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ION MOVEMENTS AND VOLUME CHANGES INDUCED BY CATECHOLAMINES IN ERYTHROCYTES OF RAINBOW TROUT: EFFECT OF pH

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SUMMARY

1. Trout red cells suspended in an isotonic medium containing catecholamines or adenosine 3′,5′-phosphate (cyclic AMP) enlarge rapidly to reach a new steady-state volume which is maintained as long as hormone is present.

2. The present investigation demonstrates that the maximum swelling reached by the cells is strongly pH dependent. At pH 7·55 the cells enlarge more rapidly than at pH 7·95 and they reach a maximal volume which is much greater. It is explained by a differential effect of pH on two pathways controlling the movements of cations: K^+ loss decreases as pH becomes more acidic in a roughly linear manner. On the contrary Na^+ uptake increases as pH becomes more acidic with a maximum around pH 7·30 and then decreases. From this pH dependence it can be expected that the maximum enlargement occurs at about pH 7·30.

3. The complex relationship describing the change in the activity of the Na^+–H^+ exchanger as a function of pH (bell-shaped curve) is explained by the predominant influence of internal H^+ on the antiporter in the alkaline range of pH and by the predominant influence of external H^+ on the transporter in the acidic range.

INTRODUCTION

Addition of catecholamines to a suspension of trout red cells induces a swelling of the erythrocytes and when the hormone is in contact with the cells for a long time they reach a new steady-state volume. As previously described (Borgese, Garcia-Romeu & Motais, 1987) this new steady-state volume results from a dynamic equilibrium involving the simultaneous functioning of two regulatory processes induced by the hormone: a volume increase response that causes the cell to enlarge by giving NaCl via a Na^+–H^+ exchange, and a volume decrease response that causes cell to shrink by losing K^+ via a Cl^--dependent K^+ pathway. As it has been demonstrated that the increase response is mediated by an adenosine 3′,5′-phosphate (cyclic AMP)-dependent Na^+–H^+ exchange (Mahé, Garcia-Romeu & Motais, 1985), it was of interest to study the influence of pH on the Na^+–H^+ exchanger and thus on

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volume changes induced by catecholamines. More precisely the influence of both external pH (pH_o) and internal pH (pH_i) was estimated. It was obtained by measuring the activity of the Na^+–H^+ antiporter at a fixed pH_i but at different values of pH_o and conversely at a fixed pH_o but at different values of pH_i.

METHODS

The experimental conditions, methods for cell isolation and cation and water content determination were the same as previously described (Borgese *et al.* 1987).

$^{22}Na^+$ influx measurements. For $^{22}Na^+$ uptake measurements, 10 μl of $^{22}Na^+$ (20 μCi/ml) and isoprenaline (final concentration 5×10^{-7} M) were simultaneously added to 500 μl of cell suspension (haematocrit 15%) containing ouabain (10^{-4} M) and vigorously mixed. The suspension was incubated for 3 min. The reaction was terminated by centrifugation (20000 *g*) in a Sorval RC 2B refrigerated centrifuge. Extracellular trapping was estimated by addition of the isotope without hormonal stimulation and immediate centrifugation. For each sampling 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. Triplicate aliquots (30 μl) of the superantant were counted. The packed cell mass was expressed with a close-fitting plastic rod on to weighed aluminium foil, and dried to constant weight for 10 h at 90 °C. After weighing the pellets were counted in a well-type scintillation counter. $^{22}Na^+$ influx was expressed as μmol Na^+ g^{-1} dry cells min^{-1}.

Flux determinations as functions of pH. In some experiments cells were adapted at different external pHs and Na^+ uptake and K^+ loss were measured at these different pHs. Adaptation was carried out by washing the cells four times in the respective salines, and then incubating for 2 h at the desired pH.

In other experiments pH_i was driven to a desired value prior to the measurement of Na^+ fluxes in salines at different pH values. pH_i was manipulated simply by changing the pH of the incubation medium, activities of internal and external H^+ being distributed in accordance with a Donnan equilibrium. The cells were then incubated in this saline for 2 h. When erythrocytes were finally suspended in the experimental solution, DIDS (4,4′-diisothiocyanostilbene-2,2′-disulphonic acid, 5×10^{-4} M) was used to clamp pH_i by inhibiting pH readjustment through the anion exchanger.

Determination of pH_i and pH_o. pH_o 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 analyser.

For measurement of pH_i, red cell pellets, obtained by centrifuging 600 μl of suspension in nylon tube at 20000 *g* for 10 min, were frozen, thawed during 5 min and then frozen again. To prevent an acid shift observed when samples are kept unfrozen, measures of pH_i were made immediately after a second thawing of each lysate. Triplicate measures were made on each sample.

RESULTS

pH dependence of cell volume changes induced by catecholamines

Fig. 1 illustrates the changes in cell volume when isoprenaline is added to trout red cells suspended in saline either to pH 7·55 (buffered with 15 mM-HEPES, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulphonic acid) or at pH 7·95 (buffered with 15 mM-Tricine, *N*-tris[hydroxymethyl]methylglycine). These experiments were made on cells equilibrated for pH with their respective suspension medium (see Methods). The experiment was started by addition of 5×10^{-7} M isoprenaline with or without ouabain (10^{-4} M).

