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ION EXCHANGE MECHANISMS ON THE ERYTHROCYTE MEMBRANE OF THE AQUATIC SALAMANDER, *AMPHIUMA TRIDACTYLUM*

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SUMMARY

The effects of different pharmacological agents and incubation media on the intracellular pH and water content of *Amphiuma* erythrocytes were investigated *in vitro*.

Adrenaline had no significant effect on the intracellular pH or cell water content. DIDS caused an intracellular alkalinization that could be abolished by amiloride, ouabain or removal of sodium from the incubation medium. In addition, amiloride and DIDS both caused a decrease in cell water content. The data indicate that sodium/proton and chloride/bicarbonate exchangers are present on the membrane of *Amphiuma* erythrocytes and these exchangers are active under steady-state conditions.

INTRODUCTION

Cala (1980, 1985) has demonstrated that *Amphiuma* erythrocytes are capable of regulating their volume after osmotic perturbations. The volume regulatory decrease after osmotic swelling is associated with a net loss of erythrocyte potassium and chloride, whereas the volume regulatory increase after osmotic shrinkage involves loosely coupled sodium/proton and chloride/bicarbonate exchange mechanisms. During such volume regulatory increases, the extracellular medium is acidified. Thus, the observed volume changes may be associated with changes in intracellular pH. In addition, *Amphiuma* is found in environments which are often hypoxic and hypercapnic (Heisler, Forcht, Ultsch & Anderson, 1982). It is therefore possible that this animal may possess a mechanism for the regulation of erythrocyte pH in order to regulate haemoglobin oxygen-affinity under these conditions.

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In teleost fish erythrocyte volume increases following adrenergic stimulation are associated with intracellular pH changes, and involve an activation of sodium/proton exchange (Nikinmaa, 1982; Nikinmaa & Huestis, 1984; Cossins & Richardson, 1985; Heming, Randall & Mazeaud, 1987). Furthermore, in the lamprey *Lampetra fluviatilis* erythrocyte pH is actively regulated by a sodium-requiring process, even in the absence of adrenergic stimulation (Nikinmaa, Kunnamo-Ojala & Railo, 1986).

In the present experiments, we investigated the role of different ion exchanges on the membrane of *Amphiuma* erythrocytes in controlling erythrocyte volume and pH. Several different transport and metabolic inhibitors and ion substitutions were used. We also examined the sensitivity of these ion exchange mechanisms to beta-adrenergic stimulation.

MATERIALS AND METHODS

Animals

Specimens of *Amphiuma tridactylum* (450–1600 g) were obtained from Charles Sullivan Co. (Nashville, TN, USA). The salamanders were kept in large fibreglass aquaria (60 × 50 × 25 cm) containing approximately 50 l of dechlorinated Vancouver tap water at the experimental temperature (25 °C) for at least a week prior to the experiments.

Surgery

Animals chosen at random were anaesthetized in tricaine methane sulphonate (MS-222; Sigma) and chronically cannulated in one of the coeliac arteries with PE 50 tubing according to the method of Heisler *et al.* (1982). Cannulated salamanders were then isolated in smaller aquaria (25 × 50 × 30 cm) in 10 l of water and allowed to recover overnight.

Experimental protocol

Samples of arterial blood were collected from cannulated salamanders and centrifuged in heparinized 1·5-ml Eppendorf centrifuge tubes. The plasma was discarded following centrifugation. The erythrocytes were then washed in Mackenzie's amphibian saline (de la Lande, Tyler & Pridmore, 1962) adjusted to 30 mmol l^{-1} bicarbonate, with the exception of one set of experiments in which the saline contained the impermeant cation choline instead of sodium. The protocol for the washing of the cells was variable depending on the experiment. In experiments in which choline replaced sodium, the cells were left suspended for 10 min during each wash to improve the removal of sodium. If ouabain (10^{-4} mol l^{-1}) or 2,4-dinitrophenol (2,4-DNP; 10^{-4} mol l^{-1}) were to be used in the experiment, the respective drug would be present in all washings. If amiloride (10^{-3} mol l^{-1}) or DIDS (4,4-diisothiocyanostilbene 2,2-disulphonic acid; 10^{-4} mol l^{-1}) were to be used, they would only be added to the final suspension. In all cases, the erythrocytes were resuspended to approximately 15 % haematocrit following the second wash in

