The Control of Na\(^+\)/H\(^+\) Exchange by Molecular Oxygen in Trout Erythrocytes

A Possible Role of Hemoglobin as a Transducer

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ABSTRACT It has previously been shown that addition of catecholamines to a suspension of trout erythrocytes induces an enlargement of the cells owing to an uptake of NaCl mediated by a cAMP-dependent, amiloride-sensitive Na\(^+\)/H\(^+\) exchange. In this article, we show that the change in cell volume induced by catecholamines is much greater when the erythrocytes are incubated in N\(_2\) than when they are in O\(_2\). This difference is explained by an inhibition of the cAMP-dependent Na\(^+\)/H\(^+\) exchange by O\(_2\). The inhibition is not reversed in cells incubated in O\(_2\) but poisoned with cyanide. It cannot be explained by a difference in the content of cAMP in O\(_2\) and in N\(_2\). In a CO atmosphere, in which the cells are anoxic, swelling and Na permeability are not increased as they are in N\(_2\): in CO, the cells behave as they do in O\(_2\). Moreover, cells previously exposed to CO and then put in an N\(_2\) atmosphere do not show the expected increase in Na\(^+\)/H\(^+\) exchange. This strongly indicates that the binding of CO to hemoglobin, which persists during the subsequent exposure to N\(_2\), is the primary event responsible for the inhibition. As CO substitutes for O\(_2\) in binding to hemoglobin, the effect of O\(_2\) in the control of Na\(^+\)/H\(^+\) exchange is probably explained by this interaction with heme. (Allen and McManus [1968, Biophysical Journal, 8:125a] previously described a similar effect of CO on passive Na permeability in duck red cells.) It is proposed that the hemoglobin, by interacting differently, according to its degree of oxygenation, with the cytoplasmic segment of band 3 protein, may influence some transport function, such as Na\(^+\)/H\(^+\) exchange. The physiological significance of a control of Na\(^+\)/H\(^+\) exchange by molecular O\(_2\) is discussed.

INTRODUCTION

The addition of catecholamines to a suspension of trout erythrocytes induces an enlargement of the cells owing to an uptake of NaCl and osmotically obligated water. This accumulation of salt and water is cAMP dependent (Mahé et al., 1985). Catecholamines stimulate an amiloride-sensitive, cAMP-dependent Na\(^+\)/H\(^+\) antiporter, which allows external Na\(^+\) to enter according its chemical gradient...
in exchange for internal H\(^+\), with a stoichiometry of 1:1 (Baroin et al., 1984b; Cossins and Richardson, 1985). The exchange of Na\(^+\) for H\(^+\) results in an acidification of the external medium and an alkalinization of the intracellular medium (Nikinmaa, 1983; Baroin et al., 1984b; Cossins and Richardson, 1985; Borgese et al., 1986, 1987b). This pH disequilibrium drives net flux via Cl\(^-\)/HCO\(_3\)^\(-\) exchanges normally occurring via the anion exchanger located in band 3 protein. The result is a considerable uptake of Na\(^+\) and Cl\(^-\) with water following osmotically, causing cell swelling.

During the above studies, we observed that the frequent mixing of the suspension or washing of cells with fresh saline, each of which increases the O\(_2\) content of the suspension, was sufficient to cause inhibition of Na\(^+\)/H\(^+\) exchange. Consequently, we decided to study the effect of oxygen on the activity of the Na\(^+\)/H\(^+\) antiporter in trout erythrocytes. The results reported here show that the activity of the Na\(^+\)/H\(^+\) exchange is controlled nonmetabolically by O\(_2\) and strongly suggest that the triggering factor is the binding of O\(_2\) to heme. It is interesting to note that Tosteson and Robertson (1956) and Allen and McManus (1968) showed that switching duck red blood cells from N\(_2\) to O\(_2\) also reduces the passive permeability to Na. The results presented in this article have previously been published in abstract form (Borgese et al., 1987a).

**MATERIALS AND METHODS**

**Preparation of Cells**

Rainbow trout, *Salmo gairdneri*, were obtained from a commercial hatchery and kept for 1 wk in the laboratory in tanks provided with running tap water (water temperature, 15\(^\circ\)C). Fish were stunned by a sharp blow to the head and blood was removed from the caudal vein by a heparinized syringe. The blood of several animals was pooled. The cells were washed three times in saline solution (pH 7.90) to remove catecholamine released in the blood at the time of removal. They were then suspended at a hematocrit of 15% and left overnight at 4\(^\circ\)C in the saline solution to ensure that they had reached a steady state with respect to ion and water content and were no longer in a catecholamine-stimulated condition.