Two important features of the results can be noted. First, the volume response is strongly pH dependent: at pH 7·95 the cells enlarge more slowly than at pH 7·55 and they reach a new steady-state volume which is lower (40% inhibition). It should be pointed out that the initial volume of the two batches of cells is significantly

different. This is due to the osmotic effect associated with the chloride shift induced by the difference in ionization of haemoglobin at the two pH values.

Secondly, as we previously observed in experiments performed at pH 7·95 (Borgese *et al.* 1987) ouabain had no appreciable effect either on the time course or on the magnitude of the cell volume changes at pH 7·55.

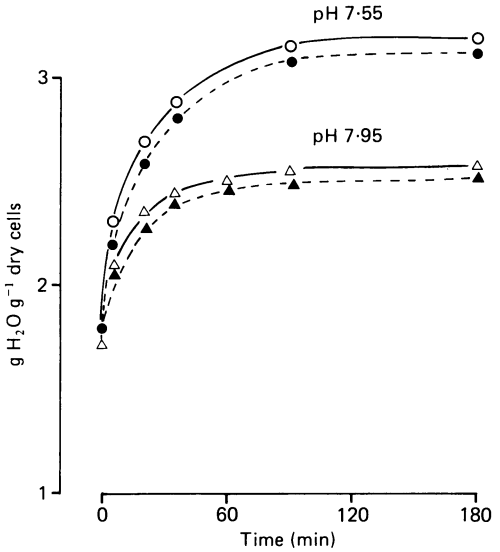

Fig. 1. Time course of cell volume changes after stimulation of trout red cells by isoprenaline ($5{\cdot}5 \times 10^{-7}$ M) at two different pHs. ●, ▲: without ouabain. ○, △: with ouabain.

Fig. 2 shows the changes in cation content associated with the cell increase in the same experiment as depicted in Fig. 1. From these data the following salient observations warrant emphasis.

(1) At a given pH (7·55 or 7·95), the comparison between ouabain-treated cells and control cells shows that ouabain blocks the exchange of Na^+ for K^+ without substantially affecting the $Na^+ + K^+$ content. This result explains why ouabain has no appreciable effect on changes in cell size, since it has been shown that water movement during volume increase is satisfactorily explained by the movements of only Na^+, K^+ and Cl^- (Borgese *et al.* 1987).

(2) At both pHs it can be seen that the net Na^+ uptake continues for several hours, in fact for as long as the cells are in contact with hormone. Thus at pH 7·55, as previously shown at pH 7·95 (Borgese *et al.* 1987), the cells reach a new stable volume not as a consequence of an inhibition of Na^+ entry but because of the establishment of a dynamic equilibrium between the two regulatory processes induced by the hormone: the volume increase response mediated by cyclic AMP, that causes cells to enlarge by gaining Na^+, and the volume decrease response, triggered by cell enlargement, that causes cells to shrink by losing K^+.

(3) The difference observed in the volume changes in pH 7·55 and 7·95 must therefore reflect a differential effect of pH on Na^+ uptake and/or K^+ loss. Fig. 2

clearly shows that at pH 7·55 the net Na^+ influx is greater than at pH 7·95. By contrast the opening of the K^+ channel occurs later at pH 7·55 that at pH 7·95; thus in the first hour the net K^+ loss is smaller at the more acidic pH. These opposite effects of pH on net Na^+ and K^+ fluxes explain why at pH 7·55 the cells enlarge more rapidly.

