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EFFECT OF CATECHOLAMINES ON DEFORMABILITY OF RED CELLS FROM TROUT: RELATIVE ROLES OF CYCLIC AMP AND CELL VOLUME

BY GILLES CHIOCCHIA AND RENÉ MOTAIS

From the Laboratoire Jean Maetz, Département de Biologie du Commissariat à l'Energie Atomique, B.P. 68, 06230 Villefranche-sur-Mer, France

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SUMMARY

1. In the presence of catecholamine the nucleated red blood cells of trout show a large increase in cell volume as a result of an accumulation of sodium and chloride due to activation of an amiloride-sensitive, cyclic AMP-dependent Na^+–H^+ exchanger allowing Na^+ to enter in exchange for internal H^+.

2. The activation of this cyclic AMP-dependent Na^+–H^+ exchange is considered to be involved in an adaptive response to hypoxia by increasing the oxygen-carrying capacity of erythrocytes. But cell swelling could increase resistance to blood flow and thus impair the expected physiological advantages for oxygen transport. The effect of catecholamine on the deformability properties of the red blood cells has been studied by measuring the rate at which blood flows through a Nucleopore filter (5 μM).

3. The results show that stimulation by catecholamine in fact increases the erythrocyte deformability, a response which must favour the supply of oxygen at the tissue level.

4. Hormonal stimulation increases the cellular cyclic AMP content (and cyclic AMP-dependent phosphorylation of cytoskeleton proteins could influence cell deformability) and the cell volume. It has been shown that when cellular cyclic AMP content is increased under conditions where the cell cannot swell, the erythrocyte becomes more rigid and not more deformable. Conversely the results show a systematic coincidence between cell swelling and deformability increase. The precise way in which volume change and deformability are interrelated needs more study.

INTRODUCTION

The addition of catecholamines to a trout red blood cell suspension promotes a large increase in cell volume (Baroin, Garcia-Romeu, Lamarre & Motais, 1984*a*; Bourne & Cossins, 1982; Nikinmaa, 1982; Borgese, Garcia-Romeu & Motais, 1987*a*) as a result of an accumulation of sodium and chloride, part of the entering sodium then being rapidly exchanged for potassium via the sodium–potassium pump (Baroin *et al.* 1984*a*).

It has been demonstrated that catecholamines activate an amiloride-sensitive

Na^+–H^+ antiporter which allows Na^+ to enter in exchange for internal H^+ with a stoichiometry of 1:1 (Baroin, Garcia-Romeu, Lamarre & Motais, 1984*b*). This stimulation is mediated by cyclic AMP since it is prevented by propranolol, and forskolin or exogenously added cyclic AMP mimic the effect of catecholamines (Mahé, Garcia-Romeu & Motais, 1985). The exchange of Na^+ for H^+ results in an acidification of the external medium (Baroin *et al.* 1984*b*; Cossins & Richardson, 1985; Borgese, Garcia-Romeu & Motais, 1986) and an alkalinization of the red blood cell (Cossins & Richardson, 1985; Borgese, Garcia-Romeu & Motais, 1987*b*). This pH disequilibrium activates Cl^-–HCO_3^- exchanges normally occurring via the anion exchanger located in band 3 protein. The result of the parallel functioning of the two exchangers is the considerable and simultaneous uptake of Na^+ and Cl^-, with water following osmotically, causing much cell swelling.

The activation of this cyclic AMP-dependent Na^+–H^+ exchange could be involved in an adaptative response to a respiratory stress as suggested by the following observations: (1) *in vitro*, the addition of catecholamines to a trout red blood cell suspension increases the oxygen-carrying capacity of the erythrocytes, as a result of intracellular alkalinization (Nikinmaa, 1982, 1983; Cossins & Richardson, 1985); (2) *in vivo*, when a trout is submitted to a deep and rapidly developed hypoxia, a well-defined metabolic acidosis occurs which is due to the β-adrenergic-controlled release of H^+ from erythrocytes via the Na^+–H^+ exchanger (Fievet, Claireaux, Thomas & Motais, 1988; Thomas, Fievet, Claireaux & Motais, 1988), and simultaneously the haemoglobin oxygen affinity is increased (Tetens & Lykkeboe, 1985; Claireaux, Thomas, Fievet & Motais, 1988) and (3) the activity of the Na^+–H^+ exchanger is controlled by the partial pressure of oxygen in the saline. This control is triggered by the binding of molecular oxygen to haeme which, via the modification in the quaternary structure of the haemoglobin molecule, could affect the exchanges (Motais, Garcia-Romeu & Borgese, 1987). This control of Na^+–H^+ exchanges by the partial pressure of oxygen in the blood appears as a regulatory loop in a process serving to increase the carrying capacity of erythrocytes for oxygen in deep hypoxia.