**Experimental Solutions**

The saline solution contained (millimolar): 145 NaCl, 5 CaCl\(_2\), 1 MgSO\(_4\), 4 KCl, 15 HEPPS [N-(hydroxy-2-ethyl) piperazine N'-propane sulfonic acid], 5 glucose, pH 7.90. In the experiments in which the pH of the solution had to be modified, HEPPS was replaced by the most appropriate buffer: BES, MES, HEPES, or tricine. Solutions were flushed with 100% O\(_2\), N\(_2\), or CO.

**Cell Ion and Water Contents**

After the overnight period of incubation, the red cells were washed four times in the appropriate experimental solution. They were kept in this saline (15\(^\circ\)C) for 3-4 h to ensure that they had reached a steady state with respect to ion and water content. Cells incubated in O\(_2\) or in N\(_2\) were gently stirred under a stream of gas; cells in CO were exposed to a CO atmosphere in a slowly rotating tonometer. Experiments were started by the addition of isoprenaline (5 \(\times\) 10\(^{-7}\) M) to the suspension. At intervals, samples of whole suspension were poured into nylon tubes, which were centrifuged at 20,000 g for
10 min in a Sorvall (Newton, CT) RC2B refrigerated centrifuge. These specially prepared tubes contain up to 0.7 ml.

**Cell water.** The packed cell mass was pushed out of the nylon tube with a close-fitting plastic rod onto weighed aluminum foil. After weighing, the packed cells were dried to constant weight for 10 h at 90°C and reweighed. Cell water content is expressed as grams of water per gram cell solid. Samples were studied in triplicate.

**Ion content.** The dry cells were suspended in 10 ml MgSO₄ solution (1 mM) and mixed carefully for 4 h. 200 μl of 70% (vol/vol) perchloric acid was then added to the suspension. After centrifugation at 20,000 g for 15 min, the clear supernatant was saved for analysis of cations and Cl⁻. Measurements were made as previously described (Baroin et al., 1984a). A trapping correction of 1.5% was routinely applied to the final calculation (Borgese et al., 1987b). Ion contents are expressed as micromoles per gram cell solid.

**Determination of pHi and pHv.**

\( \text{pH}_i \) was measured on 40-μl samples with a Radiometer pH glass capillary electrode maintained at 20°C and linked to a Radiometer PHM 72 acid-base analyzer.

For measurement of \( \text{pH}_v \), red cell pellets, obtained by centrifuging 600 μl of suspension in a nylon tube at 20,000 g for 10 min, were frozen, thawed for 5 min, and then frozen again. To prevent an acid shift, which occurs when samples are kept unfrozen, measurements of \( \text{pH}_i \) were made immediately after a second thawing of each lysate. Triplicate measurements were made on each sample.

**Cyclic AMP Level and ATP Measurement.**

Cyclic AMP was measured by radioimmunoassay as previously described (Mahé et al., 1985). ATP was measured by the firefly bioluminescence method with a Chemglow photometer (American Instrument Co., Silver Spring, MD). Typically, 100 μl of red cell suspension (hematocrit 20%) was hemolyzed in 2 ml of 10 mM HEPES buffer, pH 7.5, and placed in a boiling water bath for 10 min. After centrifugation, the extract was stored at -18°C. Firefly lantern extracts and ATP were purchased from Sigma Chemical Co. (St. Louis, MO) for the assay procedure.

**RESULTS**

**Effect of \( O_2 \) on the Activity of the \( Na^+/H^+ \) Exchange System**

Fig. 1A presents the changes in cell volume when isoprenaline is added to cells preincubated either in 100% \( O_2 \) or in 100% \( N_2 \). First, it can be seen that the initial cell volume differs in \( O_2 \) and in \( N_2 \). This is due to the anion shift induced by the different degree of ionization of hemoglobin in the two gasses. Second, it is clear that the change in cell volume induced by addition of isoprenaline is much greater in \( N_2 \) than in \( O_2 \). This difference is explained by a greater net uptake of Na in \( N_2 \) than in \( O_2 \) (220 ± 1.2 [\( n = 3 \)] and 81.6 ± 1.8 [\( n = 3 \)] μeq·g⁻¹·dry cells·35 min⁻¹, respectively). Furthermore, the swelling is fully inhibited by amiloride in both atmospheres (data not shown), which indicates that stimulation of the \( Na^+/H^+ \) exchange is responsible for the cell enlargement in both experimental conditions.