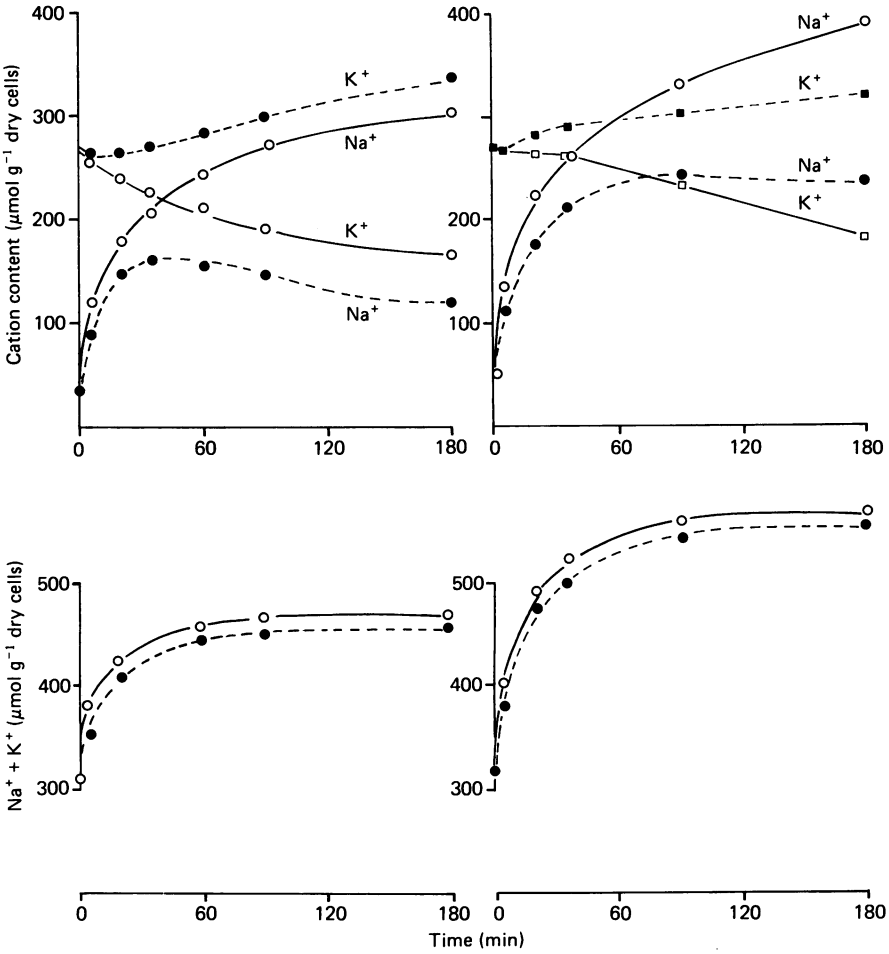

Fig. 2. Changes in cation content, and time course of $Na^+ + K^+$ content in the experiment of which the volume changes are depicted in Fig. 1. ○, with ouabain; ●, without ouabain. The right part of the Figure illustrates the data obtained at pH 7·55; the left part illustrates those obtained at pH 7·95.

pH dependence of Na^+ uptake and K^+ loss

To evaluate the effects of pH on Na^+ uptake and K^+ loss, cells were incubated in a normal saline containing different buffers (15 mM): MES (2-[*N*-morpholino]ethanesulphonic acid), BES (*N*,*N*-bis[2-hydroxyethyl]-2-aminoethanesulphonic acid), HEPES and Tricine. Care was taken to ensure that the cells were equilibrated at the pH at which the subsequent determination of flux was performed. Ouabain (10^{-4} M) was added just before the beginning of the experimental period.

Fig. 3 illustrates the pH dependence of net Na^+ influx and net K^+ loss measured for 35 min after hormonal stimulation. Two important features of the data can be noted. First, the net K^+ loss increases with pH, the rate of K^+ loss increasing about tenfold between pH 6·25 and 8·12. Secondly, the net Na^+ influx shows a bell-shaped pH dependence with a maximum at about pH 7·3–7·6.

This effect of pH on both Na^+ and K^+ net fluxes clearly explains why the swelling induced by catecholamines is so strongly pH dependent, as illustrated in Fig. 1. The maximum enlargement can be predicted to occur around pH 7·30.

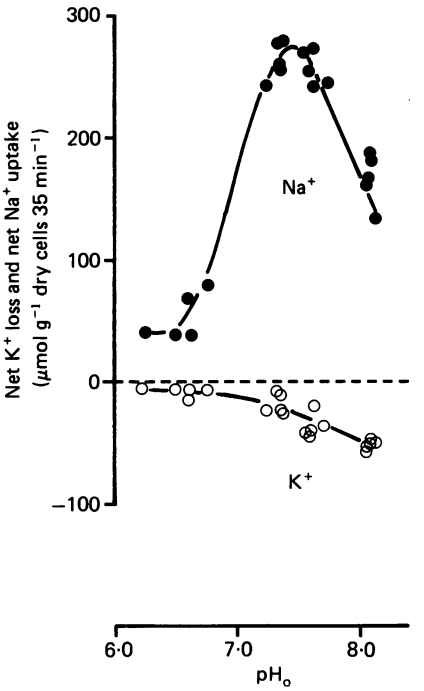

Fig. 3. Effect of pH on net Na^+ uptake and net K^+ loss in trout red cells after stimulation by isoprenaline ($5{\cdot}5 \times 10^{-7}$ M). The cells were pre-incubated in saline at the indicated pH, and net ion changes were measured for 35 min in the medium of incubation. Ouabain (10^{-4} M) was added just before hormonal stimulation.

pH dependence of $^{22}Na^+$ uptake in short-term experiments

As previously reported, the Na^+ uptake is mediated by a Na^+–H^+ exchanger which is activated by isoprenaline (Baroin, Garcia-Romeu, La Marre & Motais, 1984*b*; Borgese, Garcia-Romeu & Motais, 1986; Cossins & Richardson, 1985). To investigate further the pH dependency of the Na^+–H^+ exchange system Na^+ uptake was measured in short-term experiments using labelled Na^+ ($^{22}Na^+$).