The above interpretation of the respiratory function of Na^+–H^+ exchange in red cells raises a problem. As reported above, the stimulation of Na^+–H^+ exchanges *in vitro* causes a large increase in red blood cell volume (up to 50% of the initial volume). Such cell swelling may increase resistance to blood flow and thus impair the expected physiological advantages for oxygen transport. Indeed numerous authors using various methods, such as filtration (Schlick & Schmid-Schonbein, 1975; Leblond & Coulombe, 1979; Koutsouris, Delatour-Hanss & Hanss, 1985), micropipettes (Leblond & Coulombe, 1979; Meir, Kucera, Lerche & Baumler, 1983) and ektacytometer (Mohandas, Clark & Shohet, 1980) have already shown in mammalian erythrocytes a reduced deformability of the cell as its volume increases. By contrast Hughes & Kikuchi (1984), using a filtration method, demonstrated a greater deformability of erythrocytes in blood sampled from hypoxic than from normoxic trout, a result suggesting that the catecholamine-induced swelling, which also occurs *in vivo* in response to hypoxia (Fievet, Motais & Thomas, 1987; Fievet *et al.* 1988), does not have a negative effect on the flow properties of fish blood.

The aim of the present work was to determine first whether catecholamines added *in vitro* induce an increased deformability of fish nucleated red cells, and then to analyse the factor(s) involved in this modification.

METHODS

Preparation of cells

Rainbow trout, *Salmo gairdneri*, were obtained from a commercial hatchery and kept for 1 week in the laboratory in tanks provided with running tap water (water temperature, 15 °C). Fish were anaesthetized by immersion in neutralized tricaine (MS 222: 0·1 g l^{-1}) in water after which blood

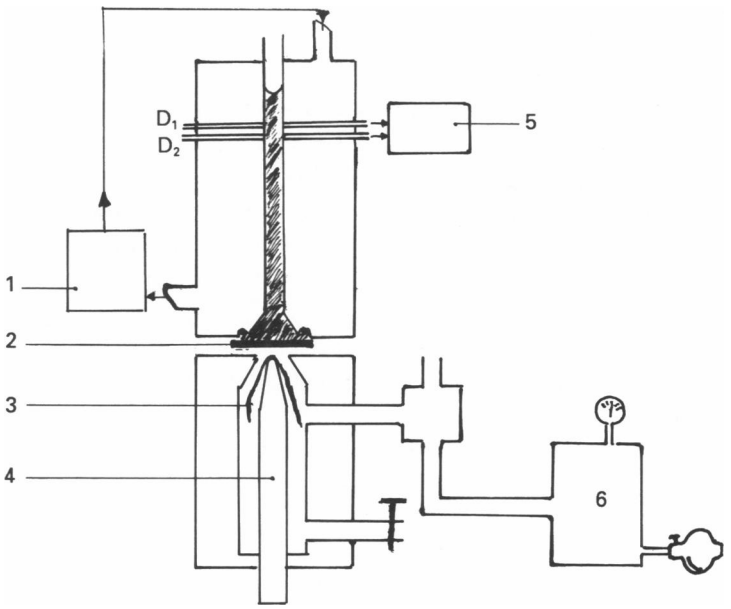

Fig. 1. Diagram of apparatus used for measurement of red cell deformability. Explanation in the text. 1, thermostat; 2, Nucleopore filter; 3, paper filter; 4, drop-catching device; 5, chronometer; 6, over-pressure ballast.

was removed from the caudal vein using 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 haematocrit of 15% and left overnight at 4 °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.

Water content

Experiments were started by the addition of catecholamines or isoprenaline to the suspension. At intervals, samples of the whole suspension (haematocrit 15%) were poured into tubes, which were centrifuged at 20000 *g* for 10 min in a Sorvall (Newton LT) RC_2B refrigerated centrifuge. These specially prepared tubes contained up to 0·7 ml. The packed cell mass was pushed out of the nylon tube with a close-fitting plastic rod onto pre-weighed aluminium 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 of cell solids. Samples were studied in triplicate.