Fig. 2 gives the net Na uptake of cells preincubated in different mixtures of \( O_2 \) and \( N_2 \). It can be seen that the magnitude of the Na uptake depends on the partial pressure of \( O_2 \), which suggests that the degree of hypoxia controls the activity of the catecholamine-stimulated \( Na^+/H^+ \) exchange.
Figure 1. (A) Time course of cell volume changes after stimulation of trout red cells by isoprenaline ($5 \times 10^{-7}$ M). Temperature, 15°C; pH 7.95. (●) Cells incubated in an O$_2$ atmosphere for 4 h. (○) Cells incubated in an N$_2$ atmosphere for 4 h. The initial volumes of cells incubated in O$_2$ and N$_2$ are different owing to the anion shift induced by the difference in degree of ionization of hemoglobin in the two gases. The 4-h incubation was adopted in order to be certain that cell volume was in a steady state. (B) Time course of cAMP accumulation in the same batches of cells.

Figure 2. Net Na uptake induced by addition of isoprenaline as a function of the oxygen content in the gas phase (in percent). The cells were preincubated and gently stirred in different mixtures of O$_2$ and N$_2$ for 4 h. Ouabain ($10^{-4}$ M) was added just before hormonal stimulation. Net Na uptake was measured for 35 min after stimulation.
A modulation by $P_{O_2}$ of the amount of hormonal messenger accumulated in response to hormonal stimulation could explain the regulating effect of the gas on Na⁺/H⁺ exchange. This possibility can be ruled out since, after hormonal stimulation, the amount and the time course of accumulation of intracellular cAMP are similar in red cells incubated either in O₂ or in N₂ atmosphere (Fig. 1B). Nor do the changes in the activity of the Na⁺/H⁺ exchange appear to be related to the shift from aerobic respiration to glycolysis, because when the cells are poisoned with 1 mM cyanide under an O₂ atmosphere, the inhibition of the net Na uptake is not reversed (data not shown).

**Effect of CO on the Activity of the Na⁺/H⁺ Exchange System**

The question was further elucidated by studying cells in 100% CO. In such an atmosphere, erythrocytes are anoxic, as in N₂. In CO, however, the Na permeability is much lower than in N₂ (Fig. 3): the cells in CO behave like cells in O₂. In other words, in such an anoxic system, CO substitutes for O₂ in maintaining a low rate of Na⁺/H⁺ exchange. It is well known that CO substitutes for O₂ in binding to the iron porphyrin rings (in the heme molecule) and that this binding is difficult to reverse. As shown in Fig. 3 (filled triangles), cells previously exposed to CO and then stirred under a stream of N₂ for 4 h show the same inhibition of the Na⁺/H⁺ exchange system as the cells in a CO atmosphere. This interesting result strongly suggests that the binding of CO to heme, which persists even when N₂ has flushed CO out of the gas and aqueous phases (see legend to Fig. 3), is the determining factor inhibiting the Na⁺/H⁺ antiporter. If the action of
CO is via such a substitution of O2, this implies a nonmetabolic role for molecular O2 in regulating Na+/H+ exchange.

**pH Dependence of the Na+/H+ Exchange System in O2, N2, and CO**

We have previously shown (Borgese et al., 1987b) that the activity of the catecholamine-stimulated Na+/H+ exchanger in an N2 atmosphere is strongly pH dependent. When the red cells are adapted in media covering a wide range of pH values, it appears that the change in the exchanger activity is a complex relationship described by a characteristic bell-shaped curve: the Na uptake increases as pH becomes more acid, with a maximum around pH 7.3, and then decreases (see Fig. 4A). Since in red cells the distribution of H+ across the membrane is passive, in accordance with a Donnan equilibrium, pHi varies considerably, with changing values of pHo over a broad range. Thus, the relationship between the activity of Na+/H+ exchange and pHi is also described by a bell-shaped curve (Fig. 4B). We demonstrated that this complex relationship is explained by the predominant influence of internal H+ on the antiporter in the alkaline range and by the predominant influence of external H+ on the transporter in the acidic range.

