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ADRENERGIC SWELLING OF NUCLEATED ERYTHROCYTES: CELLULAR MECHANISMS IN A BIRD, DOMESTIC GOOSE, AND TWO TELEOSTS, STRIPED BASS AND RAINBOW TROUT

BY MIKKO NIKINMAA* AND WRAY H. HUESTIS
Department of Chemistry, Stanford University, Stanford, California 94305, U.S.A.

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SUMMARY

The mechanism of adrenergic swelling and associated pH changes was investigated in avian (goose) and teleost (striped bass and rainbow trout) erythrocytes.

The swelling of goose red cells was probably caused by $Na^+/K^+/Cl^-$ co-transport and consecutive osmotic flow of water into the cell. Goose red cells swelled when exposed to isoproterenol in the presence of elevated extracellular K^+, but not at physiological K^+ concentrations. The swelling was quantitatively inhibited by furosemide, and by removing Cl^- from the incubation medium, but was not affected by DIDS.

The isoproterenol-induced swelling of fish erythrocytes may be due to loosely coupled Na^+/H^+ and Cl^-/HCO_3^- exchanges. Furosemide did not completely inhibit the swelling of striped bass red cells. The cell volume increased even if K^+ was completely removed from the incubation medium. In contrast, both DIDS and amiloride treatment, and the removal of Na^+ from the incubation medium, inhibited the volume changes. In fish red cells the swelling is associated with a clear acidification of the medium and alkalinization of the red cell contents. This phenomenon was most pronounced when the cells were treated simultaneously with DIDS and isoproterenol; the intracellular pH became higher than the extracellular one. Both amiloride and removal of Na^+ from the incubation medium prevented the reversal of the transmembrane pH gradient in cells treated simultaneously with DIDS and isoproterenol.

INTRODUCTION

Beta-adrenergic stimulation of avian red cells causes cell swelling at high extracellular K^+ concentration as first demonstrated by Riddick, Kregenow & Orloff (1971). The mechanism of this swelling involves furosemide-sensitive $Na^+/K^+/Cl^-$ co-transport and osmotic inflow of water (for review see Palfrey & Greengard, 1981). The transport of Cl^- seems to take place at a site different from the normal anion

* Permanent address: Division of Physiology, Department of Zoology, University of Helsinki, Arkadiankatu 7, SF-00100 Helsinki 10, Finland.
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exchange channel, since DIDS (diisothiocyanostilbene disulphonic acid), inhibitor of band 3 mediated anion exchange, has no effect on the stimulated co-transport (Palfrey, Feit & Greengard, 1980*a*; Haas, Schmidt & McManus, 1982).

Adrenergic stimulation also induces swelling in fish red cells (Fugelli & Reiersen, 1978; Nikinmaa, 1982, 1983), but little is known about the mechanism of this volume increase. Nikinmaa (1982) has shown that the swelling of rainbow trout red cells occurs at normal plasma K^+ concentrations but seems to be enhanced by increased extracellular K^+ concentrations. Bourne & Cossins (1982) have shown that catecholamines increase both the ouabain- and the furosemide-sensitive influx of K^+ in rainbow trout red cells. Additionally, adrenalin decreases the pH-gradient across rainbow trout red cell membrane (Nikinmaa, 1982, 1983).

In the present study we have further investigated the mechanisms of adrenergic swelling in nucleated red cells. The adrenergic volume and pH changes in avian and teleost red cells were determined in media with varying ion composition, and in the presence and absence of the transport inhibitors, amiloride, furosemide and DIDS.