Measurement of erythrocyte deformability

One of the most satisfactory means of estimating erythrocyte deformability is the filtration method, based on the continuous flow of individual cells through narrow channels: microsieving can thus be considered a 'dynamic' deformability method which depends on the membrane and cell interior viscosity. Whole-blood filtration flow rate cannot be considered as an effective erythrocyte deformability measurement, because it depends on numerous factors such as plasma viscosity, haematocrit, leucocyte count, etc.

The method used in this work is the technique developed by Hanss (1983). Using a diluted

erythrocyte suspension and based on an initial flow rate measurement through 5 μm Nucleopore membranes, it is largely insensitive to well-known secondary phenomena (erythrocyte sedimentation, and/or aggregation) which are commonly present in many filtration techniques.

The Hanss 'hemorheometre' (Fig. 1) is built around two plastic units. The upper one contains a vertical transparent tube surrounded by circulating temperature-regulated water. This tube ends below as a small conical chamber; to its upper part are fitted two adjacent level detectors (D_1 and D_2) connected to an electronic timer. The lower unit of the apparatus is removable and has a large chamber which can be connected either to an over-pressure circuit or to the atmosphere. A Nucleopore filter (13 mm diameter) can be fitted over the circular opening (9 mm) of this chamber.

Once the filter is in place, the two units are held together and the tubing is filled with a liquid (saline or red cell suspension in saline). A small positive pressure in the lower chamber prevents any flow. The positive pressure is suddenly released and the liquid descends by gravity. The liquid–air meniscus actuates successively the two level detectors which start then stop the timer. The time interval thus recorded is proportional to the resistance of the filter to the liquid flow. As the level sensors are located at the upper end of the tubing and as the distance between them is small in relation to the total tube length, the measurements indicate the *initial flow rate*. As the volume needed to obtain the time interval (t) is small (about 60 μl), inaccuracies due to the filter becoming blocked by large rigid particles are kept to a minimum. Similarly, since the filtered volume is small, the time interval is short (typically about 0·5–1 s), and any spurious sedimentation effects are therefore avoided.

To obtain standardized erythrocyte rheological data, the following rigidity index, RI, was used:

$$\mathrm{RI} = \frac{t_s - t_b}{t_b H}.$$

where H is the haemotocrit in decimal form and t_s and t_b are the time intervals for the suspension and for the saline alone, measured with the same filter. As previously shown (Hanss, 1983), RI is independent of H in the 0–0·1 range. This shows that what is measured depends only on the rheological property of the average individual cell and not on interactions between cells. The haemotocrit used was about 0·04. The 5 μm Nucleopore membranes used in this series of experiments were from the same lot (51 A 5A13). The saline solution in which red blood cells were suspended contained (in mM): 145 NaCl, 5 $CaCl_2$, 1 $MgSO_4$, 4 KCl, 15 HEPPS(*N*-(hydroxy-2-ethyl)-piperazine-*N'*-propane sulphonic acid) and 5 glucose, pH 7·90. In the experiment in which the pH of the solution had to be modified, HEPPS was replaced by the most appropriate buffer, either MES or HEPES.

RESULTS

Effect of catecholamines on red blood cell deformability and swelling

Figure 2 presents the time dependence of cell volume (upper graph) and cell deformability expressed as the rigidity index (lower graph) when isoprenaline is added to red blood cell suspension. The lower graph represents two successive experiments performed on the same batch of blood. These results are representative of ten similar experiments.

It can be observed that addition of isoprenaline (5×10^{-7} M), as previously described, induces a rapid increase in cell volume. Simultaneously and with a similar time course there is a decrease in the rigidity index.

Addition of adrenaline (10^{-5} M) and noradrenaline (10^{-5} M) gave identical results (not shown).

Effect of exogenously added cyclic AMP

The stimulation of Na^+–H^+ exchanges is mediated by cyclic AMP and can be mimicked by addition of cyclic AMP to the external medium (Mahé *et al.* 1985).

Figure 3 illustrates the effect of such an addition on cell volume (upper graph) and cell deformability (lower graph). It can be seen that the responses are very similar to the responses observed after addition of isoprenaline, i.e. a simultaneous increase in cell volume and decrease in rigidity.