the appropriate incubation media and 10 μl of 1 μCi ml^{-1} [^{14}C]DMO (5,5-dimethyl-2,4-oxazolidinedione; New England Nuclear, specific activity 50 mCi mmol^{-1}) was added to the suspension for later determination of intracellular pH. The suspensions were then incubated in 50-ml tonometers at 25°C and gassed with humidified mixtures of either 4% CO_2/96% air or 8% CO_2/92% air. The CO_2/air mixtures were delivered by Wösthoff gas-mixing pumps (Wösthoff Digamix, Types M-300a and M-30f, Bochum, FRG). Following a 30-min equilibration period, two 0·4-ml samples were taken from the tonometer and centrifuged. The pH of the extracellular fluid was measured immediately from the first sample tube using a Radiometer PHM 72 and associated micro-pH unit (Radiometer, Copenhagen, Denmark). A 100-μl sample of extracellular fluid was taken from the second sample tube for the determination of DMO levels for subsequent calculation of intracellular pH (pH_i). The erythrocytes from the first sample tube were saved for the pH_i determination and those from the second sample tube were saved for the determination of cellular water content.

At this point, the experiment was either terminated or, in those experiments in which the effects of adrenaline were to be assessed, 10^{-5} mol l^{-1} adrenaline was added to all tonometers except the control. In the adrenaline experiments, the sampling procedure was then repeated following another 30 min of equilibration.

Analyses

The erythrocyte water content was determined by weighing the wet cell pellet, drying it to a constant mass at 90°C and reweighing it (Nikinmaa & Huestis, 1984). The erythrocyte pH was determined from the distribution of DMO between the erythrocytes and the incubation medium as described by Nikinmaa & Huestis (1984), with the exception that the erythrocytes were haemolysed and deproteinized in 0·6 mol l^{-1} perchloric acid to reduce any quenching caused by haemoglobin. The pK for DMO was taken as 6·245 (Albers, Usinger & Spaich, 1971). Trapped extracellular water (1–3%; see, for example, Emilio & Shelton, 1980) was not taken into account. Chloride analyses of the incubation medium and the erythrocytes were performed on a Radiometer CMT 10 chloride titrator (Radiometer, Copenhagen, Denmark).

Statistics

Significance of the results was assessed using a Student's paired *t*-test to evaluate the effects of adrenaline or a Student's unpaired *t*-test to evaluate the effects of incubation in the different media.

RESULTS

The pH and volume of *Amphiuma tridactylum* erythrocytes were not influenced by the incubation time (30 min and 60 min values were similar). The presence of the anion exchange inhibitor DIDS, however, caused a marked intracellular alkalization and decrease in erythrocyte water content at both CO_2 tensions studied

Table 1. *Effects of adrenaline, DIDS and amiloride on the water content, extracellular pH (pH_e), intracellular pH (pH_i), and on the pH gradient (ΔpH) of* Amphiuma tridactylum *erythrocytes equilibrated with 4% CO_2/96% air*

Incubation medium	30 min	Treatment	60 min
Saline (control)			
cell water	74·0 ± 0·6 (8)	A	74·0 ± 0·7 (8)
pH_e	7·62 ± 0·01 (10)		7·63 ± 0·02 (10)
pH_i	7·39 ± 0·02 (10)		7·37 ± 0·02 (10)
ΔpH	+0·23 (10)		+0·26 (10)
Saline			
cell water	73·2 ± 0·6 (7)	B	75·3 ± 0·09 (7)
pH	7·64 ± 0·01 (9)		7·66 ± 0·01 (9)
pH_i	7·40 ± 0·02 (9)		7·42 ± 0·01 (9)†
ΔpH	+0·24 (9)		+0·24 (9)
Saline + DIDS			
cell water	70·8 ± 0·4 (8)†	B	72·0 ± 0·8 (8)*
pHe	7·64 ± 0·02 (9)		7·64 ± 0·02 (9)
pH_i	7·64 ± 0·03 (9)†		7·62 ± 0·03 (9)†
ΔpH	+0·01 (9)		+0·02 (9)
Saline + amiloride			
cell water	68·5 ± 0·8 (8)†	B	70·5 ± 0·9 (8)†
pH_e	7·68 ± 0·02 (10)†		7·66 ± 0·01 (10)
pH_i	7·39 ± 0·03 (10)		7·42 ± 0·03 (10)
ΔpH	+0·29 (10)		+0·24 (10)

Values are means ± 1 S.E.M. (number of individual preparations).
* denotes significant ($P < 0·05$) difference from 30 min value. † denotes significant ($P < 0·05$) difference from treatment A.
Treatment A, control; treatment B, 5×10^{-5} mol l^{-1} adrenaline.

