents the flux at 1.5 min, and that between 10 and 13 min represents the flux at 11.5 min. In order to avoid an Na⁺/K⁺ exchange mediated by Na⁺/K⁺-ATPase, ouabain (10⁻⁴ M) was added to the suspensions before experimentation. In certain experiments, this was not done; in those cases, the glycoside was added to each aliquot to inhibit the radioactive return flux for the 3 min of flux measurement.

Extracellular and Intracellular pH

pH measurements were made with a Radiometer (Copenhagen, Denmark) BMS3 MK2 blood microsystem linked to a Metrohm (Herisau, Switzerland) 632 pH meter. Aliquots of suspension were centrifuged in 600-μl-capacity nylon tubes for 10 min at 30,000 *g*. The supernatant was used for the extracellular pH (pH_o) measurements. The red cell pellets for intracellular pH (pH_i) determinations were frozen, thawed for 5 min, and refrozen. An acid shift occurs when samples are left unfrozen; the pH_i measurements were therefore made immediately after a second thawing of each pellet. Three samples were measured from each aliquot.

Modification of Internal Ionic Content

Nystatin, a polyene antibiotic, greatly increases the monovalent ion permeability. It can thus be used to increase the internal Na concentration of the cells with a corresponding diminution of K, thereby maintaining the same cellular volume. Techniques used on blood cells of other species (Cass and Dalmark, 1973; Schmidt and McManus, 1977; Haas et al., 1982) were modified to give a good reversibility of permeabilization after washing free from nystatin. Cells were suspended at 2% hematocrit in loading solution (145 mM NaCl or KCl plus 50 mM sucrose) buffered with 15 mM HEPES (pH 7.80). Nystatin was added to a final concentration of 10 μg/ml, and the cells were incubated for 10 min at 0°C: higher doses or longer contact with the antibiotic can cause an irreversible increase of the monovalent cation permeability. To restore impermeability, the nystatin-treated cells were washed six times in nystatin-free loading solution at 25°C in the presence of 1% bovine serum albumin (fraction V) and 10⁻⁴ M ouabain, buffered with HEPES, and titrated at pH 7.80. Before adjusting the hematocrit to 20%, the cells

were washed three more times with N₂-gassed normal saline (145 mM NaCl, buffered with HEPES, pH 7.50) with 50 mM saccharose and 10⁻⁴ M ouabain at 16°C.

Cyclic AMP

Cyclic AMP was measured by radioimmunoassay as previously described (Mahé et al., 1985).

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 and added to the experimental suspensions to give a final concentration of 5.5 × 10⁻⁷ M. In a certain number of experiments, lower concentrations were used. Forskolin (Calbiochem-Behring Corp., La Jolla, CA) as a 1.5 × 10⁻² M ethanol solution, was added to the suspensions to give a 1.5 × 10⁻⁴ M concentration. Nystatin (Mycostatin, Sigma Chemical Co.) was dissolved in dimethylsulfoxide (Fluka Chemie, Buchs, Switzerland) at a concentration of 20 mg/ml and this stock solution was added to suspensions to give a final concentration of 10 µg/ml. Ouabain (Sigma Chemical Co.) was dissolved in dimethylsulfoxide (Fluka Chemie) at a concentration of 10⁻¹ M. This solution was added to the suspensions to give a concentration of 10⁻⁴ M.

The following buffers were obtained from Merck, Sharp & Dohme (West Point, PA): *N,N*-bis(2-hydroxyethyl)-2-aminoethanesulfonic acid (BES; pK_a at 20°C = 7.15); HEPES (pK_a at 20°C = 7.55); *N*-(hydroxy-2-ethyl)-piperazine *N'*-propane sulfonic acid (HEPPS; pK_a at 20°C = 8.00); *N*-[tris-(hydroxymethyl)methyl]glycine (tricine; pK_a at 20°C = 8.15).

RESULTS

Evolution of Unidirectional Na Fluxes after Hormonal Stimulation

Fig. 1 shows the time dependence of the unidirectional Na influx (J_{in}^{Na}) and efflux (J_{out}^{Na}) after stimulation of the Na⁺/H⁺ antiporter by isoproterenol in the presence of ouabain, which had been added to block active Na extrusion by the Na⁺/K⁺ pump. There was first a very rapid activation of Na influx, which reached a maximum at 1.5 min, with an abrupt reversal of slope and a subsequent diminution; in all cases, this could be expressed as a simple exponential equation of the type $J_{in}^{Na} = be^{-at}$. In Fig. 1, $J_{in}^{Na} = 40.42e^{-0.021t}$ ($r = 0.99$; $t_{1/2} = 34$ min). Measurements made each minute for the first 10 min after the addition of isoproterenol showed that the maximum J_{in}^{Na} occurred before the second minute. Ouabain had no effect on the evolution of J_{in}^{Na} (results not shown).

Since [Na⁺]_o is constant during the experiment, this rapid diminution of J_{in}^{Na} could be explained by a decrease in [H⁺]_i, the partner ion involved in the exchange. Indeed [H⁺]_i can limit the activity of the antiport either thermodynamically (i.e., acting as substrate for the Na_o⁺/H_i⁺ antiporter) or kinetically (i.e., as a modulator ion) interacting with an internal modifier site (Aronson et al., 1982; Grinstein et al., 1984; Grinstein and Rothstein, 1986).

Fig. 2 shows the time dependence of the effect of isoproterenol upon pH_i and J_{in}^{Na} for red blood cells adapted over a wide range of pH_o (6.93–8.22) corre-

sponding to a pH_i range of 6.77–7.70. In the most acid erythrocyte suspension, the [H⁺]_i before hormonal stimulation was 8.5 times that of the most alkaline suspension and this difference persisted after the addition of isoproterenol. At the start of inactivation, 1.5 min after isoproterenol addition, the pH_i values of the *a* and *c* cell batches were 7.78 and 6.83, respectively, a difference of almost one pH unit. If the factor responsible for the reduction of J_{in}^{Na} were simply the drop in the [H⁺]_i, one would expect (i) slight variations of Na influxes after their maxima in aliquots *a* and *c*, since at this time the pH_i was constant (*a*) or almost so (*c*), and (ii) an increased J_{in}^{Na} in preparation *b* when the pH_i tended to return