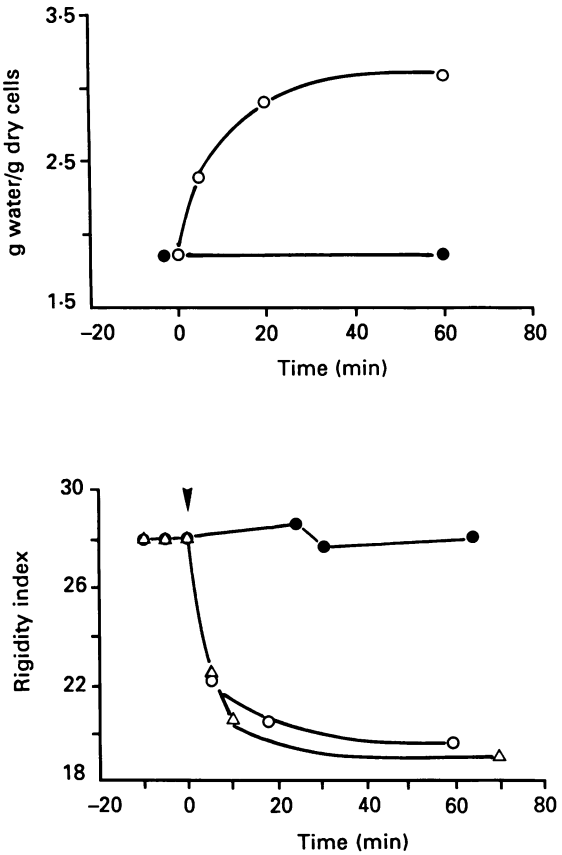

Fig. 2. Upper graph, time dependence of cell volume changes after stimulation of trout red blood cells by isoprenaline (5×10^{-7} M). Temperature 15 °C, pH = 7·9. ●, control cells; ○, isoprenaline-treated cells. Lower graph, time dependence of cell deformability, expressed as the rigidity index, after stimulation by isoprenaline. ●, control; ○ and △, two successive experiments with isoprenaline.

Relationship between deformability, cell volume change and cell cyclic AMP content

From the above results it can be seen that cell rigidity decreases when cell volume increases. This cannot be a direct relationship however since cell swelling is induced by an increase in the cell cyclic AMP content and cyclic AMP-dependent protein phosphorylation is likely to control interactions between proteins of the red cell cytoskeleton. The following experiments were therefore performed to evaluate the relative roles of cell volume change and cell cyclic AMP concentration on red blood cell deformability.

Effect of catecholamine on red cells suspended in choline chloride medium

Addition of catecholamine to red blood cells suspended in sodium-free medium stimulates adenylate cyclase and thus induces an increase in the cell cyclic AMP content (not shown) and activation of the Na^+–H^+ antiporter. In the absence of

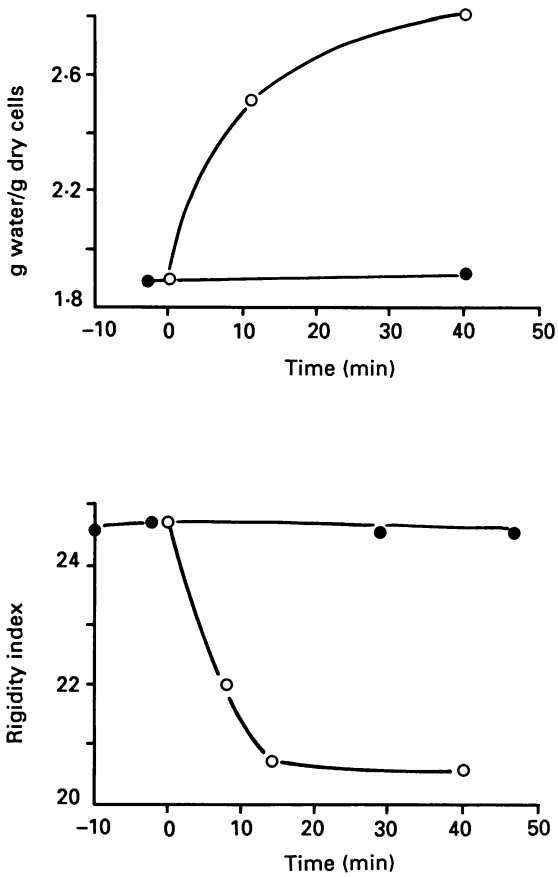

Fig. 3. Upper graph, time dependence of cell volume changes after addition of cyclic AMP (10^{-3} M) to red blood cell suspension. IBMX (10^{-4} M) was present in the suspension to inhibit phosphodiesterase (Mahé *et al.* 1985). Lower graph, time dependence of cell deformability after addition of cyclic AMP (this experiment is representative of four similar experiments). Symbols and experimental conditions as in Fig. 2.

