

# Glutaraldehyde Fixation of the cAMP-dependent $\text{Na}^+/\text{H}^+$ Exchanger in Trout Red Cells

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**ABSTRACT** It has been shown that the addition of a  $\beta$ -adrenergic catecholamine to a trout red blood cell suspension induces a 60–100-fold increase of sodium permeability resulting from the activation of a cAMP-dependent  $\text{Na}^+/\text{H}^+$  antiport. Subsequent addition of propranolol almost instantaneously reduces the intracellular cAMP concentration, and thus the Na permeability, to their basal values (Mahé et al., 1985). If glutaraldehyde (0.06–0.1%) is added when the  $\text{Na}^+/\text{H}^+$  exchanger is activated after hormonal stimulation, addition of propranolol no longer inhibits Na permeability: once activated and fixed by glutaraldehyde, the cAMP dependence disappears. Glutaraldehyde alone causes a rapid decrease in the cellular cAMP concentration. In its fixed state the antiporter is fully amiloride sensitive. The switching on of the  $\text{Na}^+/\text{H}^+$  exchange by cAMP is rapidly (2 min) followed by acute but progressive desensitization of the exchanger (Garcia-Romeu et al., 1988). The desensitization depends on the concentration of external sodium, being maximal at a normal Na concentration (145 mM) and nonexistent at a low Na concentration (20 mM). If glutaraldehyde is added after activation in nondesensitizing conditions (20 mM Na), transfer to a Na-rich medium induces only a very slight desensitization: thus the fixative can “freeze” the exchanger in the nondesensitizing conformation.

$\text{NO}_3^-$  inhibits the activity of the cAMP-dependent  $\text{Na}^+/\text{H}^+$  antiporter of the trout red blood cell (Borgese et al., 1986). If glutaraldehyde is added when the cells are activated by cAMP in a chloride-containing medium, the activity of the exchanger is no longer inhibited when  $\text{Cl}^-$  is replaced by  $\text{NO}_3^-$ . Conversely, after fixation in  $\text{NO}_3^-$  medium replacement of  $\text{NO}_3^-$  by  $\text{Cl}^-$  has very little stimulatory effect. This indicates that the anion dependence is not a specific requirement for the exchange process but that the anion environment is critical for the switching on of the  $\text{Na}^+/\text{H}^+$  exchanger and for the maintenance of its activated configuration.

## INTRODUCTION

Glutaraldehyde is a covalent cross-linking agent introduced as a fixative in electron microscopy by Sabatini et al., (1963). It was subsequently shown that, used in conditions leading to a submaximal fixation of the tissue, the vasopressin-dependent osmotic permeability of the frog urinary bladder was preserved after withdrawal of

the hormone (Jard et al., 1966; Eggena 1972, 1983). These results indicate that the hormone-dependent water channels "frozen" by the fixative in their activated state, remained functional. Similarly, Parker (1984) demonstrated that glutaraldehyde treatment of dog red blood cells fixes the  $\text{Na}^+/\text{H}^+$  exchanger, which is activated by cell shrinkage, in either the activated or inactivated state depending on the cell volume at which the fixation took place. Parker's work suggests a use for such a fixative agent in a study of the functional properties of a regulatable transport pathway.

Trout erythrocytes possess a cAMP-dependent, amiloride-sensitive  $\text{Na}^+/\text{H}^+$  exchanger, physiologically activated by  $\beta$ -adrenergic catecholamines (Baroin et al., 1984b; Cossins and Richardson, 1985; Borgese et al., 1986, 1987). After the addition of agonist, sodium influx is greatly increased due to the activation of the exchanger. Then, 2 min later, it abruptly decreases in an exponential manner towards its basal value. It was demonstrated that this rapid drop in  $\text{Na}^+$  uptake reflects a desensitization of the exchanger that is triggered by the external sodium concentration (Garcia-Romeu et al., 1988).

The purpose of this study was to define more precisely, using glutaraldehyde, the relationships between the exchange mechanism and the processes involved in its regulation.

#### MATERIAL 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 laboratory 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 the red cells were washed four times in the experimental solution; the hematocrit was adjusted to 25%.

The washing saline contained (in millimolar): 145 NaCl, 5  $\text{CaCl}_2$ , 1  $\text{MgSO}_4$ , 4 KCl, 5 glucose. The experiments were performed at 15°C with the saline buffered with HEPES (15 mM; pH 7.50). KCl replaced the sodium in experiments in which the sodium concentration of the saline was reduced. Because the rates of swelling and sodium absorption are influenced by the oxygen concentration of the medium (Motais et al., 1987), all experiments were performed in solutions flushed with  $\text{N}_2$ .

##### *Measurements of Cell Volume, Ion Content, Unidirectional Sodium Fluxes and cAMP Concentration*

The techniques used for measuring cell volume, intracellular sodium, potassium and chloride concentrations, unidirectional sodium influxes ( $J_{\text{inNa}}$ ), intracellular pH, and cAMP concentration have been described in various publications from our laboratory (Mahé et al., 1985; Borgese et al., 1986, 1987; Garcia-Romeu et al., 1988). The  $J_{\text{inNa}}$  are expressed in micromoles per minute per gram dry cell solids (d.c.s.; micromoles per minute  $\times$  gram d.c.s) and the ionic contents in micromolar/gram dry cell solids (micromolar/gram d.c.s.).

### Materials

A fresh aqueous solution of  $2.75 \times 10^{-5}$  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  $5.5 \times 10^{-7}$  M.

Ouabain (Sigma Chemical Co.) was dissolved in DMSO (Fluka AG, Buchs, Switzerland) at a concentration of  $10^{-1}$  M. In all experiments, this solution was added to the suspension to give a concentration of  $10^{-4}$  M; the addition was made 5 min before stimulation with isoproterenol.

Glutaraldehyde (25% in water, Fluka AG) was diluted 2.5 times in water and this intermediate solution was added to the suspensions to yield a final concentration of 6 mM. The purity of the glutaraldehyde solutions was periodically assayed by ultraviolet spectrophotometry (Anderson, 1967). Preliminary experiments showed that the effects of glutaraldehyde were similar whether the solutions were buffered with phosphate buffer or HEPES; the latter was therefore used in all experiments.