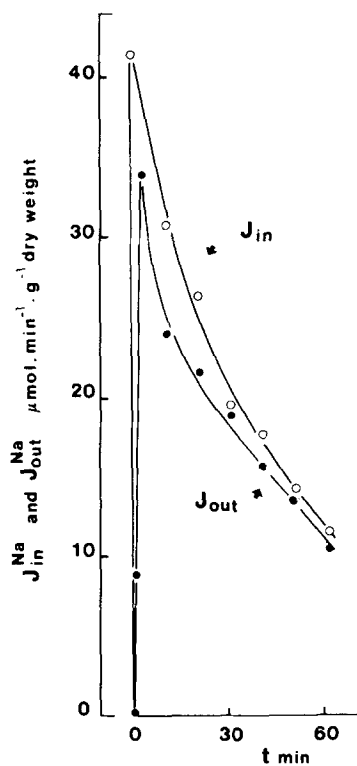


FIGURE 1. Na influxes (○) and effluxes (●) of trout erythrocytes as functions of time after stimulation with isoproterenol (5.5×10^{-7} M). The efflux was calculated as the difference between influx and net absorption of Na. Ouabain: 10^{-4} M, pH 7.50. Note the rapid increase of both fluxes and their abrupt decrease after a maximum at 1.5 min for J_{in}^{Na} and 4.5 min for J_{out}^{Na} .

to its original, more acid, value. Nevertheless, after an initial, rapid activation, J_{in}^{Na} abruptly decreased in a very similar way for the three batches of cells: the sudden changes of the J_{in}^{Na} slopes were simultaneous in the three samples and the $t_{1/2}$ of the diminution was ~ 14 min. The only striking difference was between the maximum flux values, but this was due to their pH dependence (Borgese et al., 1987b). It can be concluded from these results that the rapid drop in J_{in}^{Na} is not caused by a decrease of the [H⁺]_i and thus by a limitation of one of the antiporter substrates. Neither can it be attributed to a control of Na permeability by a modulator site sensitive to protons, as will be discussed below.

The unidirectional Na effluxes, calculated as the difference between J_{in}^{Na} and the simultaneous net absorption, are also shown in Fig. 1. The maximum J_{out}^{Na} was found to occur at ~ 4.5 min; the efflux then diminished rapidly and progressively with a time dependence similar to that of J_{in}^{Na} , whereas $[Na^+]_i$ increased 10-fold (Fig. 3). Indeed, it appears that the two fluxes are inactivated quasi-simultaneously and the slight delay of the efflux inactivation may be only apparent. The Na permeability reduction could be partially masked by the large increase of $[Na^+]_i$, which occurs very rapidly after addition of the hormone.

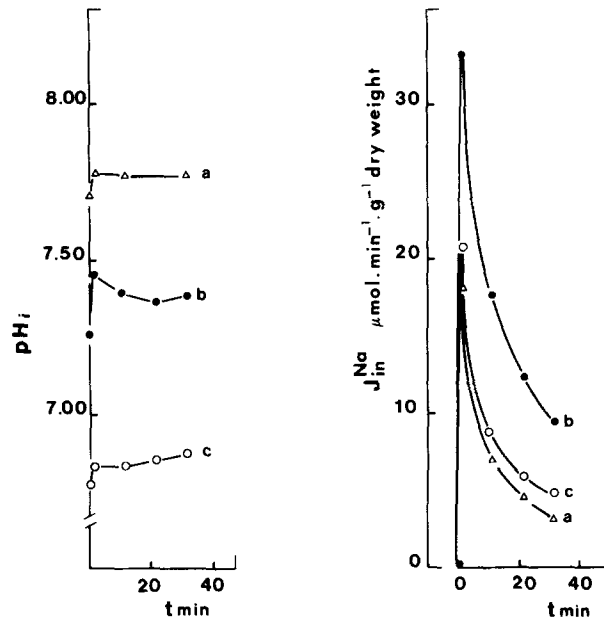


FIGURE 2. Evolution of pH_i and J_{in}^{Na} after addition of isoproterenol (5.5×10^{-7} M) in the presence of ouabain (10^{-4} M). Three preparations of erythrocytes were equilibrated at different pH values and the measurements were made at each pH. (a) Tricine buffer; (b) HEPES buffer; (c) BES buffer. The pH_o and pH_i , respectively, of each preparation immediately before isoproterenol addition are: (a) 8.22 and 7.70; (b) 7.51 and 7.26; (c) 6.93 and 6.77.

In conclusion, the concentration of ions involved in the exchange is not a limiting factor responsible for the reduction of unidirectional Na fluxes. Thus, the reduction in the transport rate of Na after the activation phase is due to a diminution in the activity of the transporter per se.

Permeability Changes and Time Dependence of the Cellular cAMP Concentration

Na^+/H^+ exchanger activity is controlled by cAMP (Mahé et al., 1985). A diminution of Na permeability could result from a reduction of the intracellular cAMP concentration after desensitization of the β -adrenergic receptor (see

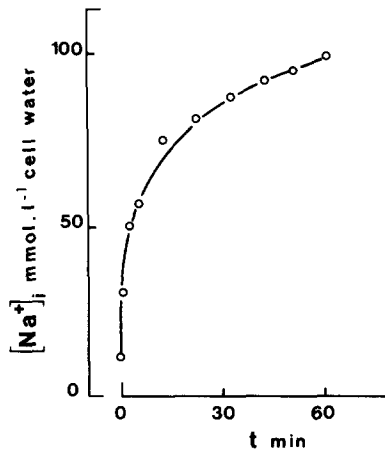


FIGURE 3. $[Na^+]_i$ as a function of time after isoproterenol addition (same experiment as in Fig. 1). Note that $[Na^+]_i$ increases throughout the experiment but that the effluxes (Fig. 1) do not follow this increase.

Harden, 1983; Stiles et al., 1984; Hertel and Perkins, 1984; Levitsky, 1986). Alternatively, above a certain nucleotide concentration, cAMP may inactivate the antiporter.

The former possibility was investigated in the experiments recorded in Fig. 4. Fig. 4 A represents the time dependence of J_{in}^{Na} and the cAMP concentration per

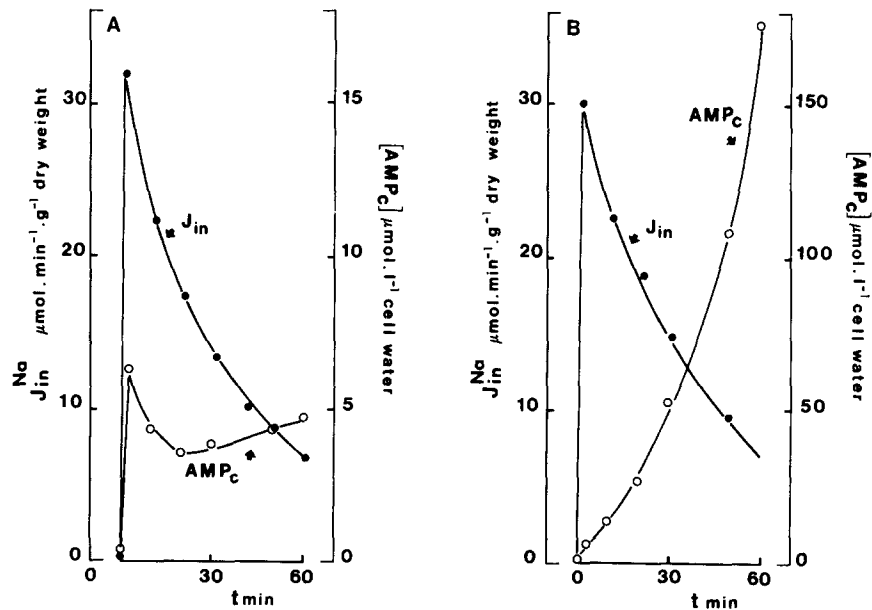


FIGURE 4. J_{in}^{Na} and cellular cAMP concentration as a function of time after stimulation with either isoproterenol (A) or forskolin (B). Note the 10-fold difference in cAMP concentration scale between A and B and the similarity of the time dependence of the J_{in}^{Na} values, which indicate that the rapid fall in unidirectional influxes after stimulation cannot be due to a diminution of the cAMP concentration. Isoproterenol: 5.5×10^{-7} M; forskolin: 1.5×10^{-4} M; ouabain: 10^{-4} M; pH_i: 7.50.