MATERIALS AND METHODS

Inulin, DMO (5,5-dimethyloxazolidine-2,4-dione), and isoproterenol were obtained from Sigma Chemical Company, and DIDS was a product of Aldrich. Domestic goose (*Anser anser*) blood was obtained through Microbiological Media (San Ramon, CA) or from the State Veterinary Institute (Helsinki, Finland), and striped bass (*Morone saxatilis*) blood from the University of California at Davis. Rainbow trout (*Salmo gairdneri*) blood was drawn by cardiac puncture from fish obtained from a commercial fish farm (Savon Taimen OY, Finland). Blood was centrifuged, plasma discarded, and red cells washed three times by resuspension in the different buffers used (Table 1). After the final wash the red cells were suspended in the buffer, and allowed to equilibrate overnight at 4 °C (goose and striped bass red cells), or for 1 h at 23 °C (rainbow trout cells). After equilibration the medium was supplemented with 175 μCi l^{-1} methoxy-[^{3}H]-inulin (New England Nuclear, specific activity 303·8 mCi g^{-1}) for determinations of extracellular space, and/or with 0·25 mmol l^{-1} unlabelled DMO plus 25 μCi l^{-1} ^{14}C-DMO (New England Nuclear, specific activity 55·4 mCi $mmol^{-1}$) for determinations of intracellular pH. All the experiments were carried out within 48 h from sampling and only on samples showing no detectable haemolysis.

Table 1. *The composition of buffers used in the experiments*

Buffer	HEPES	Na	K	Choline	Cl	NO_3^-	Mg
A	30	140	0	0	141	0	1
B	30	135	5	0	141	0	1
C	30	125	15	0	141	0	1
D	30	135	5	0	0	141	1
E	30	125	15	0	0	141	1
F	30	15	15	110	141	0	1
G	30	0	15	125	141	0	1

Concentrations in mmol l^{-1}.

Red cell suspensions were then incubated at 20 % haematocrit (sample size 1 ml) for 1 h at 23 °C (striped bass and rainbow trout red cells) or 40 °C (goose cells). Incubations were carried out in the buffers of Table 1 with or without 10^{-5} mol l^{-1} DL-isoproterenol. The following transport inhibitors were added to the incubations in some of the experiments: (a) 10^{-3} mol l^{-1} amiloride, (b) 10^{-4} mol l^{-1} DIDS and (c) 10^{-4} mol l^{-1} furosemide.

After incubation, the extracellular pH of each sample was measured using a Radiometer PHM 72 or 82 pH meter, and incubation medium and red cells were separated by centrifugation (3 min, Eppendorf Centrifuge 3200 or 5413). The cell pellets were weighed, dried at 78 °C to a constant weight and reweighed. Distilled water (four volumes) was added to both incubation medium and dried red cell pellet, and the samples were extracted by agitation at room temperature for 96 h. The extracts were transferred to liquid scintillation vials and bleached by addition of 0·5 volumes 30 % H_2O_2. Samples were analysed for ^3H and/or ^{14}C by liquid scintillation counting (Beckman LS 3150T, LKB Wallac 1211 Minibeta). Cell volume was measured from entrapped cell water, calculated from the wet and dry weight of cells as follows:

$$\% \ H_2O = 100 - (100 \times \text{dry weight/wet weight}).$$

The wet weight of the samples was corrected for trapped extracellular water, indicated by inulin space:

$$\text{trapped } H_2O \ (\%) = \frac{(\text{d.p.m. } ^3H/\text{ml cell pellet})}{(\text{d.p.m. } ^3H/\text{ml supernatant})} \times 100.$$

The intracellular pH was determined from the transmembrane distribution of the weak acid DMO using the following formulae:

$$pH_i = pK_{DMO} + \log \{DMO_i/DMO_e \ (10^{pH_e - pK_{DMO}} + 1) - 1\},$$

$$DMO_i = (DMO_{tot} - DMO_e \times Q_e)/(1 - Q_e).$$

(Deutsch *et al.* 1979; Heisler, 1975), where pK_{DMO} at 23 °C was taken as 6·23 and Q_e is the fractional extracellular volume.