external sodium, however, the exchange does not occur and the cells do not swell. This is illustrated in the upper part of Fig. 4. Nevertheless, in these conditions, there is an increase in cell rigidity (Fig. 4, lower graph) suggesting that cyclic AMP *per se* has a negative effect on deformability.

Effect of amiloride

In the experiment illustrated in Fig. 5, red blood cells suspended in normal saline were stimulated at time zero by isoprenaline which, as described above, induces an

increase in cell volume and a decrease in cell rigidity. Then, 33 min later, amiloride (5×10^{-4} M) was added to the suspension. Amiloride is known to inhibit the Na^+–H^+ antiporter in trout erythrocytes (Mahé *et al.* 1985), thus blocking Na^+ entry and cell swelling (Baroin *et al.* 1984*a*). As cell enlargement promotes the opening of a pathway allowing K^+ leakage (Borgese *et al.* 1987*a*, *b*) addition of amiloride in fact

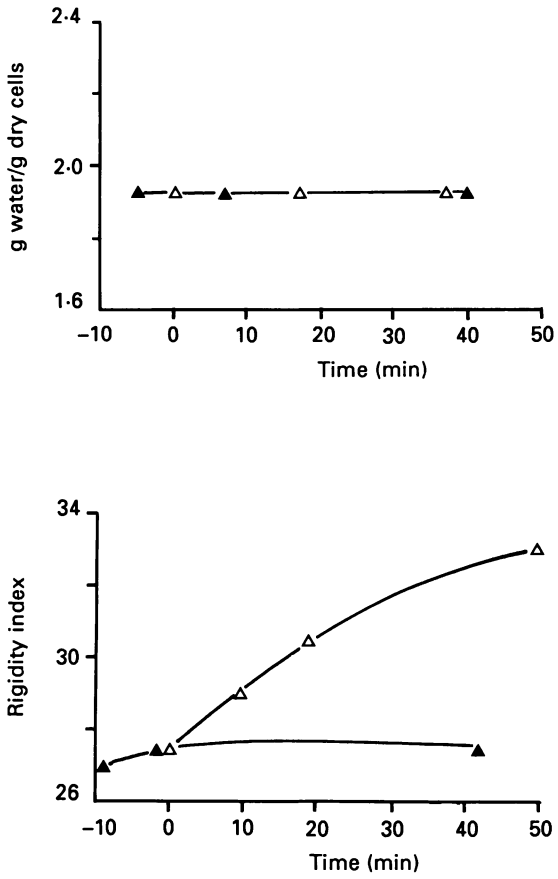

Fig. 4. Upper graph, effect of isoprenaline (5×10^{-7} M) on cell volume when red blood cells are suspended in a choline chloride medium (sodium-free medium). Lower graph, time dependence of cell deformability after stimulation by isoprenaline of red blood cells suspended in a choline chloride medium. Three similar experiments have been made. ▲, control cells; △, isoprenaline-treated cells.

results in a cell volume decrease due both to the instant blockage of Na^+ entry and to the permanent K^+ leakage. The cell shrinkage following the addition of amiloride can be observed in the upper part of Fig. 5. Simultaneously an increase in cell rigidity was measured (lower graph Fig. 5) again suggesting a relationship between cell volume change and cell deformability.

This conclusion is supported by the fact that addition of amiloride promotes a very rapid fall of cellular cyclic AMP content due to both the blockage of cyclic AMP synthesis by amiloride and the rapid metabolism of cyclic AMP by phosphodiesterase

activity (Mahé *et al.* 1985). Thus in the experiment illustrated in Fig. 5 the negative effect of cyclic AMP on deformability cannot be advanced to explain the change in cell deformability. It should also be noted that amiloride alone, i.e. added to control cells, does not influence cell deformability (not shown).