liter cell water after isoproterenol stimulation. During the first 20 min after isoproterenol stimulation, J_{in}^{Na} and cellular cAMP changed in parallel; then the cAMP concentration increased slightly, while J_{in}^{Na} continued to fall. These results are not sufficiently clearcut to exclude a possible correlation between the cAMP level and the reduction of Na permeability. To obtain an unequivocal answer to this question, we stimulated maximal cAMP synthesis by adding forskolin to give a concentration of 1.5×10^{-4} M in the erythrocyte suspension, the diterpene short-circuiting the receptor and acting directly on the adenylate cyclase. The results are shown in Fig. 4 B: the kinetics of J_{in}^{Na} are the same as after isoproterenol stimulation. The reduction of the J_{in}^{Na} was not prevented by the presence of very high intracellular concentrations of cAMP, the level of which rose during

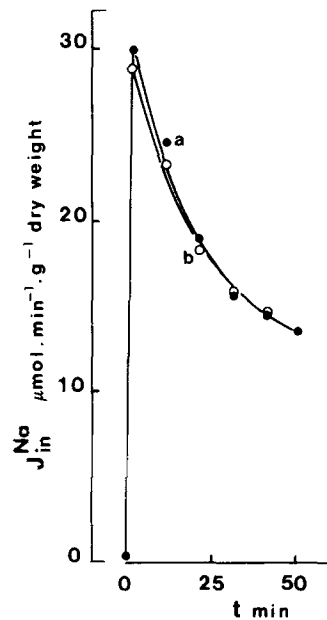


FIGURE 5. J_{in}^{Na} of two preparations of erythrocytes incubated for 1.5 h in choline saline in the presence (a) and absence (b) of forskolin (5×10^{-5} M). After the incubation period, the choline saline was replaced by normal Na saline plus forskolin (5×10^{-5} M). At this point, the cAMP concentration of the preparation incubated without forskolin (b) was $0.7 \mu\text{M liter}^{-1}$ cell water and that of the preparation incubated with forskolin (a) was $25 \mu\text{M liter}^{-1}$ cell water, more than twice the maximum concentration normally obtained after isoproterenol stimulation. Since external Na is lacking during incubation, these cells do not swell during this period; the water content passes from 1.75 to 1.70 ml/g dry wt after 1.5 h incubation. Ouabain: 10^{-4} M; pH_o: 7.50.

the experiment to a concentration 27 times the maximum after hormonal stimulation (note that the scales of the ordinates of A and B differ 10-fold). The evolution of cell swelling in the two cases was identical despite the large differences in cAMP concentration (not shown). These results demonstrate that the reduction of the Na permeability of the membrane after activation is not caused by diminution of the cellular cAMP concentration, as would occur after desensitization of the β -adrenergic receptor.

The second possibility was studied in the experiments illustrated in Fig. 5. The erythrocytes were incubated in choline saline with or without forskolin for 1.5 h. In the cells incubated with forskolin (5×10^{-5} M), the cellular cAMP concentration was found to be $25 \mu\text{M/liter}$ cell water, i.e., more than twice the max-

imum concentration after isoproterenol stimulation. Cell volumes remained unchanged since there was no Na in the incubation solution. At the end of the incubation period, the cells of the two batches were resuspended in Na saline plus forskolin. Fig. 5 shows that the high intracellular cAMP concentration maintained throughout the long incubation period did not modify the J_{in}^{Na} kinetics and is therefore not the causal factor in the Na permeability reduction after the activation phase.

Processes Involved in Antiporter Desensitization

From the results described above, it can be concluded that the sudden drop in Na permeability occurring soon after the activation phase is the result of a cAMP-independent change in the activity of the Na⁺/H⁺ antiporter. We have called this process desensitization of the antiporter, and we have investigated the parameters involved.

The nature of the Na⁺/H⁺ antiporter inactivation suggests a regulatory phenomenon controlling Na entry and consequently net water absorption. It is reasonable to suppose that cell volume changes and/or an increase of [Na⁺]_i owing to antiport activity are the stimuli for a decrease in J_{in}^{Na} by a feedback mechanism. One method of inducing variations of the net Na and water absorption in order to study the effects of changes of these parameters on the desensitization is to subject the erythrocytes to different Na concentrations before stimulation.

Fig. 6 illustrates the time dependence of J_{in}^{Na} at different [Na⁺]_o values. The flux curves differ considerably according to [Na⁺]_o. The initial unidirectional Na influx increases with [Na⁺]_o. As illustrated in the inset to Fig. 11, this activation phase is a saturable function of [Na⁺]_o. The dependence of the fall in J_{in}^{Na} on [Na⁺]_o follows a different pattern. When erythrocytes are suspended in solutions containing little Na (5–20 mM), there is no influx inactivation during the 60-min experimental period. When the external medium contains 145 mM Na, however, at 60 min the influxes fall to 31% of the maximum value measured at 2 min. As expected (see the inset to Fig. 6), net water absorption is very different under the three different [Na⁺]_o conditions. At 5, 20, and 145 mM [Na⁺]_o, the $\Delta gH_2O/g$ dry wt values between 0 and 58 min were 0.04, 0.43, and 1.16, respectively. The total amounts of Na having penetrated into the cells during the same period of time, measured as unidirectional influx, were 210, 296, and 340 $\mu\text{mol/g}$ dry wt at these three [Na⁺]_o values.

Thus, the reduction of water absorption at reduced [Na⁺]_o is proportionally far greater than that of J_{in}^{Na} . This is due to the fact that [Na⁺]_i in normal saline is 10–15 mM/liter cell water: at 5 mM [Na⁺]_o, the chemical gradient of Na is thus reversed and is very small at 20 mM. With such slight gradients, J_{in}^{Na} must be rapidly compensated by J_{out}^{Na} , with the result that net Na and water absorptions become zero.