(Tables 1, 2). The intracellular alkalization could be prevented or reduced by (1) removing sodium from the incubation medium, (2) treating the cells with amiloride, or (3) blocking the sodium/potassium pump with ouabain (Table 3). The results indicate that there is a sodium/proton exchange mechanism on the erythrocyte membrane which is functional under steady-state conditions. This exchange is driven by the sodium gradient produced by the sodium/potassium pump (secondarily active). In the absence of bicarbonate movements, as occurs in the DIDS experiments (Tables 1, 2), the intracellular pH is controlled solely by the sodium/proton exchange.

The presence of amiloride alone in the incubation medium caused only slight changes in the pH gradient across the erythrocyte membrane, but did cause a reduction in the erythrocyte water content (Tables 1, 2). These results indicate that the chloride/bicarbonate exchange is capable of equilibrating acid equivalents under normal circumstances.

The sodium/proton exchange mechanism on the erythrocyte membrane did not appear to be sensitive to beta-adrenergic stimulation. Adrenaline alone had no

Table 2. *Effects of adrenaline, DIDS and amiloride on the water content, extracellular pH (pH_e), intracellular pH (pH_i), and on the pH gradient (ΔpH) of* Amphiuma tridactylum *erythrocytes equilibrated with 8 % CO_2/92 % air*

Incubation medium	30 min	Treatment	60 min
Saline (control)			
cell water	72·6 ± 0·4 (7)	A	73·8 ± 0·2 (7)
pH_e	7·43 ± 0·02 (9)		7·44 ± 0·02 (9)
pH_i	7·26 ± 0·02 (9)		7·32 ± 0·04 (9)
ΔpH	+0·17 (9)		+0·11 (9)
Saline			
cell water	73·0 ± 0·6 (8)	B	74·1 ± 0·3 (8)
pH_e	7·45 ± 0·02 (10)		7·43 ± 0·02 (10)
pH_i	7·31 ± 0·04 (10)		7·27 ± 0·02 (10)
ΔpH	+0·14 (9)		+0·16 (10)
Saline + DIDS			
cell water	72·0 ± 0·02 (8)	B	72·4 ± 0·4 (8)†
pHe	7·43 ± 0·03 (10)		7·41 ± 0·02 (10)
pH_i	7·48 ± 0·02 (10)†		7·45 ± 0·02 (10)†
ΔpH	−0·05 (10)		−0·04 (10)
Saline + amiloride			
cell water	71·7 ± 0·6 (8)	B	71·8 ± 0·8 (8)†
pH_e	7·48 ± 0·02 (10)		7·46 ± 0·02 (10)*
pH_i	7·29 ± 0·04 (10)		7·31 ± 0·03 (10)
ΔpH	+0·19 (10)		+0·14 (10)

Values are means ± 1 S.E.M. (number of individual preparations).
*denotes significant ($P < 0·05$) difference from 30 min value. †denotes significant ($P < 0·05$) difference from treatment A.
Treatment A, control; treatment B, 5×10^{-5} mol l^{-1} adrenaline.

significant effects on any of the measured parameters. In the presence of DIDS or amiloride, significant differences were seen between control and adrenaline samples. In the presence of DIDS, adrenaline caused a slight increase in the cell volume in cells equilibrated with 4 % CO_2/96 % air, but this effect was absent in 8 % CO_2/92 % air. In addition, in cells treated with amiloride, adrenaline caused a slight decrease in the extracellular pH in 8 % CO_2/92 % air, but no significant changes in either the intracellular pH or the pH gradient. This effect was absent in 4 % CO_2/96 % air. There was no consistent trend, therefore, in the effect of adrenergic stimulation on cells treated with DIDS or amiloride.