From the results illustrated in Fig. 4, it is clear that for any chosen pHo or pHi, the activity of the Na+/H+ exchanger is greatly inhibited in an O2 or CO atmosphere when compared with the activity in an N2 atmosphere. Qualitatively, however, the relationship between pH and activity of the exchange system is similar in the three gasses.
DISCUSSION

Inhibitory Effect of O₂ on the Na⁺/H⁺ Exchange System and the Possible Role of Hemoglobin as a Transducer of the Signal

The data reported above show that (a) the change in cell volume induced by isoprenaline is much greater when the erythrocytes are incubated in N₂ than when they are in O₂ (Fig. 1A), and (b) the Na uptake depends on the partial pressure of O₂ (Fig. 2). These data indicate that the degree of hypoxia modulates the activity of the catecholamine-stimulated, amiloride-sensitive Na⁺/H⁺ exchange.

The inhibition of the activity of the exchange system by O₂ cannot be explained by a difference in the content of cAMP since the amount and the time course of accumulation of intracellular cAMP are similar in red cells whether they are incubated in O₂ or in N₂ (Fig. 1B). Neither can it be related to the shift from respiration to glycolysis, because in the presence of cyanide, swelling and Na uptake are not increased as they are in N₂. The experiments performed in CO (Fig. 3) again discount a possible metabolic role for O₂ in the inhibition of Na⁺/H⁺ exchange: when CO is bound to hemoglobin, even in an N₂ atmosphere, the cells behave like cells in O₂. Since CO and O₂ compete for the same binding site on hemoglobin, these results strongly suggest that hemoglobin is involved in the control of the exchange system via an interaction of the heme with O₂ or CO.

How can such a regulatory effect of hemoglobin be envisaged? It is well established that binding of O₂ and CO molecules with ferrous iron induces a change in the quaternary structure of the hemoglobin molecule. Starting from this consideration, three possibilities can be considered.

(a) The O₂-linked change in quaternary structure involves release of H ions, i.e., hemoglobin becomes a stronger acid when it is in the oxygenated form than it is in the reduced form. As a consequence, at an identical pH, the red cells in O₂ or CO atmospheres are more acid than red cells in N₂. The variation we observed in the activity of the Na⁺/H⁺ exchange system could reflect such a difference in pH (although a more acid pH would be expected to stimulate rather than inhibit the Na⁺/H⁺ exchange). This possibility can be ruled out since the results in Fig. 4 clearly show that, for any chosen pH, the activity of the exchange system is greatly inhibited in O₂ and in CO atmospheres as compared with the activity in an N₂ atmosphere.

(b) ATP is the major organic phosphate in trout red cells (Brunori, 1975). Its role as an allosteric modifier of fish hemoglobin is similar to that of 2,3-diphosphoglycerate (2,3-DPG) in mammalian erythrocytes: it interacts more strongly with deoxyhemoglobin than with oxyhemoglobin. Thus, the concentration of free ATP in the cell varies as a function of oxygenation and could have a role in regulating Na⁺/H⁺ exchange. Under our experimental conditions, the ATP contents of cells in O₂, N₂, and CO (and thus the free ATP concentration) differ widely owing to the 4-h preincubation in the gas atmospheres (3.44, 1.83, and 0.17 mM/liter cells, respectively). The fact that cells in CO respond to hormone as do cells in O₂ and not as cells in N₂ cannot be explained in terms of regulation of Na⁺/H⁺ exchange by the free ATP concentration.
Finally, let us consider a third possibility. It is well established that hemoglobin binds to the cytoplasmic side of the erythrocyte membrane in a reversible manner. The predominant sites of association are on the cytoplasmic segment of the band 3 protein (Shaklai et al., 1977; Salhany and Shaklai, 1979; Salhany et al., 1980; Cassoly, 1983), which is involved in the transport of anions (Rothstein et al., 1978; Steck, 1978; Knauf, 1979) and presumably of other compounds (Toon et al., 1985), including cations (Jones and Knauf, 1985). Deoxyhemoglobin has a higher affinity for band 3 than does oxyhemoglobin (Walder et al., 1984; Chetrite and Cassoly, 1985). The physiological significance of this specific interaction is unknown. It is tempting to speculate that hemoglobin, by interacting differently, according to its degree of oxygenation, with the major integral membrane protein, may influence some specific transport function, such as Na⁺/H⁺ exchange.