We have thus found conditions that block the expression of the Na⁺/H⁺ antiporter desensitization. However, in the experiment of Fig. 6, there are three closely interrelated variables: [Na⁺]_i, [Na⁺]_o, and cell swelling. A further series of experiments was carried out to define the individual roles of these variables.

It is possible that cell swelling, beyond a certain threshold, acts as a signal inactivating the unidirectional Na influxes. This being so, cells previously swollen by hypotonic shock should show an accelerated desensitization, and conversely this should be retarded in cells shrunken by hypertonic shock.

In Fig. 7, the kinetics of cell swelling and unidirectional Na influx of a control sample of cells (320 mosmol) are compared with those of a sample of cells swol-

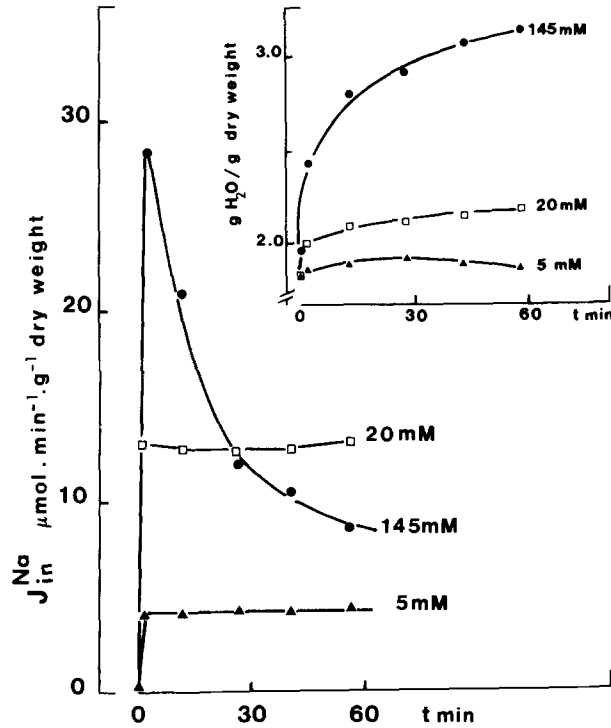


FIGURE 6. J_{in}^{Na} as a function of time after isoproterenol stimulation in erythrocyte preparations with three different external Na concentrations (145, 20, and 5 mM). At the two lower concentrations, NaCl was replaced by choline chloride in the solutions. The inset shows the net water absorptions as a function of time; before isoproterenol addition, the water contents (g H₂O/g dry wt) of the three samples were very similar: 1.85, 1.86, and 1.98 at 5, 20, and 145 mM, respectively.

len by hypotonic shock (220 mosmol), the Na concentration of the suspension being identical in the two cases (see the legend to Fig. 7). The initial volume of the cells after hypotonic shock is 30% greater than that of the control cells. It is even higher than that of cells in isotonic saline at the moment of the fall in the unidirectional fluxes. Nevertheless, J_{in}^{Na} inactivation is initiated simultaneously in the two samples. Since net water absorption after isoproterenol treatment was equivalent in the two samples, the cell volume in the hypotonic medium was always much greater than that in the isotonic medium. In spite of this, the rate of decrease in J_{in}^{Na} in the swollen hypotonically shocked cells was

no greater, the $t_{1/2}$ of the J_{in}^{Na} of these cells being 19 min, while that of control cells was 18 min. From these results, one can conclude that the increase of cell volume is not the factor responsible for the sudden desensitization of the Na⁺/H⁺ antiporter or for its subsequent kinetics.

The independence of Na⁺/H⁺ antiporter desensitization and cell volume was confirmed by the experiment illustrated in Fig. 8, in which cells had been shrunk by hypertonic shock. After such a shock, trout red cells adjust their volume by Na uptake, but this volume-regulatory increase, initiated only by strong shocks, is of small amplitude and is partly insensitive to amiloride (results not shown).

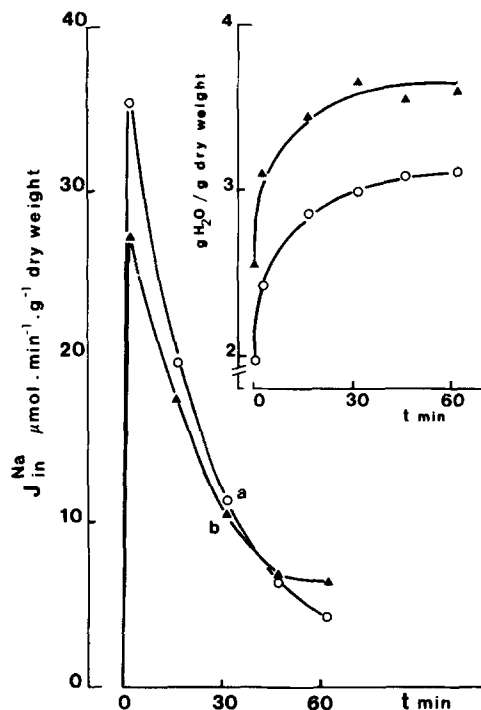


FIGURE 7. J_{in}^{Na} and net water absorption (inset) of two erythrocyte preparations, one control (a) and the other subjected to hypotonic shock just before isoproterenol treatment (b). The osmotic pressures of the suspension media were 320 mosmol (a) and 220 mosmol (b), established by addition of water buffered with 15 mM HEPES (pH 7.55). In the 320 mosmol solution, the Na content was diluted with an isotonic choline chloride solution to a concentration of 100 mM as in the hypotonic solution. Isoproterenol: 5.5×10^{-7} M; ouabain: 10^{-4} M; pH_o: 7.54.

is of small amplitude and is partly insensitive to amiloride (results not shown). Inactivation of J_{in}^{Na} occurs, but the curve as a function of time differs from that described here after adrenergic stimulation. In order to avoid the complication of two stimulating factors with different kinetics and an ill-defined relationship, isoproterenol was added 50 min after hypertonic shock in the experiment illustrated in Fig. 8. During this 50-min period, the unidirectional Na influxes stimulated by the hypertonic shock fell to the nonstimulated level and the cell volume re-equilibrated at 20% below the control level. The experiment shows that: (a) In spite of the big difference in the volume of the two cell preparations at the moment of isoproterenol addition, the hormone-stimulated J_{in}^{Na} values decrease simultaneously; this would not have been the case if volume had been

the stimulus initiating inactivation. (b) Contrary to what would be expected from a simple volume dependence, the speed of fall in J_{in}^{Na} was greater in the volume-reduced cells ($t_{1/2} = 24$ min) than in the control cells ($t_{1/2} = 31$ min). (c) The total J_{in}^{Na} during the 53-min period between isoproterenol addition and the end of the experiments was 1.26 mmol/g dry wt in the control cells and 1.10 mmol/g dry wt in the hypertonically shocked cells, i.e., 87% of the control value. In the latter cells, swelling was only 43% of that of the control cells, because in 587 mosmol medium, each millimole of Na is associated with 1.7 ml of water, while at 320 mosmol, each millimole of Na is associated with 3.13 ml of water.