Table 4 compares the erythrocyte pH determined by the DMO method in several experiments with the erythrocyte pH predicted by the distribution of chloride across the erythrocyte membrane using the Donnan equation. These results showed that there was a significant difference (0·05–0·07 pH units) between the two determinations, even in the controls which were not affected by amiloride. However, this difference was exacerbated (0·50–0·52 pH units) in the presence of DIDS, and completely abolished when the ionophore 2,4-DNP was added to the medium.

Table 3. *Effect of DIDS, amiloride, ouabain and absence of extracellular sodium on the water content, extracellular pH (pH_e), intracellular pH (pH_i) and on the pH gradient (ΔpH) of* Amphiuma tridactylum *erythrocytes equilibrated (30 min) with 4% CO_2/96% air*

	Saline control	Saline + DIDS	Saline + DIDS + amiloride
Cell water	73·8 ± 0·6 (4)	70·9 ± 0·6 (4)*	67·0 ± 1·0 (8)*
pH_e	7·54 ± 0·01 (4)	7·51 ± 0·04 (4)	7·54 ± 0·01 (8)
pH_i	7·36 ± 0·02 (4)	7·53 ± 0·05 (4)*	7·35 ± 0·03 (8)
ΔpH	+0·17 (4)	−0·02 (4)	+0·20 (8)
	Saline control	Saline + ouabain	Saline + ouabain + DIDS
Cell water	75·5 ± 1·1 (6)	73·9 ± 0·6 (6)	72·2 ± 0·7 (6)*
pH_e	7·52 ± 0·01 (6)	7·54 ± 0·01 (6)	7·46 ± 0·01 (6)*
pH_i	7·32 ± 0·04 (6)	7·24 ± 0·03 (6)	7·33 ± 0·02 (6)
ΔpH	+0·20 (6)	+0·29 (6)	+0·13 (6)
	Saline (no sodium) control	Saline (no sodium) + DIDS	
Cell water	67·7 ± 0·4 (20)	67·9 ± 0·4 (24)	
pH_e	7·63 ± 0·03 (4)	7·66 ± 0·01 (8)	
pH_i	7·32 ± 0·06 (4)	7·32 ± 0·03 (8)	
ΔpH	+0·32 (4)	+0·34 (8)	

Values are means ± 1 S.E.M. (number of individual preparations).
* denotes value significantly ($P < 0·05$) different from the saline control.

Table 4. *Comparison of intracellular pH determined by the DMO distribution and intracellular pH predicted from the chloride distribution in* Amphiuma tridactylum *erythrocytes*

Incubation medium	pH_i (DMO)	pH_i (Cl^-)
4% CO_2/96% air		
saline	7·37 ± 0·02 (10)	7·32 ± 0·03 (10)*
saline + DIDS	7·60 ± 0·04 (6)	7·08 ± 0·05 (6)*
saline + amiloride	7·38 ± 0·04 (6)	7·32 ± 0·05 (6)*
saline + 2,4-DNP	7·26 ± 0·02 (7)	7·28 ± 0·02 (7)
8% CO_2/92% air		
saline	7·23 ± 0·02 (10)	7·16 ± 0·03 (10)*
saline + DIDS	7·43 ± 0·01 (6)	6·93 ± 0·08 (6)*
saline + amiloride	7·22 ± 0·03 (6)	7·14 ± 0·06 (6)

Values are means ± 1 S.E.M. (number of individual preparations).
* denotes significant difference between pH_i (DMO) and pH_i (Cl^-).

DISCUSSION

Cala (1980, 1985) has demonstrated that *Amphiuma* erythrocytes use loosely coupled, electrically silent, sodium/proton and chloride/bicarbonate exchange