Propranolol (DL-propranolol ClH; Sigma Chemical Co.) was dissolved in the DMSO at a concentration of  $10^{-2}$  M and added to the relevant experimental samples to give a concentration of  $10^{-5}$  M, in which case it was added 5 min after isoproterenol stimulation (2 min after glutaraldehyde fixation).

A  $10^{-2}$  M solution of amiloride (Merck, Sharp, and Dohme-Chibert, Riom, France) in DMSO was added to the suspensions to a final concentration of  $5 \times 10^{-4}$  M. A freshly made  $10^{-2}$  M solution of 4,4'-diisothiocyano-2,2'-stilbene desulfonic acid (DIDS) (Sigma Chemical Co.) in saline adjusted to pH 7.55 was used to give a suspension concentration of  $5 \times 10^{-4}$  M. The buffers HEPPS (pKa at 20°C = 8.00) and HEPES (pKa at 20°C = 7.55) were obtained from Merck-Schuchardt (Darmstadt, FRG).

## RESULTS

### *Characteristics of the cAMP-dependent Na<sup>+</sup>/H<sup>+</sup> Exchange*

Fig. 1 A shows that the sodium influx is rapidly and greatly increased (60–100 times) by addition of isoproterenol to a trout red blood cell suspension and that simultaneously the cellular cAMP content increases, phenomena that have already been reported by Mahé et al. (1985). These results suggest that cAMP may activate the Na<sup>+</sup>/H<sup>+</sup> exchanger, a view confirmed by the fact that exogenously added cAMP or forskolin also activates the Na<sup>+</sup>/H<sup>+</sup> exchange system (Mahé et al., 1985). Fig. 1 B shows, furthermore, that the subsequent addition of a  $\beta$ -adrenergic antagonist, propranolol, immediately inhibits sodium uptake and causes a simultaneous decrease in the intracellular concentration of cAMP, indicating that the trout erythrocyte has sufficient phosphodiesterase activity to metabolize cAMP rapidly. The above experiments thus demonstrate that the switching on and off of the Na<sup>+</sup>/H<sup>+</sup> exchange system is controlled by the cellular cAMP concentration.

The experiment illustrated in Fig. 1 A shows, however, that the activity of the Na<sup>+</sup>/H<sup>+</sup> exchanger is also controlled by other factors in a more subtle manner. It can be seen that the unidirectional sodium influx reaches a maximum 1.5 min after stimulation and then abruptly decreases in an exponential manner ( $t_{1/2} \sim 30$  min). This reduction in the activity of the Na<sup>+</sup>/H<sup>+</sup> exchanger cannot be ascribed to a decrease in the cellular cAMP concentration via a receptor desensitization, for example, as there was a constant increase in cellular cAMP content during the first

30 min of this experiment, after its concentration stabilized. The same reduction in sodium permeability occurs when the  $\text{Na}^+/\text{H}^+$  exchanger is switched on by exogenously added cAMP or forskolin, which acts independently of the hormone receptor and promotes a constant increase in cAMP content. We called this phenomenon "desensitization of the antiporter" and demonstrated that the triggering factor of

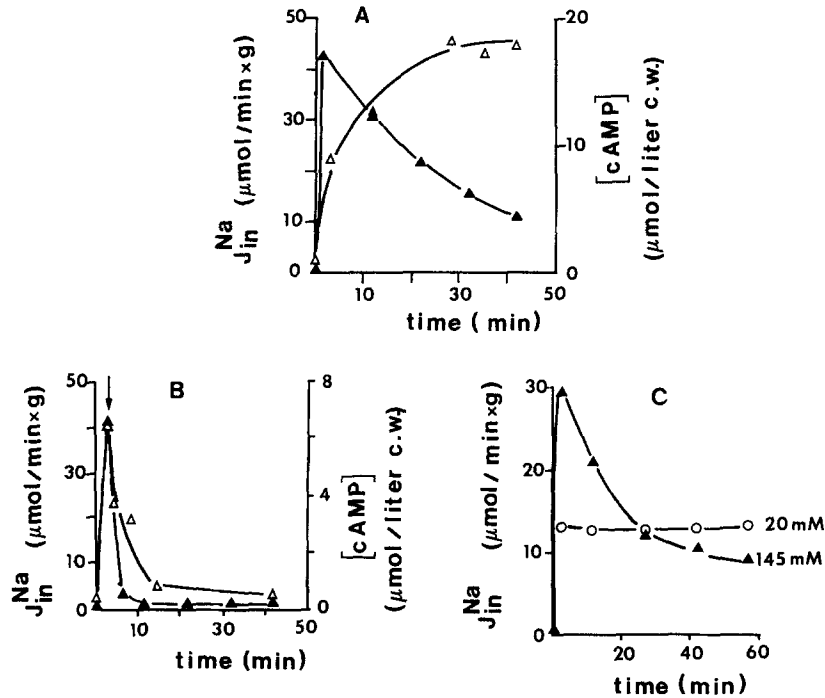


FIGURE 1. Evolution of unidirectional sodium influxes ( $J_{in}^{Na}$ ) ( $\blacktriangle$ ) and cAMP concentrations ( $\triangle$ ) after stimulation of trout red blood cells by isoproterenol ( $5 \times 10^{-7}$  M). It can be seen (A) that the cAMP concentration increased for the first 30 min and then stabilized, while the  $J_{in}^{Na}$ , after an initial rapid increase in the first 1.5 min, decreased exponentially. B shows that the  $J_{in}^{Na}$ , stimulated by isoproterenol, was rapidly inhibited by the  $\beta$  blocker propranolol ( $10^{-5}$  M) administered at the arrow. The cAMP concentration also returned to its basal level. C illustrates the effect of external sodium concentration on the desensitization of the  $\text{Na}^+/\text{H}^+$  antiporter after isoproterenol stimulation. At a low  $\text{Na}^+$  concentration (20 mM) the  $J_{in}^{Na}$  remained constant after an initial moderate activation, whereas at 145 mM sodium concentration the activation and desensitization processes developed normally. As in all the following figures the  $J_{in}^{Na}$  are expressed in micromoles per minute per gram dry cell solids.

the desensitization process is the level of the external sodium concentration (Garcia-Romeu et al., 1988). In other words, when red cells are hormonally stimulated in media containing low  $\text{Na}^+$  (20 mM), the activation of  $\text{Na}^+/\text{H}^+$  exchange is not followed by its desensitization, whereas when the red cells are suspended in a normal saline (145 mM  $\text{Na}^+$ ), desensitization occurs. These two conditions are illustrated in Fig. 1 C.