Fig. 4*A* shows the time course of $^{22}Na^+$ accumulation in red cells suspended in a saline buffered at pH 7·50; ouabain (10^{-4} M) was present in the incubation medium to prevent the efflux of $^{22}Na^+$ catalysed by Na^+–K^+-ATPase. In the absence of amiloride $^{22}Na^+$ accumulated rapidly in the cells. In the presence of amiloride (5×10^{-4} M) the accumulation of ^{22}Na was very slow and in fact virtually undetectable during the first 3 min after addition of isoprenaline, i.e. during the period furnishing

the value for the initial $^{22}Na^+$ uptake rate in the experiment described below. Thus isoprenaline induces a considerable $^{22}Na^+$ uptake which is largely inhibited by amiloride. This effect of amiloride on $^{22}Na^+$ influx was expected since it has previously been shown that amiloride completely blocks the net Na^+ uptake induced by isoprenaline both by directly inhibiting Na^+–H^+ exchange and by inhibiting the synthesis of the hormonal messenger (cyclic AMP) which is involved in the activation of the Na^+–H^+ exchange (Mahé *et al.* 1985).

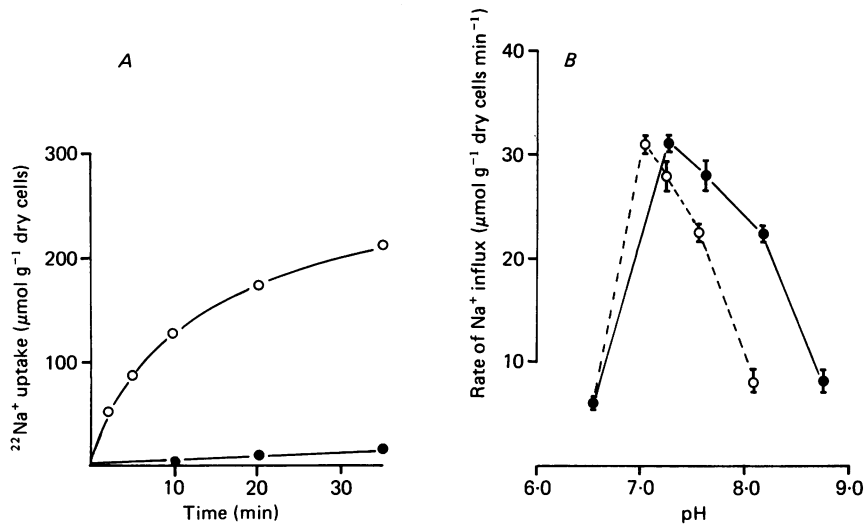

Fig. 4. *A*, time course of $^{22}Na^+$ accumulation in trout red cells after stimulation by isoprenaline. The pH of the saline was 7·5. Na^+ uptake was performed in the presence (●) or in the absence (○) of 0·5 mM-amiloride. 10^{-4} M-ouabain was added just before hormonal stimulation. *B*, effect of pH on initial rate of Na^+ influx induced by isoprenaline. As in Fig. 3, the cells were pre-incubated in saline at the indicated pH and $^{22}Na^+$ influx was measured for 3 min in the medium of incubation in the presence of ouabain. Continuous line: Na^+ influx as a function of pH_o; interrupted line: Na^+ influx as a function of pH_i. Data are means ± S.E. of means of three experiments.

The dependence on pH of the initial rate of $^{22}Na^+$ uptake is presented in Fig. 4 *B*. In this experiment the rate of $^{22}Na^+$ influx was determined in cells equilibrated at the pH at which the determination of flux was performed, i.e. in the same experimental conditions as in the experiment depicted in Fig. 3. It can be seen that changing pH_o from 6·5 to 8·75 produced a characteristic bell-shape curve (continuous line) which is similar to that depicted in Fig. 3.

Thus the pattern of pH dependence of Na^+ uptake is similar when entry of Na^+ is measured during the first 3 min (initial rate) or the 35 min following hormonal stimulation.

In Fig. 4 *B* the dependence of the initial rate of $^{22}Na^+$ uptake on pH_i is also depicted (interrupted line). Indeed, as discussed below, when red cells are equilibrated with saline at different pHs, pH_i varies with pH_o. It can be seen from Fig. 4 *B* that the general patterns of pH dependence of Na^+ uptake are similar, the curve only being shifted to the left when expressed as a function of pH_i. The question therefore arises as to whether the observed effect of pH on Na^+ permeability is due to external and/or to internal pH.

H^+ distribution between trout red cells and the external medium

It is now well established (see Funder & Wieth, 1966) that in mammalian red cells the distribution of H^+ is in accordance with a Gibbs–Donnan equilibrium at different pHs, i.e. the internal H^+ activity is that expected from a purely passive distribution across the cell membrane. Such a distribution is presumed to occur in fish erythrocytes but has never been demonstrated. For measurements of pH_i, red cell pellets were repeatedly frozen and thawed and the pH measured directly on the lysate and assumed to be representative of pH_i. It must be pointed out, however, that fish erythrocytes are nucleated; thus their intercellular compartment is heterogeneous and the effect of this heterogeneity of intracellular distribution is unknown. It is interesting to note that, in a comparative study, Milligan & Wood (1985) showed that both the cell homogenate technique and the DMO (5,5-dimethyloxazolidine-2,4-dione) method gave the same values of intracellular pH in trout erythrocytes.