RESULTS

Goose red cells

The effects of isoproterenol on goose red cell volume are illustrated in Fig. 1 and Table 2. At low extracellular K^+ concentration, cells incubated with isoproterenol had the same water content as controls at all the pH values studied (Fig. 1). When extracellular K^+ concentration was increased to 15 mmol l^{-1}, the volume of isoproterenol-treated cells increased by 7–9 %. This increase was completely inhibited by furosemide at all pH values.

Further studies were carried out at pH 7·2 (Table 2). Isoproterenol treatment at this pH induced a significant ($P < 0·001$) increase of 7 % in goose red cell volume, but only for samples in 15 mmol l^{-1} extracellular K^+. Again, no volume changes were observed at the lower K^+ concentration. The isoproterenol-induced volume increase

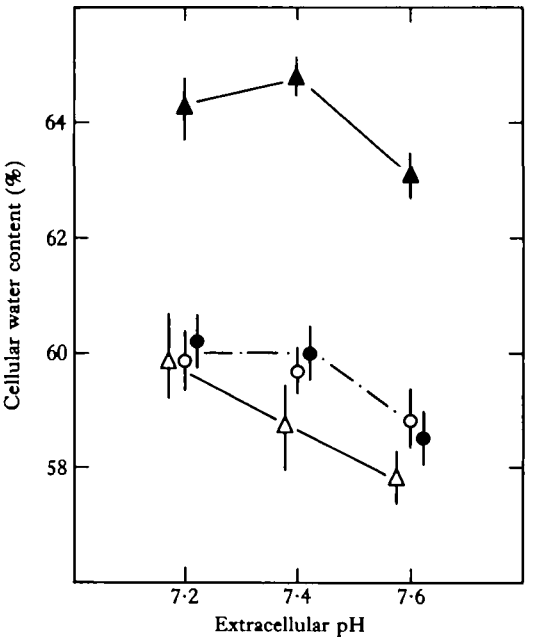

Fig. 1. Effects of isoproterenol on the water content of goose red cells, as functions of extracellular pH. Control cells (○), $N = 15$; cells plus isoproterenol (10^{-5} mol l^{-1}) at 5 mmol l^{-1} extracellular K^+ (●), $N = 12$; cells plus isoproterenol at 15 mmol l^{-1} extracellular K^+ (▲), $N = 7$; cells plus isoproterenol and furosemide (0·1 mmol l^{-1}) at 15 mmol l^{-1} extracellular K^+ (△), $N = 5$. Error bars indicate ±S.E.M. At all pH values tested, cells treated with isoproterenol at 15 mmol l^{-1} extracellular K^+ had significantly ($P < 0·001$) higher water content than cells under other conditions. Differences among the other three groups were not statistically significant. Comparisons were made using the Wilcoxon two-sample rank sum test.

could be inhibited completely by furosemide, but was not affected by DIDS (Table 2). Neither DIDS nor furosemide had any effect on cell volume in the absence of an adrenergic agonist at either K^+ concentration. When the incubation medium contained nitrate instead of chloride, isoproterenol had no effect on the cell volume at 15 mmol l^{-1} extracellular K^+ concentration.

In goose red cells, isoproterenol had little effect on the pH gradient across the red cell membrane. In control incubations (K^+ 15 mmol l^{-1}) the extracellular pH was

Table 2. *Effects of isoproterenol on the water content (in %) of goose red cells*

Treatment	Control	Isoproterenol (10^{-5} mol l^{-1})	% Change	$P <$
Buffer B (K^+ 5 mmol l^{-1})	59·6 ± 0·38(19)	59·6 ± 0·30(18)	0	NS
Buffer C (K^+ 15 mmol l^{-1})	59·6 ± 0·30(15)	63·8 ± 0·25(15)	7·2	0·001
Buffer C + DIDS	59·9 ± 0·62(12)	64·0 ± 0·64(6)	6·8	0·01
Buffer C + furosemide	59·1 ± 0·45(12)	58·8 ± 0·44(15)	−0·5	NS
Buffer E (Cl^- 0 mmol l^{-1})	60·4 ± 0·55(6)	59·8 ± 0·69(7)	−1·0	NS

Means ± S.E.M. (N) and the percentage change in volume are given.
Statistical significances of the differences between the means were tested using the Wilcoxon matched-pairs signed-ranks test or the Wilcoxon two-sample rank sum test.
NS, not significant.