The following experiments, using glutaraldehyde as a cross-linking agent, were designed to study the ON-OFF switching and desensitization processes.

*Glutaraldehyde Protects against the Switching-Off Effect of Propranolol*

As illustrated in Fig. 1 B, the Na<sup>+</sup>/H<sup>+</sup> exchanger switched on by isoproterenol is rapidly switched back to the off mode by addition of propranolol, which induces a rapid drop in cAMP concentration. We observed that if glutaraldehyde was added to a cell suspension 2 min before addition of propranolol, the Na<sup>+</sup>/H<sup>+</sup> exchanger was thereafter practically insensitive to propranolol, i.e., was fixed in the activated state. Fig. 2 shows the concentration dependence of this protective effect of glutaraldehyde. An optimum effect is seen at a concentration of 6–10 mM (0.06–0.1%) with an hematocrit of 25%. We did not try to determine the optimum time of fixation of glutaraldehyde; we only know that 2 min after addition of the cross-linker, propranolol no longer inhibited Na<sup>+</sup> influx.

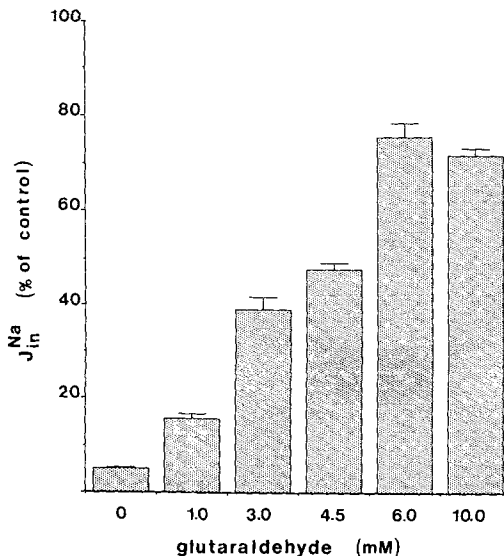


FIGURE 2. Protective effect of various concentrations of glutaraldehyde against inhibition of unidirectional sodium influxes ( $J_{in}^{Na}$ ) by propranolol after isoproterenol stimulation. Increasing concentrations of glutaraldehyde were added to different cell suspension aliquots 3 min after isoproterenol addition ( $5.5 \times 10^{-7}$  M). 5 min after stimulation and 2 min after glutaraldehyde treatment, propranolol (to give  $10^{-5}$  M) was added to each suspension. The  $J_{in}^{Na}$  were measured 11.5 min after stimulation and compared with fluxes of a control group without glutaraldehyde and propranolol treatments. Suspension pH, 7.55; ouabain,  $10^{-4}$  M.

The protective effect of glutaraldehyde could be due to the cross-linker locking in the "ON" position either the adenylate cyclase system furnishing cAMP or the Na<sup>+</sup>/H<sup>+</sup> exchanger itself.

*Cellular cAMP Concentration and Glutaraldehyde*

Fig. 3 A illustrates that when glutaraldehyde was applied to red cells previously stimulated by isoproterenol, the cellular cAMP concentration rapidly decreased to a value close to the basal level: glutaraldehyde, therefore, does not fix the adenylate cyclase in the activated state. Nevertheless, as shown in Fig. 3 B, the evolution of sodium fluxes in these cells is similar to that observed in cells stimulated by isoproterenol but not fixed by glutaraldehyde and therefore containing a higher cellular concentration of cAMP.

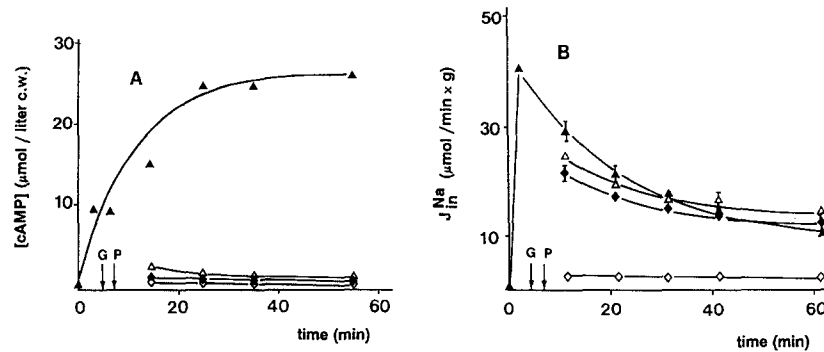


FIGURE 3. (A) Effect of glutaraldehyde and propranolol on the cAMP concentration in red blood cells stimulated by isoproterenol. This figure shows a single experiment representative of three others. Glutaraldehyde (6 mM) was added 5 min after hormonal stimulation (arrow G). Propranolol ( $10^{-5}$  M) was added 8 min after hormonal stimulation (arrow P). ▲, isoproterenol; △, isoproterenol + glutaraldehyde; ◆, isoproterenol + glutaraldehyde + propranolol; ◇, isoproterenol + propranolol. It can be seen that glutaraldehyde alone, propranolol alone, and glutaraldehyde plus propranolol resulted in the same pattern of cAMP concentration decrease. (B) Effect of glutaraldehyde and propranolol on  $J_{in}^{Na}$  in red blood cells stimulated by isoproterenol. Means  $\pm$  SE for eight studies. In some cases, the error bars are smaller than the data points. Glutaraldehyde (6 mM) was added 5 min after hormonal stimulation (arrow G). Propranolol ( $10^{-5}$  M) was added 8 min after hormonal stimulation (arrow P). Symbols are the same as in A. It can be seen that the  $J_{in}^{Na}$  of the glutaraldehyde-fixed cells with or without propranolol treatment behaved in the same way as the control cells in spite of the cAMP concentration being at its basal level. pH of suspensions, 7.55; ouabain,  $10^{-4}$  M.