This interpretation does not necessarily mean that band 3 mediates the isoproterenol-stimulated Na⁺/H⁺ exchange: the hemoglobin–band 3 interaction, directly or via the cytoskeleton, could induce secondary changes of other structures that permit the passage of cations. The possibility still remains that hemoglobin could also interact with another membrane protein that mediates or controls Na⁺/H⁺ exchange. In other words, from the present data, the suggestion arises that hemoglobin, by binding differently, according to its quaternary structure, to a membrane protein could act as a transducer, allowing molecular O₂ to control membrane permeability.

In light of the data obtained with trout red cells, it is worthwhile to consider earlier investigations carried out in other nucleated erythrocytes. Klahr et al. (1969) observed that, in turtle red cells, anaerobic conditions stimulate an ouabain-insensitive Na flux that has the characteristics of exchange diffusion. Similarly, Tosteson and Robertson (1956) observed that the rate of Na and K transport across the cell membrane in avian red cells was stimulated upon shifting the gas phase from O₂ to N₂, an unexpected result since the cells are expected to be at a metabolic disadvantage in an N₂ atmosphere. From energetic considerations, they proposed that this permeability change could be due in part to an increase in exchange diffusion. Allen and McManus (1968) confirmed and extended this work. Using ouabain, they showed that N₂ causes an increase in the passive permeability of the cell membrane to Na but not to K (the increase in K influx described by Tosteson and Robertson results from the stimulation of the Na-K pump by the increase in cell Na). It appeared, therefore, that a specific permeability change was triggered by anoxia or by some degree of hypoxia. However, in an atmosphere of CO₂, the Na permeability did not increase as it did in N₂, which indicates a nonmetabolic role of O₂ and the involvement of a heme in Na permeability. Thus, hemoglobin appears to be involved in the control of some specific Na pathways in unstimulated as well as in hormonal-stimulated nucleated erythrocytes.

Physiological Significance of Inhibition of Na⁺/H⁺ Exchange by O₂

It has been shown that the addition of catecholamine to a suspension of trout red blood cells increases the O₂-carrying capacity of the erythrocytes (Nikinmaa,
Thus, stimulation of the Na\(^+\)/H\(^+\) exchange by catecholamines could be involved in an adaptative response to a respiratory stress. Until recently, the evidence of such a possibility was circumstantial rather than direct. We recently described results (Fievet et al., 1987) obtained in vivo that present evidence that a respiratory stress induces the stimulation, mediated by catecholamines, of the red cell Na\(^+\)/H\(^+\) exchange system. It was shown that when trout are abruptly exposed to deep hypoxia, a well-defined metabolic acidosis occurs, which was attributed to a release of lactic acid (Thomas and Hughes, 1982). Using an extracorporeal blood circulation that permitted a continuous recording of the acid-base parameters, we observed that the very rapid fall in arterial pH occurring at the onset of hypoxia is biphasic in nature (Thomas et al., 1986). We demonstrated (Fievet et al., 1987) that the first phase of acidification coincided with an increase in the plasma concentration of adrenaline and was inhibited by \(\beta\)-blocker agents such as propranolol. Since the slight increase of blood lactic acid occurring during this rapid step of acidification is unaffected by propranolol, it follows that the pH drop results from the \(\beta\)-adrenergic-controlled release of another acid. We showed, by studying ionic movements in red blood cells during this period, that the fall in pH is due to an extrusion of H\(^+\) from red blood cells in exchange for external Na\(^+\) and that this exchange is under \(\beta\)-adrenergic control. In other words, we demonstrated that the Na\(^+\)/H\(^+\) exchanges that were described in vitro are physiologically stimulated when the fish is submitted to deep and rapidly developed hypoxia (\(P_{\text{water} \cdot O_2} = 40\) Torr), which suggests that the countertransport is indeed involved in an adaptative response to a respiratory stress. This view is reinforced by the fact that such a stimulation is not observed in vivo in response to only moderate hypoxia (\(P_{\text{water} \cdot O_2} = 60\) Torr). The mechanism responsible for this putative respiratory function of the Na\(^+\)/H\(^+\) exchange is actually unknown (see Motais and Garcia-Romeu, 1987). If Na\(^+\)/H\(^+\) exchange is really involved in an adaptative response to a hypoxic stress, the control of this exchange by the partial pressure of \(O_2\) in the blood appears as a regulatory loop in a process serving to increase the \(O_2\)-carrying capacity of erythrocytes.

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