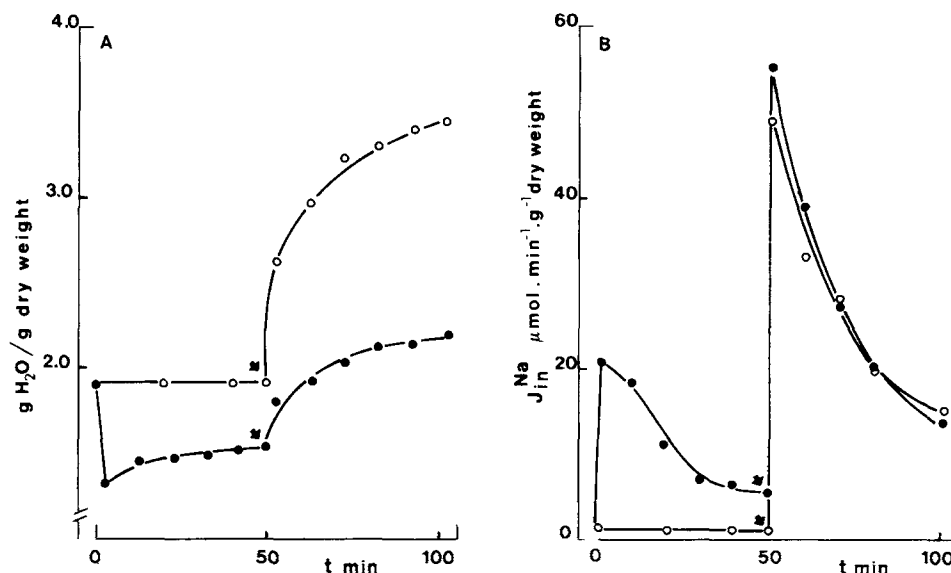


FIGURE 8. Net water absorption (A) and J_{in}^{Na} (B) of two erythrocyte preparations, one in isotonic solution (320 mosmol; ○) and the other in hypertonic solution (587 mosmol; ●) made from 2.9 M NaCl diluted 22 times. Isoproterenol was added (at arrows) 50 min after the beginning of the experiments, by which time the measured parameters of the cells in hypertonic shock had attained their new equilibria. Isoproterenol: 5.5×10^{-7} M; ouabain: 10^{-4} M; pH_i: 7.55.

Since cell swelling after isoproterenol treatment is a function of net Na absorption, the lack of correlation between cell swelling and the kinetics of the Na⁺/H⁺ antiporter desensitization points to the independence of desensitization and [Na⁺]_i. More direct confirmation of this was obtained by studying the kinetic behavior of the J_{in}^{Na} of cells whose [Na⁺]_i had been modified by nystatin treatment before isoproterenol stimulation.

Table I gives the Na and K concentrations and water content of two aliquots of cells incubated in presence of nystatin in salines containing either 145 mM Na or 145 mM K, washed to remove the antibiotic, and resuspended in normal saline (145 mM Na, 4 mM K) with ouabain (10^{-4} M) but without isoproterenol.

After rinsing, the Na and K permeabilities were found to be low, the [Na⁺]_i and [K⁺]_i remaining stable over 60 min. The unidirectional Na influxes of these nystatin-treated cells were low (Fig. 9, *c* and *d*).

If the Na⁺/H⁺ antiporter inactivation stimulated by isoproterenol is triggered by an increase in [Na⁺]_i, one would expect a more rapid reduction of J_{in}^{Na} in cells loaded with Na than in those with a normal Na content. Fig. 9 illustrates the time dependence of the unidirectional Na influxes of a cell sample with high [Na⁺]_i (*a*) and one with low [Na⁺]_i (*b*).

The fall in the J_{in}^{Na} of the two experimental preparations was simultaneous, irrespective of their [Na⁺]_i (248 mM and 13 mM Na/liter cell water, respectively). It can be seen that the rate of decay of J_{in}^{Na} was much slower in preparation *a*, the $t_{1/2}$ being 79 min as compared with 32 min in *b*. We have no explanation for the differences in maximal flux and the rate of desensitization between the two samples. Perhaps the large changes in [Na⁺]_i and [K⁺]_i result in a complex interference in the cellular metabolism.

TABLE I
Na, K, and Water Contents

	145 mM Na ⁺ saline		145 mM K ⁺ saline	
	5 min	60 min	5 min	60 min
	<i>μmol/g dry wt</i>		<i>μmol/g dry wt</i>	
[Na ⁺]	247.8 ± 1.9	251.5 ± 2.1	12.9 ± 1.5	11.9 ± 1.8
[K ⁺]	72.2 ± 0.7	71.8 ± 0.3	357.0 ± 1.6	348.1 ± 8.5
g H ₂ O/g dry wt	1.65 ± 0.01	1.70 ± 0.01	1.74 ± 0.01	1.70 ± 0.01

Cells were incubated in the presence of nystatin (10 μg/ml) in a saline containing either 145 mM Na or 145 mM K, washed, and resuspended in Na saline (10⁻⁴ M ouabain) without isoproterenol (see Materials and Methods). The water and ionic contents were measured 5 and 60 min after washing free of antibiotic. $n = 3$. The Na and K contents of cells before nystatin treatment were 21.2 ± 0.7 (Na) and 251.6 ± 4.6 (K) μmol/g dry wt. The water content was 1.98 g H₂O/g dry wt.

The experiments illustrated in Fig. 6 showed that the reduction of the isoproterenol-stimulated J_{in}^{Na} could be dependent on [Na⁺]_o, [Na⁺]_i, or cell volume and that the process could be completely inhibited. The experiments described above have eliminated some of the possible factors involved. Thus, neither the cell volume nor [Na⁺]_i was the factor stimulating desensitization or controlling its kinetics. This conclusion was confirmed by a different approach. Experiments were designed to obtain the same net Na absorption and thus the same cell volume change from solutions with different Na concentrations. The concentrations selected were 145 mM, in which inactivation is very rapid, and 20 mM, in which there is no inactivation (Fig. 6). In these two media, the same Na uptake and cell volume change can be obtained by varying the dose of isoproterenol, using a low concentration at 145 mM and a high one at 20 mM.

If desensitization of the Na^+/H^+ antiporter is a direct function of cell swelling and the $[\text{Na}^+]_i$ increase but only an indirect function of $[\text{Na}^+]_o$, the influxes of the two samples should be inactivated in the same way since water absorption and $[\text{Na}^+]_i$ are equal. If, on the other hand, inactivated is a direct function of $[\text{Na}^+]_o$ but is independent of swelling and $[\text{Na}^+]_i$, only the influx of the cells in normal saline (145 mM) should be desensitized, as in the experiment illustrated in Fig. 6. The conclusions from the previous series of experiments can thus be confirmed.