Thus from these experiments it appears that if the cross-linking agent is applied when the cells have been stimulated by catecholamines, the  $Na^+/H^+$  antiporter is activated and remains so regardless of subsequent variations in cAMP concentration: glutaraldehyde seems to act by locking the antiporter itself in the ON position.

#### Glutaraldehyde and the ON-OFF Switching Process

The data reported above show that the  $Na^+/H^+$  exchanger, when it has been switched on by hormonal stimulation, can be frozen in this activated state by treatment with glutaraldehyde. Even after this fixation, however, the exchanger can always be inhibited by amiloride (Fig. 4).

Fig. 5 shows that if the fixative is applied to unstimulated cells, subsequent addition of cAMP only stimulates the Na pathway slightly (17% of the control). In other words, the fixing effect of glutaraldehyde when the antiporter is in the OFF position is considerable but not total. It must be pointed out that the fixation time used to lock the exchanger in the OFF mode, however, was longer (10 min) than that used to fix it in the ON mode (2 min). We used cAMP as the activating agent rather than isoproterenol to avoid the complication of the inhibiting effect of glutaraldehyde on cAMP production (see the preceding paragraph).

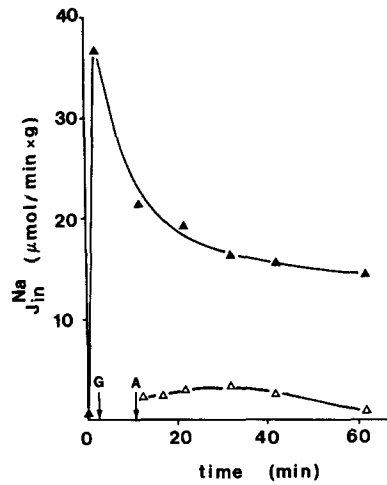


FIGURE 4.  $J_{in}^{Na}$  of blood cells stimulated by isoproterenol ( $5.5 \times 10^{-7}$  M) and then, 5 min later, fixed with glutaraldehyde (arrow G). Amiloride is added to one aliquot (arrow A): the  $J_{in}^{Na}$  of these cells was immediately inhibited. The  $Na^+/H^+$  antiporter thus remains sensitive to the diuretic in spite of the glutaraldehyde treatment. ▲, isoproterenol + glutaraldehyde; △, isoproterenol + glutaraldehyde + amiloride.

*Cell Swelling, Sodium and Chloride Absorptions, and Changes of Intra- and Extracellular pH in the Presence of Glutaraldehyde*

Fig. 6 A shows that when glutaraldehyde is added to cells previously stimulated by isoproterenol, the cell volume continues to increase in a way similar to that of cells not treated by glutaraldehyde, but at a slightly slower rate, due to slower rates of  $Na^+$  (Fig. 6 B) and  $Cl^-$  (Fig. 6 C) uptake. Thus, fixation of the antiporter in the activated state must be followed by a slight decrease either in the turnover rate or in the number of antiporters available for exchange. It should be noted that glutaraldehyde does not significantly affect cell volume or Na and Cl uptake when added to nonstimulated cells (Fig. 6 A).

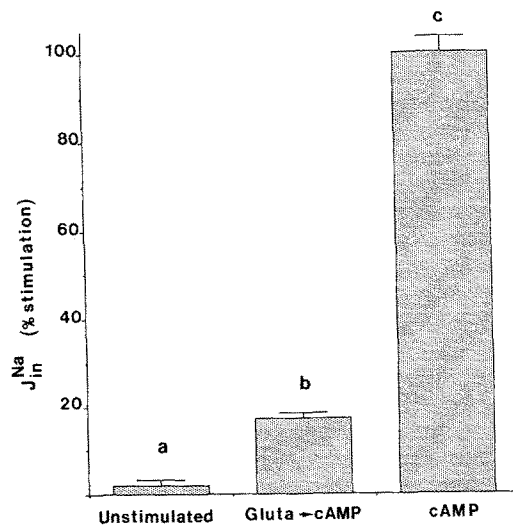


FIGURE 5. Comparison of the  $J_{in}^{Na}$  of red blood cells (a) unstimulated and (b) fixed by addition of glutaraldehyde (6 mM) 10 min before stimulation by cAMP ( $10^{-3}$  M) and (c) stimulated by cAMP ( $10^{-3}$  M). a and b shown as percentages of c. The two stimulated aliquots were also given a dose of  $10^{-3}$  M IBMX (3-isobutyl-1-methylxanthine) to inhibit phosphodiesterase activity. Stimulation of the  $Na^+/H^+$  antiporter was obtained by the addition of cAMP since the presence of glutaraldehyde inhibits cellular production of cAMP (see Fig. 3). It can be seen that glutaraldehyde added 10 min before stimulation caused considerable but not total inhibition of  $J_{in}^{Na}$ .

As previously reported, the activation of the  $\text{Na}^+/\text{H}^+$  exchanger by isoproterenol produces an acidification of the external medium (Fig. 7 A) and simultaneously an alkalization of the intracellular compartment (Fig. 7 B). It can be seen that the subsequent addition of glutaraldehyde increases the external acidification (Fig. 7 A), which could be explained by postulating an inhibitory effect of glutaraldehyde on the anion exchanger which normally mediates  $\text{Cl}^-/\text{HCO}_3^-$  countertransport and thus titrates part of the  $\text{H}^+$  excreted by the  $\text{Na}^+/\text{H}^+$  antiporter (Baroin et al. 1984b; Cossins et al., 1985; Borgese et al., 1986). Such inhibition of the anion exchanger by