Table 3. *Effects of isoproterenol on the water content of striped bass red cells*

Treatment	Control	Isoproterenol (10^{-5} mol l^{-1})	% Change	$P<$
Buffer B (K^+ 5 mmol l^{-1})	74·8 ± 0·18(9)	77·5 ± 0·40(7)	3·6	0·001
Buffer B + furosemide	75·2 ± 0·38(7)	76·8 ± 0·23(7)	2·1	0·01
Buffer B + DIDS	75·1 ± 0·47(7)	75·3 ± 0·15(7)	0·2	NS

Means ± S.E.M. (N) and the percentage change in volume are given.
Statistical significances of the differences between the means were tested using the Wilcoxon matched-pairs signed-ranks test or the Wilcoxon two-sample rank sum test.
NS, not significant.

7·245 ± 0·002(5) and intracellular pH 7·043 ± 0·017(5); in isoproterenol-treated cells the respective values were 7·242 ± 0·007(5) and 7·059 ± 0·004(5) [$\bar{x}$ ± S.E.M. (N)].

Striped bass red cells

The volume responses of striped bass red cells differed substantially from those observed with goose red cells. Bass red cell volume increased significantly ($P < 0·001$) when the cells were incubated with isoproterenol at 5 mmol l^{-1} extracellular K^+ concentration (Table 3). This swelling was inhibited almost quantitatively by DIDS, while furosemide had little effect.

The combination of DIDS and isoproterenol also had a marked effect on the transmembrane pH gradient of striped bass erythrocytes (Table 4). DIDS alone did not affect this gradient, but in combination with isoproterenol reversed the normal pH gradient. The extracellular pH decreased markedly, while the intracellular pH tended to increase (although the latter change was not statistically significant). The decrease in extracellular pH, in spite of the high buffering capacity of the medium, shows that protons (acid equivalents) are ejected from the cells in large amounts.

Rainbow trout red cells

The results closely resemble those obtained with striped bass red cells. Isoproterenol induced the swelling of rainbow trout red cells at 5 mmol l^{-1} extracellular

Table 4. *Effects of isoproterenol on the extracellular (pH_e) and intracellular pH (pH_i), and on the pH gradient (ΔpH) of striped bass red cells*

Treatment		Control	Isoproterenol (10^{-5} mol l^{-1})	$P<$
Buffer B (K^+ 5 mmol l^{-1})	pH_e	7·116 ± 0·014(7)	7·045 ± 0·034(4)	NS
	pH_i	6·794 ± 0·041(7)	6·813 ± 0·062(4)	NS
	ΔpH	+ 0·322	+ 0·232	
Buffer B + DIDS	pH_e	7·175 ± 0·018(4)	6·790 ± 0·050(4)	0·01
	pH_i	6·865 ± 0·049(4)	6·903 ± 0·045(4)	NS
	ΔpH	+ 0·310	− 0·113	

Means ± S.E.M. (N) are given.
Statistical significances of the differences between the means were tested using the Wilcoxon matched-pairs signed-ranks test or the Wilcoxon two-sample rank sum test.
NS, not significant.