Fig. 5 *A* shows the relationship between pH_i and pH_0 obtained from the experiment depicted in Fig. 4, and measured at time $t = 0$ (before stimulation) and 3 min after stimulation by isoprenaline, i.e. at the end of the period of Na^+ uptake determination.

The following salient observations warrant emphasis.

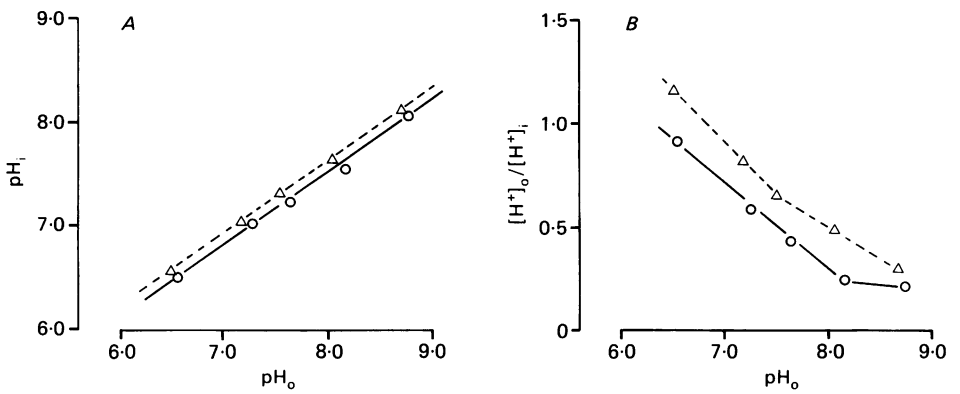

Fig. 5. *A*, relationship between pH_0 and red cell pH (pH_i). ○, pH_0 and pH_i measured before hormonal stimulation ($t = 0$). △, pH_0 and pH_i measured 3 min after hormonal stimulation ($t = 3$). Regression lines are: for $t = 0$, $pH_i = 1{\cdot}94 + (0{\cdot}70\ pH_0)$ ($n = 5$; $r = 1{\cdot}0$); for $t = 3$, $pH_i = 1{\cdot}88 + (0{\cdot}72\ pH_0)$ ($n = 5$; $r = 1{\cdot}0$). *B*, relationship between pH_0 and transmembrane distribution ratio for H^+ across red cell membrane, before stimulation (○) and 3 min after stimulation by isoprenaline (△).

(1) Over the normal range of pH_0 the intracellular pH in control erythrocytes is lower than pH_0, i.e. the transmembrane distribution of H^+ ($r_{H^+} = [H^+]_0/[H^+]_i$) is lower than 1. However, as expected if H^+ is passively distributed according to a Donnan equilibrium, this ratio varies with pH_0 being higher the lower the pH_0 (Fig. 5*B*); in other words the difference between intracellular and extracellular pH decreases with pH_0. The ratio is close to unity ($pH_i = pH_0$) when the intracellular negative fixed charges are titrated, i.e. in our experimental conditions for pH_0 of 6·5.

This ratio is a linear function of pH between pH_0 6·15 and 8·14. The observed regression relationship ($r_{H^+} = 3{\cdot}64 - (0{\cdot}42\ pH_0)$; $r = 1{\cdot}0$; $n = 4$) is very similar to

that described by Milligan & Wood (1985) for trout red cells over a limited range of pH_o (7·4–8·0) when pH_i was measured both by the DMO method and the lysate technique, but is also similar to that recorded for r_{Cl^-} and r_{H^+} in human erythrocytes by Funder & Wieth (1966).

(2) In our experimental conditions (haematocrit, 15%; buffer, 15 mM; 15 °C; $[HCO_3^-] = 0$), 3 min after addition of isoprenaline, both an acidification of the external saline and an alkalinization of pH_i are observed, irrespective of the initial value of pH_o. Over the whole pH_o range studied (6·5–8·75) the relationship between pH_i and pH_o is linear for control and stimulated erythrocytes, and the slopes are very similar (Fig. 5*A*). For control erythrocytes the regression equation is $pH_i = 1{\cdot}94 + (0{\cdot}70\ pH_o)$ ($n = 5$; $r = 1{\cdot}0$) and for three-minute-stimulated erythrocytes $pH_i = 1{\cdot}88 + (0{\cdot}72\ pH_o)$ ($n = 5$; $r = 1{\cdot}0$).