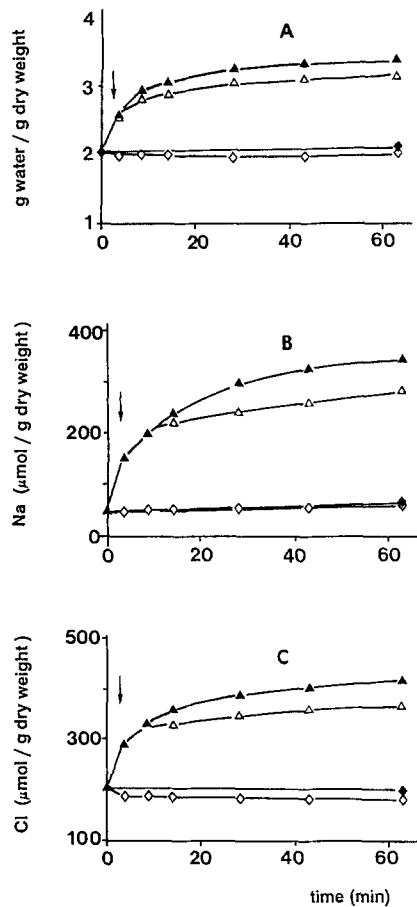


FIGURE 6. Effects of glutaraldehyde on the absorption of water (A), sodium (B), and chloride (C) by red blood cells in different conditions.  $\blacklozenge$ , unstimulated control without glutaraldehyde;  $\blacktriangle$ , control stimulated with isoproterenol;  $\triangle$ , isoproterenol + glutaraldehyde (6 mM);  $\diamond$ , unstimulated with glutaraldehyde. Glutaraldehyde was added at arrow.

glutaraldehyde, however, would promote an increased alkalisation of the red cell. This interpretation is not supported by the evidence, however: Fig. 7 B shows that conversely there is a strong acidification of the red cell after glutaraldehyde treatment.

Further experiments were made to study the role of glutaraldehyde on these pH changes. Fig. 8 shows that the addition of glutaraldehyde to an unstimulated red blood suspension induced a strong acidification of the external medium which was



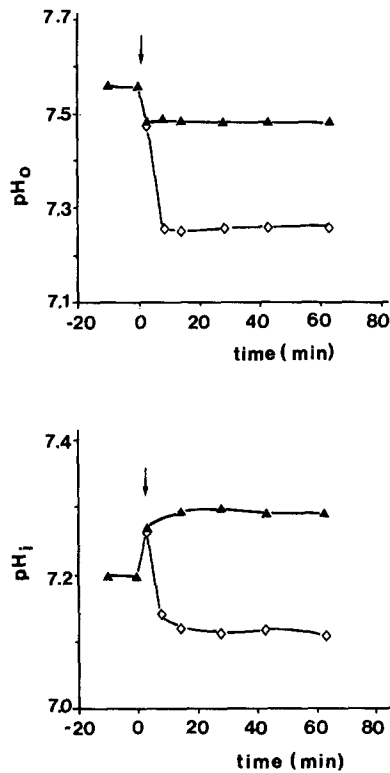


FIGURE 7. Effects of glutaraldehyde on the evolution of extracellular pH (pH<sub>o</sub>) and intracellular pH (pH<sub>i</sub>) ▲, isoproterenol (control); ◇, isoproterenol + glutaraldehyde. The hormone was added at time zero. The addition was immediately followed by acidification of the external medium and alkalization of the cytosol in the control batch; these pHs stabilized rapidly. Glutaraldehyde was added at arrow to the respective group and caused considerable acidification of both internal and external media. Isoproterenol,  $5.5 \times 10^{-7}$  M; glutaraldehyde,  $6 \times 10^{-3}$  M; ouabain,  $10^{-4}$  M.

not inhibited by amiloride, indicating again that glutaraldehyde does not activate Na<sup>+</sup>/H<sup>+</sup> exchanges. The drop in external pH, on the other hand, is considerably reduced by DIDS. As the addition of glutaraldehyde to saline alone does not promote acidification (not shown), the action of DIDS indicates that the primary event

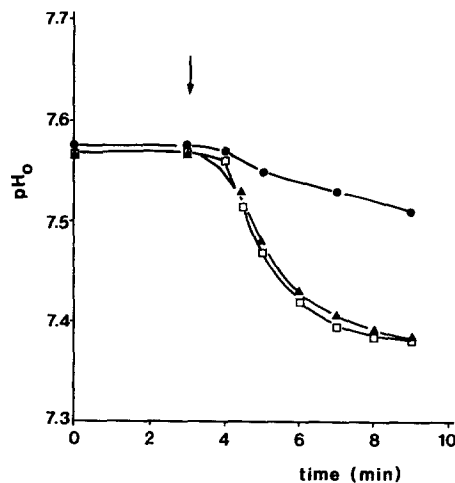


FIGURE 8. Effect of glutaraldehyde on the external pH. □, glutaraldehyde (6 mM); ▲, glutaraldehyde + amiloride ( $5 \times 10^{-4}$  M); ●, glutaraldehyde + DIDS ( $5 \times 10^{-4}$  M). The acidification resulting from addition of glutaraldehyde (arrow) was not affected by amiloride but strongly inhibited by DIDS.

on addition of glutaraldehyde is an intracellular acidification which secondarily causes an external acidification in accordance with Donnan equilibrium. These experiments show that the anion exchanger remains functional and sensitive to DIDS in the presence of glutaraldehyde. It seems reasonable to assume that glutaraldehyde per se induces a change in the pK of hemoglobin, promoting intracellular acidification (see Discussion). Thus, the changes of intra- and extracellular pH in the presence of isoproterenol plus glutaraldehyde represent the cumulative effects of isoproterenol on  $\text{Na}^+/\text{H}^+$  exchange and glutaraldehyde on hemoglobin.