Table 5. *Effects of isoproterenol on the water content of rainbow trout red cells*

Treatment	Control	Isoproterenol (10^{-5} mol l^{-1})	% Change	$P<$
Buffer A (K^+ 0 mmol l^{-1})	72·7 ± 0·45(5)	76·3 ± 0·97(5)	5·0	0·01
Buffer B (K^+ 5 mmol l^{-1})	72·2 ± 0·65(14)	76·1 ± 0·35(14)	5·4	0·001
Buffer C (K^+ 15 mmol l^{-1})	72·0 ± 0·42(5)	76·6 ± 0·76(5)	6·4	0·01
Buffer E (Cl^- 0 mmol l^{-1})	70·5 ± 0·75(10)	72·6 ± 0·66(10)	3·0	0·01
Buffer F (Na^+ 15 mmol l^{-1})	71·0 ± 0·80(10)	73·6 ± 0·60(10)	3·7	0·01
Buffer G (Na^+ 0 mmol l^{-1})	72·2 ± 0·51(5)	71·8 ± 0·48(5)	−0·5	NS
Buffer B + DIDS	72·8 ± 1·43(4)	72·6 ± 0·66(8)	−0·3	NS
Buffer B + amiloride	72·5 ± 1·47(4)	73·1 ± 0·61(8)	0·8	NS

Means ± S.E.M. (N) and the percentage change in volume are given.
Statistical significances of the differences between the means were tested using the Wilcoxon matched-pairs signed-ranks test or the Wilcoxon two-sample rank sum test.
NS, not significant.

Table 6. *Effects of isoproterenol on the extracellular (pH_e) and intracellular pH (pH_i), and on the pH gradient (ΔpH) of rainbow trout red cells*

Treatment		Control	Isoproterenol (10^{-5} mol l^{-1})	$P<$
Buffer A (K^+ 0 mmol l^{-1})	pH_e	7·252 ± 0·006(8)	7·197 ± 0·008(8)	0·01
	pH_i	7·005 ± 0·015(8)	7·061 ± 0·009(8)	0·01
	ΔpH	+0·247	+0·136	
Buffer B (K^+ 5 mmol l^{-1})	pH_e	7·282 ± 0·009(11)	7·222 ± 0·011(11)	0·01
	pH_i	7·089 ± 0·012(11)	7·128 ± 0·013(11)	0·01
	ΔpH	+0·193	+0·096	
Buffer G (Na^+ 0 mmol l^{-1})	pH_e	7·250 ± 0·002(5)	7·231 ± 0·024(5)	0·05
	pH_i	7·007 ± 0·017(5)	7·024 ± 0·008(5)	NS
	ΔpH	+0·243	+0·207	
Buffer B + DIDS	pH_e	7·277 ± 0·011(5)	7·143 ± 0·020(8)	0·01
	pH_i	7·118 ± 0·027(5)	7·276 ± 0·023(8)	0·01
	ΔpH	+0·159	−0·133	
Buffer B + DIDS + amiloride	pH_e		7·219 ± 0·004(5)	
	pH_i		7·146 ± 0·014(5)	
	ΔpH		+0·073	
Buffer G + DIDS	pH_e	7·242 ± 0·004(5)	7·212 ± 0·004(5)	0·01
	pH_i	7·028 ± 0·035(5)	7·049 ± 0·021(5)	NS
	ΔpH	+0·214	+0·173	

Means ± S.E.M. (N) are given.
Statistical significances of the differences between the means were tested using the Wilcoxon matched-pairs signed-ranks test or the Wilcoxon two-sample rank sum test.
NS, not significant.

K^+ concentration, and even in the total absence of K^+ from the medium (Table 5). On the other hand, when sodium ions were removed from the incubation medium, isoproterenol had no effect on the cell volume. Also, the presence of either amiloride, an inhibitor of Na^+/H^+ exchange, or DIDS, an inhibitor of anion exchange, in the incubation medium prevented the isoproterenol-induced volume changes. In contras

to goose red cells, rainbow trout red cell volume increased slightly when nitrate instead of chloride was the major anion in the incubation medium.