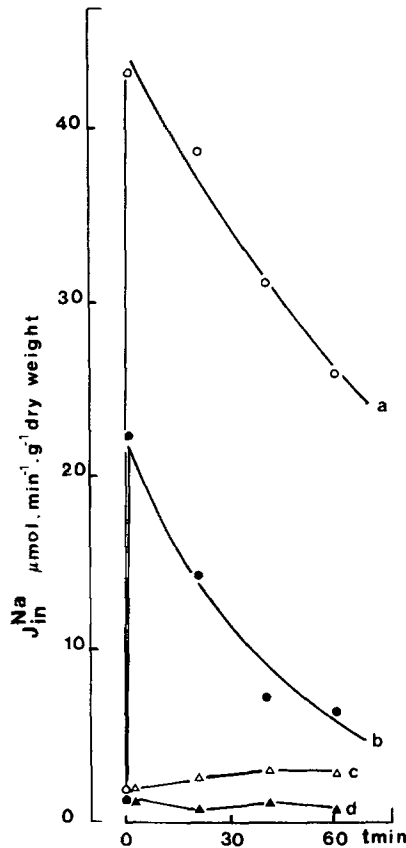


FIGURE 9. J_{in}^{Na} of erythrocytes treated with nystatin ($10 \mu\text{g}/\text{ml}$ for 10 min) to modify the intracellular Na concentration (see Materials and Methods). Loading solutions: 145 mM NaCl saline (*a* and *c*); 145 mM KCl saline (*b* and *d*). Preparations *a* and *b* were stimulated by isoproterenol; *c* and *d* acted as controls. Data for $[\text{Na}^+]_i$, $[\text{K}^+]_i$, and the water content in the controls are given in Table I. Isoproterenol (*a* and *b*): 5.5×10^{-7} M; ouabain: 10^{-4} M; pH: 7.55.

Fig. 10 shows the results of a typical experiment, which agrees with five others, based on this approach. The J_{in}^{Na} of cells in a saline with 20 mM (open circles) was not lowered, in spite of the fact that $[\text{Na}^+]_i$ and cell volume were constantly higher than in erythrocytes in 145 mM $[\text{Na}^+]_o$ (filled circles). The latter showed the typical kinetics of the desensitization process. In 20 mmol saline, the influx rates remain elevated but water absorption and $[\text{Na}^+]_i$ are similar in cells in 145 mM $[\text{Na}^+]_o$, whose J_{in}^{Na} fell. This is due to the fact that at 10 mmol $[\text{Na}^+]_i$ and 20 mmol $[\text{Na}^+]_o$, the Na gradient is rapidly dissipated after hormonal stimulation and the net flux of Na approaches zero. Thus, from the evidence of the

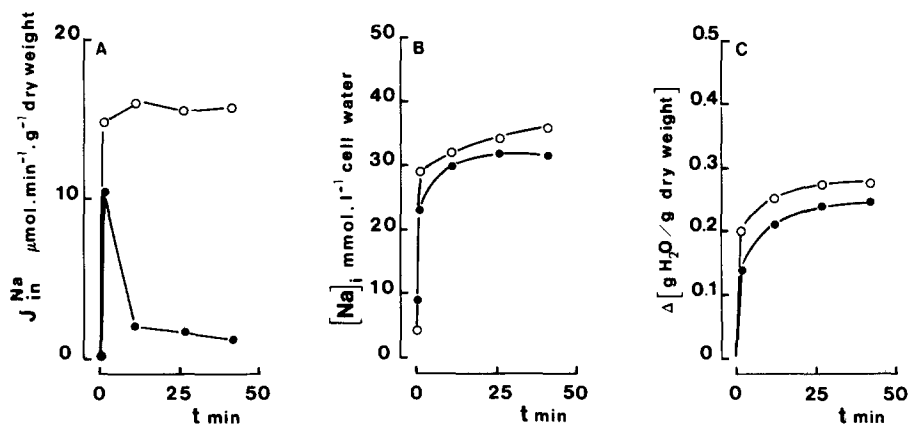


FIGURE 10. Time-dependent changes after isoproterenol addition of J_{in}^{Na} (A), net Na absorption (B), and cell swelling (C) of two erythrocyte preparations. Cells were suspended in saline containing 20 mM Na plus 125 mM choline chloride and stimulated by 5.5×10^{-7} M isoproterenol (○) and suspended in saline containing 145 mM Na and stimulated by 5.5×10^{-9} M isoproterenol (●). By modifying the dose of isoproterenol, the same Na and water absorptions can be obtained at widely different $[Na^+]_o$ values. Ouabain: 10^{-4} M; pH_o : 7.50.

experiments described above, one can conclude that desensitization of the Na^+/H^+ antiporter is dependent on the $[Na^+]_o$, but not on $[Na^+]_i$ or cell swelling.

Fig. 11 shows the percentage of reduction in J_{in}^{Na} , arbitrarily measured at 30 min, as a function of $[Na^+]_o$. It can be seen that there is no desensitization from

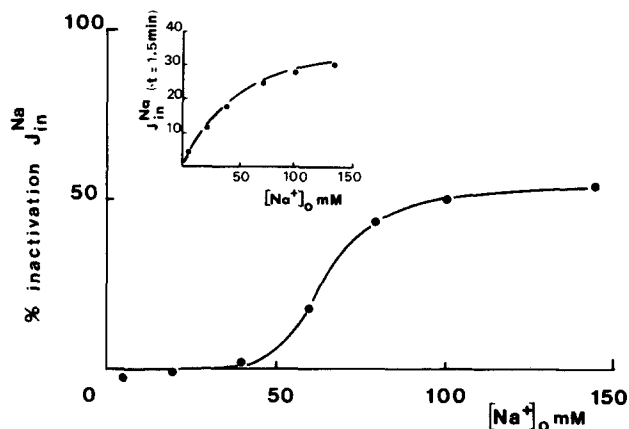


FIGURE 11. Percent decrease in J_{in}^{Na} inactivation as a function of $[Na^+]_o$. J_{in}^{Na} values were measured at 1.5 and 30 min (30 min was chosen arbitrarily) and calculated as $1 - J_{in}^{Na}(1.5 \text{ min})/J_{in}^{Na}(30 \text{ min})$. (Inset) J_{in}^{Na} at 1.5 min as a function of $[Na^+]_o$. The line connecting the points is the theoretical curve ($V_{\text{max}} = 41.7 \mu\text{mol}/\text{g}\cdot\text{min}^{-1}$ and $K_{0.5} = 47.6 \text{ mM}$). In solutions with $[Na^+]_o$ lower than 145 mM, NaCl was replaced by KCl. This gave the same result as replacing NaCl by choline chloride. Isoproterenol: 5.5×10^{-7} M; ouabain: 10^{-4} M; pH_o : 7.50.