#### Glutaraldehyde and the Antiport Desensitization

We have shown above (see "Characteristics of the cAMP-dependent  $\text{Na}^+/\text{H}^+$  Exchange") that the evolution of  $J_{\text{in}}^{\text{Na}}$  after stimulation by isoproterenol falls into

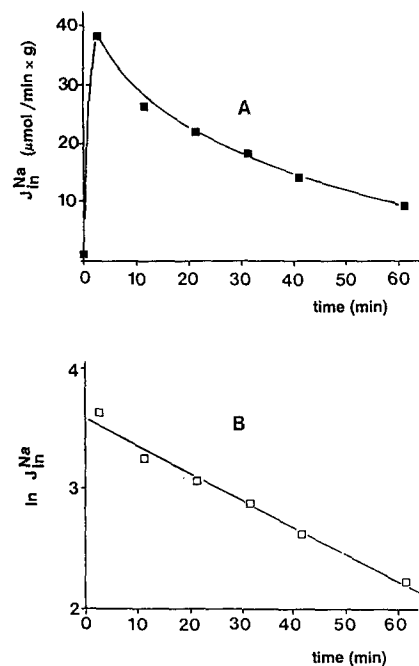


FIGURE 9. Evolution of the  $J_{\text{in}}^{\text{Na}}$  of red blood cells stimulated by isoproterenol (A). Two phases may be distinguished: the first activation, reaching a maximum  $\sim 2$  min after hormone addition and the second, desensitization. (B) Exponential representation of the desensitization phase, giving the equation:  $J_{\text{in}}^{\text{Na}} = 36.75 \times e^{-0.0228t}$ , where 36.75 is the initial rate, 0.0228 is the desensitization constant, and  $t$  is the time in minutes. Ouabain,  $10^{-4}$  M; isoproterenol,  $5.5 \times 10^{-7}$  M; pH of suspension, 7.55.

two distinct phases: the activation phase, lasting  $\sim 1.5$  min, during which the unidirectional influxes attain their maximum and the period of desensitization of the transporter during which the  $J_{\text{in}}^{\text{Na}}$  fall exponentially towards their basal level. Fig. 9 A illustrates these two phases and Fig. 9 B analyzes the exponential of the desensitization phase, from which the initial rate of activation (ordinate value at the origin) and rate constant of desensitization  $k$ , (slope of line) may be obtained.

The analysis of the exponential was used to measure the effect of glutaraldehyde on the two phases. Table I (batches 145 mM) shows that (a) Glutaraldehyde exerts a 19% inhibition of the activation phase. The  $\text{pH}_i$  and  $\text{pH}_o$  changes that it causes (see Fig. 7) cannot account for this inhibition because it is in these  $\text{pH}_o$  ranges that the

transport is maximal (Borgese et al., 1987). However, it is possible that the aldehyde changes the sodium and proton affinities of the antiporter with a shift of the transport rate curves as functions of pH. (b) Glutaraldehyde slows down the rate of desensitization by a factor of 2.21, the  $t_{1/2}$  passing from 31.5 min in controls to 70 min in the glutaraldehyde-treated cell suspension.

As previously discussed the desensitization of the antiport depends on the concentration of external sodium: at a low external sodium, the activity of the exchanger remains practically constant (see Fig. 1 C). Table I (batches 20–145 mM) shows the mean of 11 experiments in which the red blood cells suspended in a low Na saline (20 mM sodium) were first stimulated by isoproterenol and after 20 min suspended in a saline containing a normal sodium concentration (145 mM), always

TABLE I  
*Initial Rates of Na Influx (a) and Rate Constants of Desensitization (k) in the Presence (Isop + gluta) or Absence (Isop Control) of Glutaraldehyde*

Batch	n	Initial rate: a	k	$t_{1/2}$
		$\mu\text{mol} \times \text{min}^{-1} \times \text{g}^{-1}$		min
Isop control (145 mM)	11	34.36 ± 1.04	-0.0220 ± 0.0022	31.50
Isop + gluta (145 mM)	6	27.85 ± 1.06	-0.0099 ± 0.0007	70.00
		c	d	
Isop control (20–145 mM)	6	24.75 ± 1.69	-0.0207 ± 0.0019	33.47
		e	f	
Isop + gluta (20–145 mM)	6	21.53 ± 3.54	-0.0064 ± 0.0004	108.28
		g	h	

The equation expressing the unidirectional sodium influx during desensitization is:  $J_{in}^{Na} = ae^{-kt}$ ; ( $t$  = time in minutes). For the batches treated with glutaraldehyde, the procedure adopted was as follows: one batch (145 mM) was incubated in 145 mM of sodium saline, glutaraldehyde being added 3 min after hormonal stimulation. The other batch (20–145 mM) was incubated in 20 mM sodium saline, isoproterenol, and glutaraldehyde being added as in the first batch and then, after 20 min incubation, transferred to 145 mM sodium saline. Control groups were submitted to the same treatment but were not fixed by glutaraldehyde. Significance of differences: Initial rates between a and c:  $P < 0.001$ ; between e and g:  $0.4 > P < 0.3$ . Rate constants between b and d:  $P < 0.001$ ; between f and h:  $P < 0.001$ ; between d and h:  $P < 0.0001$ .

in the presence of isoproterenol. Other batches (six experiments) were treated with glutaraldehyde in the low-saline medium 3 min after hormonal stimulation. It can be seen that the desensitization, quantified by  $k$ , which is induced in the control cells after resuspension in the 145 mM sodium saline is much less marked in the glutaraldehyde-treated cells (half time 33 and 108 min, respectively, Table I). The difference between the rate constants of the two batches is highly significant, as are also the differences between the constants of the glutaraldehyde-treated batches at 20 and 145 mM sodium ( $t_{1/2}$  108 and 70 min, respectively; for significances see Table I). This shows that the desensitization process normally initiated by subjection to high concentrations of sodium is strongly inhibited by previous treatment with glutaraldehyde in a non-desensitization-producing solution (low sodium). All these fluxes are fully amiloride sensitive (not shown).