In addition to its effects on cell volume, isoproterenol caused a significant decrease in the proton gradient across the red cell membrane both in the presence and absence of K^+ in the incubation medium (Table 6). Again, the exclusion of Na^+ from the medium markedly reduced the pH changes. As in striped bass, the pH gradient across the red cell membrane was reversed when the cells were treated simultaneously with DIDS and isoproterenol. The reversal of pH gradient could be prevented by removing Na^+ from the medium or by adding the inhibitor of Na^+/H^+ exchange, amiloride, to the incubation medium.

DISCUSSION

These studies reveal several differences in the adrenergic swelling responses of avian (goose) and teleost (striped bass and rainbow trout) erythrocytes. Goose cells conformed to the previously reported pattern of adrenergic volume changes in bird red cells (see also Palfrey *et al.* 1980*a*); the volume increased only in elevated extracellular K^+ concentration, the swelling was inhibited by furosemide but not by DIDS, and nitrate could not be substituted for chloride. Thus, it is likely that as in other bird cells studied, goose red cell swelling is caused by a stimulation of furosemide-sensitive $Na^+/K^+/Cl^-$ co-transport, in which chloride movement takes place mainly through a route other than the band 3 anion exchange pathway, followed by osmotic inflow of water (for review of the mechanism see Palfrey & Greengard, 1981).

In contrast, fish red cells swell at low extracellular K^+ concentration, or even in the absence of K^+ from the incubation medium. The response is inhibited by DIDS and amiloride, and by removing Na^+ from the incubation medium, but only slightly affected by furosemide or by substituting nitrate for chloride in the incubation medium. Additionally, pronounced extra- and intracellular pH changes are associated with the incubations. These findings clearly show that the $Na^+/K^+/Cl^-$ co-transport, the driving force behind avian red cell swelling, does not play a significant role in the adrenergic volume changes of striped bass and rainbow trout red cells.

The adrenergic swelling of amphibian erythrocytes is also due to a mechanism different from that in birds. Isoproterenol stimulated the net fluxes of sodium and potassium into frog (*Rana pipiens*) red cells treated with a phosphodiesterase inhibitor, and this led to an increase in cell volume (Rudolph & Greengard, 1980). In contrast to the situation in birds, red cell swelling did not require extracellular K^+ (Rudolph & Greengard, 1980), and stimulation of ion fluxes was not inhibited by furosemide but by amiloride (Palfrey, Stapleton, Alper & Greengard, 1980*b*). Furthermore, if sodium was excluded from the medium, stimulated potassium influx did not occur. In sodium-containing medium the potassium influx could be inhibited by ouabain, the inhibitor of the Na^+/K^+ pump (see Palfrey & Greengard, 1981). On the basis of these findings Palfrey & Greengard (1981) suggested that in frog red cells beta-adrenergic drugs stimulate a Na^+/H^+ exchange across the red cell membrane, and that increased potassium influx is a secondary phenomenon resulting from sodium entry into the cell and consequent stimulation of the Na^+/K^+ pump.

Cala (1980) showed that the increase in the volume of *Amphiuma* red cells after osmotic shrinking was characterized by net cellular uptake of sodium and chloride, and consequent osmotic inflow of water. Furthermore, the sodium fluxes were sensitive to the bicarbonate level of the medium. On the basis of the above evidence Cala (1980) presented a model for the increase in cell volume in which Na^+/H^+ and Cl^-/HCO_3^- exchanges are loosely coupled. According to this model, red cell swelling would be a result of sodium and chloride influxes in exchange for protons and bicarbonate which (being in equilibrium with H_2O and CO_2) do not exert osmotic pressure (Cala, 1980).