Dependence of the Na^+–H^+ exchange system on the external pH

To estimate the influence of the extracellular pH on the activity of the Na^+–H^+ exchange system, the rate of $^{22}Na^+$ uptake was determined in cells at a constant pH_i (7·3) but at a pH_o varying from 6·15 to 8·20 (Fig. 6*A*). These experiments were performed on cells pre-equilibrated at pH_o of 7·7 (giving pH_i 7·3) and then suspended

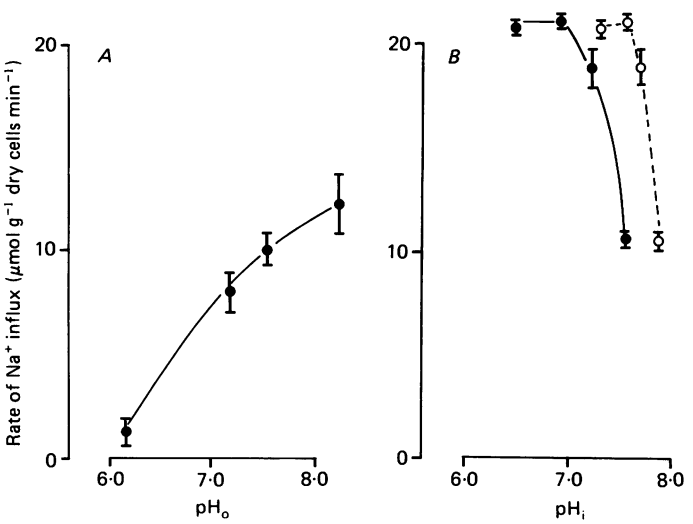

Fig. 6. *A*, effect of pH_o on initial rate of Na^+ influx induced by isoprenaline. pH_i was the same for all the batches of erythrocytes (7·3) and obtained by pre-equilibrating the cells in a saline at pH 7·7, after which they were transferred to salines at the different indicated pH_o containing ouabain and 5×10^{-4} M-DIDS to inhibit the anion exchanger (see text). $^{22}Na^+$ influx was measured for 3 min after hormonal stimulation. Data are means ± S.E. of means of three experiments. *B*, effects of pH_i on initial rate of Na^+ influx induced by isoprenaline. The cells were pre-equilibrated at different pH_os, giving different pH_is. Then they were suspended in the same saline ($pH_o = 8{\cdot}0$) containing ouabain and 5×10^{-4} M-DIDS (see text). $^{22}Na^+$ influx was measured for 3 min after hormonal stimulation. Data are means ± S.E. of means of three experiments. Continuous line: Na^+ influx as a function of pH_i measured at $t = 0$; interrupted line: Na^+ influx as a function of pH_i measured at $t = 3$ min.

during the experimental period (3 min) in saline at different pH_os. During the course of the measurements some variation of pH_i occurs for the following reasons. Once red cells are suspended in a saline at a pH different from the pH of incubation a readjustment of pH_i occurs via the anion exchanger system which allows an exchange between Cl^- and OH^- (or HCO_3^-). To minimize pH_i change due to the anion exchanger during the 3 min of experimentation, therefore, DIDS (5×10^{-4} M) was used to inhibit anion exchanges. Moreover the Na^+–H^+ exchange which is induced by addition of isoprenaline at $t = 0$ promotes some alkalinization of pH_i despite the buffering power of red cells (see Fig. 5*A*). The magnitude of this alkalinization will be even greater in the presence of DIDS which blocks the pH compensation by the anion-exchange system.

For these reasons, therefore, it is technically impossible to have a really constant pH_i during the 3 min experimentation. Bearing these limitations in mind, two features of the data expressed in Fig. 6*A* can be noted. First, the rate of $^{22}Na^+$ uptake is pH_o dependent, increasing as the pH_o becomes more alkaline. The initial rate of $^{22}Na^+$ uptake increases more than twelvefold between pH_o 6·15 and 8·2. Secondly, in this experiment the value of the $^{22}Na^+$ uptake measured at pH 7·5 represents the rate of Na^+ uptake in conditions in which practically no pH disequilbrium occurs (pH_o of pre-incubation = 7·7; pH_o during measurement = 7·5). This value, 10 μmol g^{-1} min^{-1}, is much lower than the corresponding value obtained in the experiment depicted in Fig. 4 (about 29 μmol g^{-1} min^{-1}). This difference in the magnitude of the fluxes is explained by the fact that in the presence of DIDS, the amount of Na^+ penetrating into the cell in the first minutes is considerably smaller than in the absence of the drug (Baroin *et al.* 1984 *b*).