*The Anion Requirements of the Na<sup>+</sup>/H<sup>+</sup> Exchanger*

The possibility of freezing the amiloride-sensitive Na<sup>+</sup>/H<sup>+</sup> antiporter in a functional state was used to study the anion requirement of the exchanger. It was previously shown that replacement of Cl by NO<sub>3</sub><sup>-</sup> or SCN<sup>-</sup> caused a substantial inhibition of the amiloride-sensitive Na uptake when this Na uptake is stimulated either by cell shrinkage in *Amphiuma* (Cala, 1983) or in dog (Parker, 1983) red cells or by isoproterenol in trout red cells (Borgese et al., 1986). Cala (1983) suggested that the nature of the anions directly affects the cationic antiporter since inhibition is observed in the absence of any anionic movement. The same conclusion was reached by Borgese et al. (1986) concerning the Na<sup>+</sup>/H<sup>+</sup> antiporter stimulated by isoproterenol in trout red cells. Using glutaraldehyde, Parker (1984) showed that

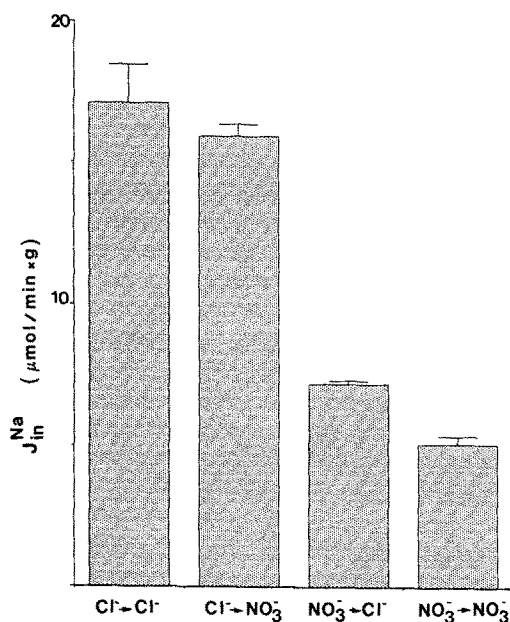


FIGURE 10. Anion requirement of the Na<sup>+</sup>/H<sup>+</sup> exchanger after glutaraldehyde treatment. The red blood cells were stimulated with isoproterenol ( $5.5 \times 10^{-7}$  M) and fixed by 6 mM glutaraldehyde in either a chloride saline or a nitrate saline with low sodium content (Na<sup>+</sup>, 20 mM; K<sup>+</sup>, 125 mM). After 40 min, each of the batches incubated in low sodium medium was divided into two aliquots that were transferred to salines containing 145 mM sodium in the following manner. Cells incubated in chloride saline transferred to (a) 145 mM NaCl (Cl → Cl) or (b) 145 mM NaNO<sub>3</sub> (Cl → NO<sub>3</sub>); cells incubated in nitrate saline transferred to (a) 145 mM NaCl (NO<sub>3</sub> → Cl) or (b) 145 mM NaNO<sub>3</sub> (NO<sub>3</sub> → NO<sub>3</sub>). The  $J_{in}^{Na}$  were measured immediately after transfer ( $t = 2$  min). pH of suspensions, 7.55; ouabain,  $10^{-4}$  M.

the activation of Na<sup>+</sup>/H<sup>+</sup> exchanges by shrinkage in dog erythrocytes is affected by anions, but once activated and fixed, this is no longer the case.

The experiment illustrated in Fig. 10, one of four similar experiments, was performed on trout erythrocytes to evaluate whether Cl<sup>-</sup> is necessary for the activation by cAMP of the antiporter or for the transport of Na<sup>+</sup> by the exchanger after it has been activated by cAMP.

In these experiments the red cells were activated by isoproterenol in Cl<sup>-</sup> or NO<sub>3</sub><sup>-</sup> saline with a low Na<sup>+</sup> concentration (20 mM Na) to avoid subsequent desensitization, then fixed by glutaraldehyde and finally resuspended in Cl<sup>-</sup> or NO<sub>3</sub><sup>-</sup> saline with a normal Na<sup>+</sup> concentration (145 mM Na<sup>+</sup>). The results indicate that the Na<sup>+</sup> influx of the cells fixed in Cl<sup>-</sup> media is high, and that the replacement of Cl<sup>-</sup> by NO<sub>3</sub><sup>-</sup> after fixation does not have an inhibitory effect. Conversely, the Na<sup>+</sup> influx of

cells fixed in NO<sub>3</sub><sup>-</sup> and resuspended in NO<sub>3</sub><sup>-</sup> saline is strongly reduced (to 32% of the control) and replacement of NO<sub>3</sub><sup>-</sup> by Cl<sup>-</sup> after fixation has very little stimulatory effect.

In brief, once activated by cAMP in a Cl<sup>-</sup>-containing medium and frozen in this state by glutaraldehyde, the exchanger is thereafter insensitive to the replacement of Cl<sup>-</sup> by NO<sub>3</sub><sup>-</sup>. On the other hand, once activated and fixed in a NO<sub>3</sub><sup>-</sup>-containing medium, the cells maintain a low sodium permeability when resuspended in a chloride saline. These results confirm that anion dependence is related to the activation mechanisms rather than to the transport mechanism itself (Adorante and Cala, 1987).

#### DISCUSSION

The capacity of glutaraldehyde to "fix" a permeability pathway in functional or non-functional states has been described in various tissues. Glutaraldehyde can freeze the epithelial barrier of frog urinary bladder in a highly permeable state to water after stimulation by vasopressin (ADH) or in a low permeable state without ADH (Jard et al., 1966; Eggena, 1972, 1983). In dog red cells the volume-activated, amiloride-sensitive Na<sup>+</sup>/H<sup>+</sup> exchanger can also be locked on or off by glutaraldehyde and when locked on, the exchanger remains amiloride sensitive (Parker, 1984). This study shows that glutaraldehyde also has a locking action on the cAMP-activated Na<sup>+</sup>/H<sup>+</sup> exchanger of trout erythrocytes that remains amiloride sensitive when fixed on. We observed that the anion exchanger also remains functional and DIDS sensitive after fixation by glutaraldehyde (Fig. 8), whereas the synthesis of cAMP is inhibited (Fig. 3).