The present results indicate that the beta-adrenergic swelling of fish red cells may also be caused by loosely coupled Na^+/H^+ and Cl^-/HCO_3^- (or Cl^-/OH^-) exchanges. Isoproterenol, and other beta-adrenergic drugs, stimulate Na^+/H^+ exchange across the red cell membrane. Because of their respective concentration gradients, there is a net influx of sodium and a net efflux of protons. As a result, the pH gradient across the red cell membrane decreases; the red cell contents are alkalinized and the incubation medium is acidified. When Na^+ is removed from the incubation medium, neither the red cell volume nor the pH gradient across the red cell membrane are affected by adrenergic stimulation. Simultaneously with the activation of Na^+/H^+ exchange, chloride ions enter the cell and bicarbonate ions come out of the cell through the anion exchange pathway. Simultaneous inward fluxes of sodium and chloride, and consecutive osmotic inflow of water cause the red cell swelling. The HCO_3^- ion coming out counteracts the effect of proton efflux on the intra- and extracellular pH. Therefore, both the swelling and the pH gradient in isoproterenol-treated cells should be affected by blocking the anion exchange pathway with DIDS. When DIDS is included in the incubation medium, the cell volume does not increase in response to adrenergic stimulation, whereas the intracellular pH increases and extracellular pH decreases markedly, reversing the normal pH gradient. These data show that the anion exchange pathway is implicated in the adrenergic volume changes in fish red cells, probably in the manner suggested above. Also, the data show that blocking the anion exchange pathway does not prevent the extrusion of protons. Thus, the two ion exchange systems are only loosely coupled. If, as suggested above, the reversal of pH gradient, induced by treating the cells simultaneously with DIDS and isoproterenol, were due to Na^+/H^+ exchange, it should not occur when sodium is removed from the medium or when Na^+/H^+ exchange is blocked using amiloride. The present data show this to be the case: in DIDS+isoproterenol-treated red cells of rainbow trout $pH_{medium}-pH_{cell}$ was $-0{\cdot}133$; when amiloride was further added to this incubation, the pH difference was $+0{\cdot}073$; and when Na^+ was removed from the medium, the pH difference was $+0{\cdot}173$.

In addition to activating the Na^+/H^+ exchange, adrenalin stimulates K^+ influx in rainbow trout red cells (Bourne & Cossins, 1982). The stimulated K^+ influx may, in addition to the loosely coupled Na^+/H^+ and Cl^-/HCO_3^- exchanges, influence the red cell volume. However, in the present experiments, such an effect was small. The stimulated K^+ influx can be divided into ouabain-sensitive and furosemide-sensitive components (Bourne & Cossins, 1982). It is possible that the ouabain-sensitive component is a secondary phenomenon, caused by the activation of Na^+/H^+ exchange, resultant Na^+ entry into the cells, and consecutive stimulation of the Na^+/K^+ pump

as in the frog (see Palfrey & Greengard, 1981). Also, the furosemide-sensitive K^+ influx and efflux are both stimulated by an increase in cell volume (Bourne & Cossins, 1984). Thus, the adrenalin-stimulated, furosemide-sensitive K^+ influx could be a secondary phenomenon to cell swelling.

The physiological role of adrenergic swelling of bird red cells is still somewhat obscure. The normal concentration of K^+ in avian plasma is approximately 5 mmol l^{-1} (Bell & Sturkie, 1965), a concentration range where isoproterenol induces no cell swelling. Neither heat stress nor dehydration has any effect on plasma K^+ levels in the fowl *Gallus domesticus* (Arad, Marder & Eylath, 1983), so it is probable that this concentration is very stable. Also, in the frog, *Rana pipiens*, cellular phosphodiesterase activity must be inhibited in order to see the adrenergic volume changes (Rudolph & Greengard, 1980).

In fish red cells, on the other hand, adrenergic swelling is observed at physiologically realistic extracellular K^+ and catecholamine concentrations, even without inhibition of cellular phosphodiesterase activity. This suggests that the catecholamine-induced changes in red cell properties play a significant role in the stress responses of these animals (Nikinmaa, 1983). Indeed, beta-adrenergic modification of red cell function appears to help maintain the arterial oxygen content of striped bass in severe exercise (Nikinmaa, Cech & McEnroe, 1984).

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