Dependence of the Na^+–H^+ exchange system on the internal pH

The influence of pH_i on the activity of the Na^+–H^+ exchange system is shown in Fig. 6*B*. In this experiment the cells were adapted at four different pHs (6·5, 7·15, 7·5 and 8·0) in saline buffered by (15 mM) MES, BES, HEPES and Tricine respectively and containing 35 mM-sucrose. After a 3 h incubation in these media, the cells were suspended in a saline at pH 8·0, highly buffered by 50 mM-Tricine and containing 5×10^{-4} M-DIDS, 10^{-4} M-ouabain and $^{22}Na^+$ (sucrose was used in the incubation media to prevent an osmotic shock due to the difference of buffer concentration in the incubation and experimental media; 50 mM-buffer was used in experimental media to minimize the external pH changes). As discussed above, during the 3 min of experimentation the pH_i is not constant because not only does the activity of the Na^+–H^+ exchanger tend to make the pH_i more alkaline but also DIDS inhibits anion exchange which partially compensates the effect of H^+ movements via the Na^+–H^+system. It is therefore impossible to plot the initial rate of $^{22}Na^+$ uptake as a function of a constant pH_i value. In Fig. 6*B* it has been plotted as a function of pH_i at $t = 0$ (continuous line) and of pH_i at $t = 3$ min after addition of isoprenaline (interrupted line). It is clear that the shape of the curve is similar in the two cases, but the curve at $t = 3$ is shifted to the right indicating that the internal pH became more alkaline during the 3 min periods of measurement. Thus the value of $^{22}Na^+$ uptake measured for each batch of cells is a time-averaged Na^+

influx controlled by the evolution of pH_i between the initial ($t = 0$) and the final ($t = 3$ min) pH_i.

Fig. 6*B* shows that the initial rate of $^{22}Na^+$ uptake increases as the pH_i becomes more acid and then levels off at pH_i 7·0 (the reference for pH_i being at $t = 0$, i.e. before hormonal stimulation). The pH_i dependence of the system is very steep, since a maximal activation of the exchange by isoprenaline can be obtained within about one pH_i unit. The half-maximum effect for activation of the exchanger by internal H^+ is observed at about pH_i 7·5–7·6.

DISCUSSION

It has previously been shown that trout red blood cells respond to β-adrenergic stimulation (Mahé *et al.* 1985) with an increase in cell volume (Bourne & Cossins, 1982; Nikinmaa, 1982; Baroin *et al.* 1984*a*, *b*) due to a large amiloride-sensitive uptake of NaCl mediated by the activation of a Na^+–H^+ exchanger operating in parallel with the anion-exchange system (Baroin *et al* 1984*b*; Borgese *et al.* 1986; Cossins & Richardson, 1985). We have recently demonstrated that this cell volume increase is controlled, the erythrocyte enlarging rapidly and then reaching a steady-state volume as long as hormone is in contact with the cells. This new steady-state volume was shown to result from a dynamic equilibrium involving the simultaneous functioning of two regulatory processes induced by the hormone: a volume increase response that causes them to enlarge by gaining Na^+ via the Na^+–H^+ exchanger and a volume decrease response that causes cells to shrink by losing K^+ via a Cl^--dependent DIDS-sensitive pathway (Borgese *et al.* 1987).

The present investigation demonstrates that both the rate of cell volume increase and the maximum enlargement reached by the cells are strongly pH dependent (Fig. 1). This is explained by a differential effect of pH on the two regulatory processes, i.e. Na^+–H^+ exchange and Cl^--dependent K^+ leak (Figs. 3 and 4): between pH 8·10 and 6·25, K^+ loss decreases as the pH_i becomes more acidic in a roughly linear manner. On the other hand Na^+ uptake increases as the pH_i becomes more acidic with a maximum around pH 7·30–7·60 and then decreases. From these different pH dependences of the K^+ and Na^+ permeabilities it is evident that the rate and the magnitude of the volume changes induced by catecholamine will depend to a large extent of the pH chosen to perform experiments. The maximum enlargement would be expected to occur at about pH 7·30.

From Fig. 3 it is clear that the rate of swelling is modulated more by the change in the activity of the Na^+–H^+ exchange system responsible for Na^+ uptake than by that of the K^+ pathway responsible for the K^+ loss. Moreover the change in the activity of the Na^+–H^+ exchanger as a function of pH is a complex relationship described by a characteristic bell-shaped curve (Figs. 3 and 4). It was therefore of interest to analyse this relationship and more precisely to deal with the influence of both pH_i and pH_o on the Na^+–H^+ exchange system. In other words the activity of the Na^+–H^+ antiporter must be measured at a fixed pH_i but at different values of pH_o and conversely at a fixed pH_o but at different values of pH_i.

Such an analysis raises some specific problems when performed on red cells. First, it must be pointed out that when red cells are adapted in media covering a wide range

of pH as in Figs. 3 and 4, the internal pH varies considerably with changing external pHs. In mammalian red cells it is well established that the distribution of H^+ across the red cell membrane is purely passive, in accordance with a Donnan equilibrium at the different pHs. This is due to the fact that the red cell membrane is quite impermeable to cations but highly permeable to anions and thus to basic equivalents (OH^-, HCO_3^-) because of the presence of a fast anion exchanger. It can be reasonably assumed that the distribution of H^+ across the cell membrane of nucleated erythrocytes (e.g. trout red cell) is governed by the same laws. In any case, the relationship between pH_i and pH_o, illustrated in Fig. 5, is similar to that described by Funder & Weith (1966) for human erythrocytes. It is therefore easy to obtain a wide range of pH_i by adapting red cells at different pH_os.