0 to 30 mM Na in the suspension medium, but at higher Na concentrations, desensitization increases in a sigmoid fashion to level off between 100 and 145 mM. At the latter concentration, the Na influx at 30 min is only 36% of that measured at 1.5 min. This curve has a very cooperative-looking Na dependence, but since the initial speed of desensitization cannot be measured, the kinetics parameters were not obtained.

In the inset of Fig. 11, the initial J_{in}^{Na} (at 1.5 min) is shown as a function of $[Na^+]_o$. It can be seen that, in opposition to desensitization kinetics, the activation phase follows first-order kinetics with respect to $[Na^+]_o$. The data are in excellent agreement with the theoretical curve as drawn, calculated from a $K_{0.5}$ of 47.6 mM and a V_{max} of 41.67 $\mu\text{mol/g/min}$.

DISCUSSION

Evidence for Desensitization of the Na^+/H^+ Antiporter

In the trout erythrocyte, β -adrenergic catecholamines activate an Na^+/H^+ antiporter and cause a subsequent absorption of Cl by the system of pH equilibration of the band 3 protein (Cl^-/HCO_3^- or Cl^-/OH^- exchange). This results in net absorptions of Na, Cl, and water osmotically drawn by these ions (Baroin et al., 1984; Cossins and Richardson, 1985; Borgese et al., 1986). The present study shows that addition of isoproterenol to a suspension of trout red blood cells results in a strong activation of the unidirectional influx and efflux of Na, followed rapidly by a sudden reduction of these fluxes.

The following observations warrant emphasis. (a) The similarity of the time dependences of the two unidirectional fluxes strongly suggests that Na influxes and effluxes are mediated by the same transport system. This conclusion is in accordance with previous results showing that when amiloride is added to isoproterenol-stimulated erythrocytes in the presence of ouabain, the intracellular Na is immediately stabilized and thereafter remains constant (Mahé et al., 1985; Borgese et al., 1987a); i.e., the only detectable pathway for net Na fluxes is the Na^+/H^+ antiporter. (b) After the activation phase, there is an abrupt reduction of J_{in}^{Na} although $[Na^+]_o$ remains constant. This decline of Na influx cannot be explained by the observed decrease in $[H^+]_i$ (see Results), the obligate partner in the exchange process. Similarly, the abrupt reduction of J_{out}^{Na} occurs despite both the large increase of $[Na^+]_i$ and the acidification of the external medium, i.e., the increase of $[H^+]_o$.

In view of the above, the rapid decrease of Na influx and efflux observed after the activation phase cannot result from a critical decrease in the concentration of ions involved in the exchange. Several other possibilities can be considered. Since the exchange is cAMP dependent, it is possible that the intracellular cAMP concentration is reduced after desensitization of the β -adrenergic receptors. The results obtained with forskolin (Fig. 4) eliminate this possibility. The effect of a possible shift of the transmembrane electrical potential on the electrochemical gradients of transported ions cannot explain the flux modifications, since the

Na⁺/H⁺ antiporter is voltage insensitive, with a stoichiometry of one Na for one proton (Aickin and Thomas, 1977; Cala, 1980, 1983; Kinsella and Aronson, 1980; Grinstein et al., 1983; Parker and Castranova, 1984; Baroin et al., 1984).

From a consideration of data obtained with other systems (Aronson et al., 1982; Paris and Pouyssegur, 1983; Grinstein et al., 1984; Thierry et al., 1985; Grinstein and Rothstein, 1986), a third possibility could be that the rapid decrease of Na fluxes is related to the fact that antiport activity is controlled by a pH_i-sensitive mechanism. It has been suggested that the interaction of internal H⁺ with a modifier site on the cytoplasmic face of the antiporter modulates transport: at a certain pH_i, the transport is practically quiescent; below this threshold pH (the "set point"), the exchanger is activated. In this context, as the activation of the antiporter by isoproterenol induces a rapid internal alkalization (Fig. 2), one could postulate that when alkalization reaches the set point, the transport is turned off, which would explain the decrease of Na fluxes. However, this possibility can be ruled out for the following reasons. (i) In preparation *c* of Fig. 2, desensitization occurs at a pH of 6.83. Thus, if this represents the fixed set point, the antiporter in preparations *a* and *b*, which had prestimulation pH's of 7.70 and 7.26, respectively, should not have been activated. (ii) To explain our results, we have to postulate that the set point of the modifier site has been adjusted upward to different levels, depending on the pH at which the cells were adapted before hormonal exposure. In trout red cells, as in mammalian cells, H⁺ is passively distributed across the cell membrane according to a Gibbs-Donnan equilibrium (Harvey et al., unpublished results): the pH_i of adapted cells is thus a continuous function of pH_o. In such a situation, involving a wide range of pH_i values, the concept of a modifier site with numerous set points, each adapted to an equilibrium pH, is untenable. Thus, the reduction in the Na fluxes cannot be explained by the functioning of a pH_i-sensitive modifier site. On the contrary, the activation phase of Na influxes seems to be controlled by an internal H⁺ modifier site with a fixed set point at pH_i 8.0 (Borgese et al., 1987b).

In conclusion, the decrease in both J_{in}^{Na} and J_{out}^{Na} cannot be explained by (a) a modification of the concentrations of the exchanged ions as a result of antiporter activity (Figs. 1–3), (b) a pH-sensitive internal modifier site, or (c) a desensitization of the β-adrenergic receptor (see Fig. 5). In view of all these limitations, it is most reasonable to conclude that the sudden decrease of Na permeability after the activation phase is due to a change in the antiporter itself, a process we have called desensitization.

Role of External Na in the Desensitization Process

The presence or absence of Na⁺/H⁺ antiporter desensitization is a function of the Na concentration of the external medium (Figs. 6, 10, and 11), a parameter that, under normal physiological conditions, remains constant. Two of the processes that are influenced by [Na⁺]_o, net Na absorption and cell swelling, cannot account for antiporter desensitization (see Results). We have seen above that a

third dependent variable, pH_i , which is influenced by Na^+/H^+ exchange stimulation, cannot be correlated with the decay of $J_{\text{in}}^{\text{Na}}$. Thus, there is no evidence of the presence of a feedback mechanism by which the transporter would be controlled by one of the consequences of its own functioning. The only remaining conclusion is that desensitization is a function of $[\text{Na}^+]_o$ per se. The mechanism by which this control is effected remains undetermined.