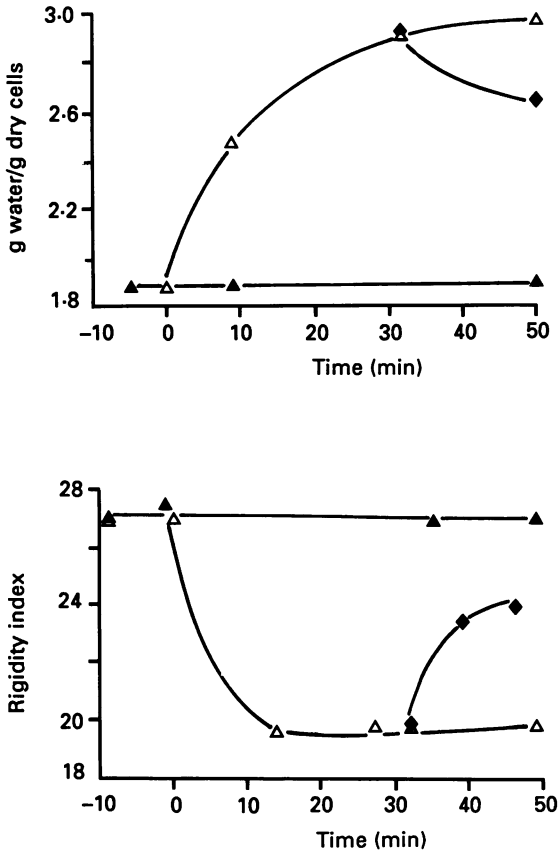

Fig. 5. Time course of cell volume change (upper graph) and cell deformability (lower graph) after stimulation of trout red cells by isoprenaline and then addition of amiloride. Three similar experiments have been made. ▲, control; △, isoprenaline; ◆, isoprenaline + amiloride.

Effect of external pH and hypotonic medium

It is well established (see review by Hladky & Rink, 1977) that the red blood cell volume varies as a function of the pH of the medium: haemoglobin at physiological pH is in the form of a polyvalent anion, and an equivalent number of K^+ ions act as the counter ions. Since the red cell membrane is freely permeable to anions but poorly permeable to cations, when haemoglobin is partly converted to the acid form, Cl^- and HCO_3^- replace it as the cell anion. The consequence of monovalent anions replacing a polyvalent anion is that the total number of ions in the cell increases, thereby

causing a raised internal osmotic pressure and an entry of water. Conversely, at a more alkaline pH, haemoglobin replaces Cl^- and HCO_3^- as the cell anion, the osmotic pressure of the cell falls and water moves out.

Thus it is possible to modify the cell volume of unstimulated control red blood cells

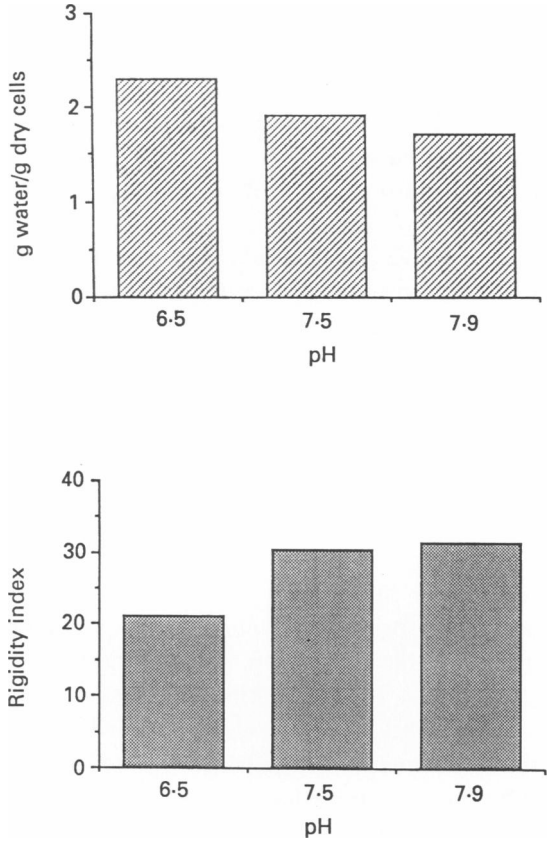

Fig. 6. Cell volume and cell deformability measured on control red blood cells adapted at three different external pH values.

without any change in cell cyclic AMP content (unpublished observations). Figure 6 shows that in such conditions, as long as the cell volume increases, the cell rigidity decreases.