Glutaraldehyde fixation induces a strong intracellular acidification in nonstimulated erythrocytes, which overrides the intracellular alkalization normally observed when red blood cells are stimulated by catecholamines. This unspecific acidification may be reasonably explained in the following way. The ε-amino group of lysine is the principal protein side chain reacting with glutaraldehyde (Reichlin, 1980). After interaction the positive charge of the ε-amino group is retained at a neutral or acid pH but the pKa of the new function is ~1 pH unit more acidic than that of the original primary amine (Peters and Richards, 1977). The glutaraldehyde binding sites being more abundant in hemoglobin than in other proteins (Corry and Meiselman, 1978), it may be assumed that a change in the pKa of the side chain amino groups of hemoglobin, induced by glutaraldehyde fixation, could be at the origin of the observed acidification after the addition of the cross-linker.

#### *The Anion Dependence of the Na<sup>+</sup>/H<sup>+</sup> Exchanger*

We have previously shown that replacement of chloride by nitrate in the suspension medium inhibits the adrenergic activated sodium influx in trout erythrocytes (Baroin et al., 1984a). In experiments performed in the absence of any anionic movement, we then showed that the inhibitory effect of NO<sub>3</sub><sup>-</sup> is due to a limitation of the activity of the cAMP-dependent Na<sup>+</sup>/H<sup>+</sup> antiporter itself (Borgese et al., 1986). In this work, we observed that once activated in a Cl<sup>-</sup>-containing medium and frozen in this state by glutaraldehyde, the antiporter is subsequently insensitive to the replacement of Cl<sup>-</sup> by NO<sub>3</sub><sup>-</sup>. Also, once activated in a NO<sub>3</sub><sup>-</sup> medium and

frozen in this state by the cross-linking agent, the antiporter then remains in a low degree of activity even if  $\text{NO}_3^-$  is replaced by  $\text{Cl}^-$ . Thus anion is not a specific requirement for exchange but the anion environment is critical for the switching on of the exchanger and for the maintenance of its activated configuration.

It is of interest that in the erythrocytes of different species the volume sensitive  $\text{Na}^+/\text{H}^+$  exchanger is also anion sensitive (Cala, 1983; Parker, 1983; Jennings et al., 1986) and Cala (1983) suggests that anion effects may reflect differences in anion interactions with the activation step and not with ion translocation. Parker (1984) showed that once activated by shrinkage and fixed by glutaraldehyde the antiporter of dog red cells is not sensitive to anions, a fact that clearly indicates that, as we report here, the anion sensitivity is linked with the activation mechanism. Similarly in *Amphiuma* erythrocytes the volume-activated  $\text{K}^+/\text{H}^+$  exchanger shows an anion sensitivity which also affects the activation process but not the translocation mechanism. (Adorante and Cala, 1987).

#### *The Switching ON-OFF Process*

The transporter becomes active when the cells are cAMP stimulated. If glutaraldehyde is added to the cells under these conditions, the transporter remains active despite considerable reduction in the cAMP concentration and also remains sensitive to amiloride (Fig. 4). Conversely, if glutaraldehyde is added when the cells are not stimulated, subsequent addition of exogenous cAMP has only a very slight action on the exchanger. Thus glutaraldehyde can irreversibly freeze the configuration of the transporter in the activated position induced by cAMP, the transporter then becoming independent of cAMP, or it can quite fully freeze the configuration of the transporter in the OFF position.

#### *The Desensitization of the Antiporter*

We have shown that 1.5 min after being activated by isoproterenol, the cAMP-dependent  $\text{Na}^+/\text{H}^+$  exchanger become progressively inactivated despite the continuous presence of cAMP, the agent responsible for the activation (Figs. 1 A and 3). This desensitization process follows an exponential time course, indicating a process occurring at random. Moreover, this inactivation depends in an allosteric manner on the external sodium concentration: it does not exist at 20–30 mM  $\text{Na}^+$ , and is maximal at the physiological  $\text{Na}^+$  concentration of 145 mM (Garcia-Romeu et al., 1988). The use of glutaraldehyde gives us further informations on the desensitization of the antiporter.

First, we observed that when glutaraldehyde is added to red blood cells stimulated in a saline containing 145 mM  $\text{Na}^+$ , the inactivation process occurs as in control cells, although its rate is reduced.

Second, when red cells are stimulated in saline containing 20 mM  $\text{Na}^+$ , and then suspended in a saline containing 145 mM  $\text{Na}^+$ , the desensitization process starts immediately with a rate identical to that of erythrocytes stimulated in the presence of 145 mM  $\text{Na}^+$ . On the other hand, if glutaraldehyde was added before the cells were transferred from 20 to 145 mM  $\text{Na}^+$  although the activation is similar to that of control preparations, the desensitization process is considerably retarded: the cells activated by isoproterenol are refractory to desensitization almost as if they

were being kept in 20 mM Na<sup>+</sup> saline. In other words, the activation and desensitization process are separate phenomena that occur in parallel and that can be regulated independently. At the same time, it can be observed that the magnitude of the initial activation measured in 145 mM Na<sup>+</sup> saline on glutaraldehyde fixed cells is practically identical whether the cells had been stimulated at 145 mM or at 20 mM Na<sup>+</sup> (Table I). From this it would appear that the functional configuration of the activated transporter is similar at 20 and 145 mM Na<sup>+</sup> but that the relationship between the antiporter and some component factor involved in the desensitization process is different and can be fixed as such by glutaraldehyde.

The presence of such a desensitization process, controlling in course of time the activity of a transport mechanism, warrants emphasis. Generally, a decrease of activity of a hormonally controlled transport mechanism is considered to be related to desensitization of the hormonal receptors. As shown and discussed in a previous paper (Garcia-Romeu et al., 1988) and as demonstrated again in this paper (Fig. 1 A and 3), the desensitization process of the Na<sup>+</sup>/H<sup>+</sup> antiporter is not due to the desensitization of the  $\beta$ -adrenergic receptor, as it is observed at a constant or even increasing cAMP concentration. Such an uncoupling between cAMP concentration and the activity of cAMP dependent cotransport (Na/K/2Cl) has been observed in turkey erythrocyte (Gardner et al., 1973).

These experiments further show that the loss of response of a transporter to an agonist need not signify receptor desensitization but may represent a decreased sensitivity of the transporter itself to some intermediate stimulus.

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