An approach to a reply to this question is indicated by the experiment illustrated in Fig. 11. We pointed out that the data do not furnish precise kinetic parameters of the desensitization process since the initial rates, for technical reasons, were not recorded. However, the differences between the true rates and those we measured experimentally would probably not be sufficient to modify the general nature of the curve profoundly. Whereas the interaction of external Na with the antiporter obeys first-order kinetics during the activation phase (Fig. 11, inset), the sigmoid curve of Fig. 11 indicates that desensitization has a greater than first-order dependence on external Na. This suggests that $[\text{Na}^+]_o$, in addition to its function as a substrate of the transfer mechanism, can act on an external modifier site and allosterically control the Na^+/H^+ antiporter. (However, for another possible interpretation of this type of curve, see Aronson, 1985.) In any case, this hypothesis does not account for the delay apparently necessary for initiating the desensitization, or for the slow rate of the process. Possibly the external Na-sensitive modifier site, by a conformational change of a transport or regulatory protein, controls an intracellular metabolic reaction responsible for the Na^+/H^+ antiporter desensitization. Actually, this appears to be the most simple hypothesis for an overall interpretation of our results.

The two most important conclusions to be drawn from the present study are the following. (a) The β -adrenergic catecholamine-sensitive Na^+/H^+ antiporter in the trout erythrocyte can show a rapid desensitization that is unrelated to a desensitization of the β -adrenergic receptor. (b) The antiporter desensitization is controlled by the Na concentration of the external medium; neither the internal Na concentration nor the cell volume participate in the transporter regulation.

The authors wish to thank N. Gabillat and B. Pellissier for their excellent technical assistance.

This work was supported by C.N.R.S. grant UA 638 to the Commissariat à l'Energie Atomique.

Original version received 30 December 1986 and accepted version received 15 October 1987.

REFERENCES

- Aickin, C. C., and C. Thomas. 1977. An investigation of the ionic mechanism of intracellular pH regulation in mouse soleus muscle fibres. *Journal of Physiology*. 273:295-316.
- Aronson, P. S. 1985. Kinetic properties of the plasma membrane Na^+/H^+ exchanger. *Annual Review of Physiology*. 47:545-560.
- Aronson, P. S., J. Nee, and M. A. Suhm. 1982. Modifier role of internal H in activating the Na-H exchanger in renal microvillus membrane vesicles. *Nature*. 299:161-163.

- Baroin, A., F. Garcia-Romeu, T. Lamarre, and R. Motais. 1984. A transient sodium-hydrogen exchange system induced by catecholamines in erythrocytes of rainbow trout, *Salmo gairdneri*. *Journal of Physiology*. 356:21–31.
- Borgese, F., F. Garcia-Romeu, and R. Motais. 1986. Catecholamine-induced transport systems in trout erythrocytes. Na⁺/H⁺ countertransport or NaCl cotransport? *Journal of General Physiology*. 87:551–566.
- Borgese, F., F. Garcia-Romeu, and R. Motais. 1987a. Control of cell volume and ion transport by β -adrenergic catecholamines in erythrocytes of rainbow trout, *Salmo gairdneri*. *Journal of Physiology*. 382:123–144.
- Borgese, F., F. Garcia-Romeu, and R. Motais. 1987b. Ion movements and volume changes induced by catecholamines in erythrocytes of rainbow trout: effect of pH. *Journal of Physiology*. 382:145–157.
- Cala, P. M. 1980. Volume regulation by *Amphiuma* red blood cells. The membrane potential and its implications regarding the nature of the ion-flux pathways. *Journal of General Physiology*. 76:683–708.
- Cala, P. M. 1983. Volume regulation by red blood cells: mechanisms of ion transport. *Molecular Physiology*. 4:33–52.
- Cass, A., and M. Dalmark. 1973. Equilibrium dialysis of ions in nystatin-treated red cells. *Nature New Biology*. 244:47–49.
- Cossins, A. R., and P. A. Richardson. 1985. Adrenalin-induced Na⁺/H⁺ exchange in trout erythrocytes and its effects upon oxygen-carrying capacity. *Journal of Experimental Biology*. 118:229–246.
- Grinstein, S., C. A. Clarke, and A. Rothstein. 1983. Activation of Na⁺/H⁺ exchange in lymphocytes by osmotically induced volume changes and by cytoplasmic acidification. *Journal of General Physiology*. 82:619–638.
- Grinstein, S., S. Cohen, and A. Rothstein. 1984. Cytoplasmic pH regulation in thymic lymphocytes by an amiloride-sensitive Na⁺-H⁺ antiport. *Journal of General Physiology*. 83:341–369.
- Grinstein, S., and A. Rothstein. 1986. Mechanisms of regulation of the Na⁺/H⁺ exchanger. *Journal of Membrane Biology*. 90:1–12.
- Haas, C., W. F. Schmidt, and T. J. McManus. 1982. Catecholamine-stimulated ion transport in duck red cells. Gradient effects in electrically neutral [Na + K + 2Cl] co-transport. *Journal of General Physiology*. 80:125–147.
- Harden, T. K. 1983. Agonist-induced desensitization of the β -adrenergic receptor-linked adenylate cyclase. *Pharmacological Reviews*. 35:5–32.
- Hertel, C., and J. P. Perkins. 1984. Receptor-specific mechanisms of desensitization of β -adrenergic receptor function. *Molecular and Cellular Endocrinology*. 37:245–256.
- Kinsella, J. L., and P. S. Aronson. 1980. Properties of the Na⁺-H⁺ exchanger in renal microvillus membrane vesicles. *American Journal of Physiology*. 238:F461–F469.
- Levitzky, A. 1986. β -adrenergic receptors and their mode of coupling to adenylate cyclase. *Physiological Reviews*. 66:819–854.
- Mahé, Y., F. Garcia-Romeu, and R. Motais. 1985. Inhibition by amiloride of both adenylate cyclase activity and the Na⁺/H⁺ antiporter in fish erythrocytes. *European Journal of Pharmacology*. 116:199–206.
- Motais, R., F. Garcia-Romeu, and F. Borgese. 1987. The control of Na⁺-H⁺ exchange by molecular oxygen in trout erythrocytes. A possible role of hemoglobin as a transducer. *Journal of General Physiology*. 90:197–207.

- Paris, S., and J. Pouyssegur. 1983. Biochemical characterisation of the amiloride-sensitive $\text{Na}^+\text{-H}^+$ antiport in chinese hamster lung fibroblasts. *Journal of Biological Chemistry*. 258:3503-3508.
- Parker, J. C., and V. Castranova. 1984. Volume-responsive movements in dog red blood cells. *Journal of General Physiology*. 84:379-401.
- Schmidt, W., and T. J. McManus. 1977. Ouabain-insensitive water movements in duck red cells. I. Kinetics of cation transport under hypertonic conditions. *Journal of General Physiology*. 70:59-79.
- Stiles, G. L., M. G. Caron, and R. J. Lefkowitz. 1984. β -Adrenergic receptors: biochemical mechanisms of physiological regulation. *Physiological Reviews*. 64:661-743.
- Thierry, J., C. Frelin, P. Vigne, P. Barbry, and M. Lazdunski. 1985. Biochemical properties of the $\text{Na}^+\text{-H}^+$ exchange system in rat brain synaptosomes. Interdependence of internal and external pH control of the exchange activity. *Journal of Biological Chemistry*. 260:9678-9684.