Secondly, when red cells having a defined pH_i from adaptation to a certain saline are transferred to a new saline with a different pH, the anion-exchange system located in band 3 allows for a very fast pH_i readjustment. Therefore, to measure the activity of the Na^+–H^+ antiport at a fixed pH_i, the anion movements must be blocked by specific inhibitors of band 3, such as DIDS. DIDS cannot completely block Cl^-–self exchange in trout red cells at 15 °C and at alkaline pH (Baroin *et al.* 1984*b*; Romano & Pasow, 1984). Nevertheless it very efficiently inhibits hetero-exchange (e.g. Cl^-–basic equivalent), i.e. pH readjustment (Borgese *et al.* 1986), and thus theoretically clamps pH_i for the period of measurement of the initial rate of Na^+ uptake.

Thirdly, in our experimental conditions the addition of catecholamines induces so great an Na^+–H^+ exchange that 3 min later both an acidification of the external saline and an alkalinization of the intracellular compartment can be recorded (Fig. 5*A*). This occurs despite the presence of the very fast anion-exchange system which tends to adjust the effects of H^+ movements occurring via the Na^+–H^+ antiporter (Borgese *et al.* 1986). Thus, in these conditions, pH_i is not really constant during the 3 min experimental period. The increase of pH_i due to the functioning of the Na^+–H^+ exchange will of course be greater in the presence of DIDS and we have to add DIDS when red cells at a defined pH_i are transferred to a saline at a different pH (see above).

For these reasons the pH_i is not really fixed during the experimental period and the initial rate of Na^+ uptake is an averaged value of the flux between the initial and the final pH_i. To give an idea of the pH shift in the presence of DIDS, the pH_i before stimulation ($t = 0$) and 3 min after stimulation, i.e. at the end of the experimental period ($t = 3$) are given in Fig. 6*B*.

In spite of these experimental limitations the following interesting results arise from the experiments performed to analyse the influence of both internal H^+ and external H^+ on the activity of the Na^+–H^+ exchanger.

From the data presented in Fig. 6*A*, it appears that the external Na^+–internal H^+ exchange is inhibited by external H^+: the maximal uptake rate we measured is found at the more alkaline saline we used (pH_o 8·2) and a decreasing pH_o at a constant pH_i decreases the activity of the Na^+–H^+ exchanger to such an extent that there is considerable inhibition at pH_o 6·2. Such an inhibitory effect of external H^+ on Na^+–H^+ exchange has previously been described for a variety of cell types and interpreted as due to competition of external H^+ with external Na^+ for the externally facing

binding site (Rindler & Salier, 1981; Aronson, 1983; Aronson, Suhm & Nee, 1983; Grinstein, Cohen & Rothstein, 1984*a*).

(2) Another important parameter determining the activity of the Na^+–H^+ exchange system in trout red cells is internal H^+ concentration, $[H^+]_i$ (Fig. 6*B*). The activity of the Na^+–H^+ exchanger is very sensitive to pH_i since a very steep increase of the rate of exchange at a constant pH_o was observed within about one pH_i unit (between pH 7·0 and 8·0 when initial pH_i are considered). At pH_i 7·0 the activity of Na^+–H^+ exchanger is maximal. The internal pH dependence of the exchanger has already been described for a variety of cell types and considered as the primary determinant of the rate of the Na^+–H^+ exchange (Aronson *et al.* 1983; Paris & Pouyssegur, 1983; Grinstein *et al.* 1984*a*; Thierry, Frelin, Vigne, Barbry & Lazdunski, 1985) The control by pH_i seems to be exerted allosterically (Aronson, Nee & Suhm, 1982; Aronson, 1983; Grinstein, Goetz & Rothstein, 1984*b*) explaining why pH_i dependence of the system is very steep.

(3) In normal conditions (i.e. in the absence of DIDS) when red cells are adapted in media over a wide range of pH_o, pH_i also varies widely in accordance with a Donnan equilibrium. In these conditions the activity of the Na^+–H^+ exchanger as a function of pH_o is described by a characteristic bell-shaped curve (Figs. 3 and 4). This curve can adequately be explained by the simultaneous influences of external and internal H^+ described above. In the alkaline pH range (pH_o varying between 8·75 and 7·25 corresponding to pH_is between 8·1 and 7·05) the activity of the Na^+–H^+ is mainly controlled by internal H^+ because the pH_i dependence is very steep. In the acidic range (pH_o varying between 7·25 and 6·5 corresponding to a pH_i range of 7·05 to 6·5) the activity of the Na^+–H^+ exchanger is no longer controlled by internal H^+ but by external H^+: at pH_o 6·5, pH_i is also 6·5; the activity of the Na^+–H^+ exchanger is potentially fully activated by internal H^+ but is strongly inhibited by external H^+.

In short, the pH dependence of the volume changes induced by catecholamines is due to a differential effects of pH on the Na^+–H^+ exchanger and on the K^+ pathway. Moreover the complex relationship of the change in activity of the Na^+–H^+ exchanger as a function of pH is explained by the predominant influence of internal H^+ in the alkaline range and of external H^+ in the acidic range.

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