Similarly when the osmolality is decreased from 30 to 230 mosmol l^{-1} by using the same saline with a lower NaCl concentration (110 mM), the cells swell (from 1·8 to 2·4 g water/g dry cells) and the cell rigidity decreases from 32 to 24.

DISCUSSION

The stimulation by catecholamines of the Na^+–H^+ antiporters located in the membrane of trout red blood cells appears to be involved in an adaptive response to

deep hypoxia though the mechanisms responsible for this putative respiratory function of the Na^+–H^+ exchange are not yet fully understood (for review, see Motais & Garcia-Romeu, 1987, 1988; Fievet *et al.* 1988). Whatever the mechanisms involved, one of the consequences of the stimulation of the exchanger is an increase of the red cell volume.

An increase in cell volume, if it corresponds to a decrease in deformability of the erythrocyte, could represent a crucial disadvantage at the microcirculation level, increasing resistance to blood flow and thus impairing the expected physiological advantages for oxygen transport.

To estimate the erythrocyte deformability we used a rheological method based on the measurement of the initial flow rate of diluted erythrocyte suspensions through 5 μm pores, which allows determination of a rigidity index having the same significance as an individual rigidity index of an average red blood cell filtering through one pore (Koutsouris *et al.* 1985).

The results reported above show that stimulation by catecholamine of trout red blood cells suspended in saline increases the erythrocyte deformability, a response which must favour the supply of oxygen at the tissue level and thus enhance the physiological advantage of an increase of the oxygen-carrying capacity of the erythrocytes induced by Na^+–H^+ exchanges. This result agrees with the conclusion of Hughes & Kikuchi (1984) that during deep hypoxia (a condition in which Na^+–H^+ exchanges are physiologically stimulated, as described by Fievet *et al.* 1987) the deformability of trout erythrocytes is increased.

The stimulation of red blood cells by catecholamines modifies at least two factors likely to affect cell deformability, i.e. the cellular cyclic AMP content and the cell volume, both of which were shown to increase. The red blood cell membrane skeleton is composed of several peripheral proteins. Cyclic AMP-dependent phosphorylation probably controls the functional roles of certain of these and thus could influence cell deformability (Kury & McConnell, 1975; Backman, 1988).

When cellular cyclic AMP content is increased but the cell cannot swell because of being suspended in a sodium-free medium, we observed that the erythrocyte becomes more rigid and not more flexible as was expected. This shows that cyclic AMP *per se* may decrease cell deformability, and thus certainly cannot explain the increase in deformability observed in normal conditions, i.e. when the Na^+–H^+ exchanger functions and the cells swell. Other data recorded above show a systematic coincidence between cell volume and deformability: (1) the cell volume decrease induced by amiloride is coincident with a decrease in deformability (Fig. 5); (2) the cell volume increase resulting from acidification coincides with an increase in deformability (Fig. 6) and (3) the cell volume increase induced by suspension in a hypotonic medium coincides with an increase in deformability.

In mammalian non-nucleated red cells it has been shown that slight changes in cell volume have little effect on the deformability of cells because of a balance between the countervailing effects of changes in intracellular viscosity and in the surface-to-volume ratio (Johnson, 1985). By contrast a reduced deformability is observed when the cells are suspended both in hypertonic and in hypotonic salt solutions (Leblond & Coulombe, 1979; Mohandas *et al.* 1980; Koutsouris *et al.* 1985); in hyperosmolar media it may be explained by an increase in internal viscosity (Chien, 1975) but in

hypotonic media the increase in cell rigidity which is observed, despite the dilution of haemoglobin which lowers intracellular viscosity, probably results from the increase in the sphericity index (Chien, 1975; Mohandas *et al.* 1980).

In fish nucleated erythrocytes the increase in cell volume does not induce an increase but rather a decrease in cell rigidity. It could be related to the fact that the nucleated erythrocytes, which are normally flat biconvex cells, tend to become more spherical as they swell and then have a smaller diameter (unpublished data).

Further detailed studies are necessary to elucidate the precise way in which volume change and deformability are interrelated.

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