mechanisms to re-establish cell volume after osmotic shrinkage. During this process, sodium and chloride enter the cell in exchange for protons and bicarbonate, respectively, with cell volume passively restored due to osmotically obligated water flow. Cala (1985) has further demonstrated that a potassium/proton exchange mechanism is involved in re-establishing cell volume after osmotic swelling. The present experiments indicate that sodium/proton and chloride/bicarbonate exchanges are involved in steady-state cell volume regulation. Inhibition of either of these exchange mechanisms causes significant reductions in cell volume (Tables 1, 2) which are most pronounced (9·2%) when both ion exchange pathways are blocked. Under control conditions, as the pH decreases in the extracellular and intracellular compartments (due to the equilibration in the CO_2/air mixtures), the chloride ratio (inside/outside) across the erythrocyte membrane will increase (Hladky & Rink, 1977; Heming *et al.* 1987) and the cells will gain water due to the osmotic influence of the increasing intracellular chloride concentration. In the presence of DIDS, however, the movements of chloride will not occur and the treated cells, therefore, have a lower water content than the control cells. The sodium/proton exchange in *Amphiuma* erythrocytes probably influences the cell volume in a manner similar to that described for adrenergically stimulated fish erythrocytes. In fish erythrocytes, adrenergic stimulation of the sodium/proton exchange mechanism results in an accumulation of sodium inside the cell, and water is drawn into the cell because of the increase in osmotic concentration (Baroin, Garcia-Romeu, Lamarre & Motais, 1984*a*; Mahe, Garcia-Romeu & Motais, 1985; Borgese, Garcia-Romeu & Motais, 1986). In the *Amphiuma* erythrocyte, the constant sodium/proton exchange maintains the cell volume at control levels. Inhibition of this exchanger with amiloride, therefore, causes a reduction in the intracellular sodium concentration and a subsequent reduction in the cell water content.

The large decrease in the transmembrane pH gradient in the presence of DIDS (Tables 1, 2) also indicates that the sodium/proton antiporter is constantly functioning and removing protons from the cell interior in exchange for sodium. DIDS blockade of chloride/bicarbonate exchange enables this proton extrusion mechanism to alkalize the intracellular compartment relative to the extracellular medium, since bicarbonate can no longer equilibrate across the erythrocyte membrane. This intracellular alkalization may occur both by the direct extrusion of protons and through a shift in the CO_2 hydration reaction towards the formation of bicarbonate. Replacement of sodium with the impermeant cation choline, or the presence of amiloride in the incubation medium, inhibits this intracellular alkalization. This supports the hypothesis that the intracellular alkalization in DIDS-treated cells occurs due to sodium-dependent proton extrusion, as is also found in teleost erythrocytes following beta-adrenergic stimulation (Nikinmaa & Huestis, 1984; Cossins & Richardson, 1985). In contrast to the situation in adrenergically stimulated fish erythrocytes, the sodium/proton exchanger of *Amphiuma* erythrocytes does not play a significant role in establishing the erythrocyte pH. In the presence of bicarbonate movements, the erythrocyte pH was independent of the presence of sodium or amiloride in the incubation medium. Thus, the role of

the sodium/proton exchanger under steady-state conditions appears to be mainly to regulate volume.

The mechanism of proton extrusion in *Amphiuma* erythrocytes is also sensitive to ouabain. The sodium/proton exchange mechanism therefore seems to be dependent on the sodium/potassium pump. The association between these two mechanisms has also been documented in the frog (Palfrey & Greengard, 1981). Our experiments indicate that the intracellular alkalization occurring in *Amphiuma* erythrocytes in the presence of DIDS is also dependent on the continuous cycling of these two mechanisms.

There is a significant difference at both carbon dioxide tensions between the measured erythrocyte pH and that calculated from the distribution of chloride (Table 4). While this difference is small, it may be removed by treatment with the protonophore 2,4-DNP, indicating that protons are not passively distributed according to a Donnan equilibrium in all cellular compartments. The reason for this difference, however, is unclear. It is not removed by treatment with amiloride and is therefore probably not due to the sodium/proton exchange mechanism.

Upon β-adrenergic stimulation, teleost erythrocytes show a marked increase in both cell water content and pH (Nikinmaa, 1982; Baroin *et al.* 1984*a*,*b*; Nikinmaa & Huestis, 1984; Cossins & Richardson, 1985; Heming *et al.* 1987). These adrenergic effects contribute to an increase in haemoglobin oxygen-affinity during stress (Nikinmaa, Cech & McEnroe, 1984; Primmett, Randall, Mazeaud & Boutilier, 1986). There have been very few studies, however, documenting the effects of β-adrenergic stimulation of amphibian erythrocytes. Rudolf & Greengard (1980) have found that *Rana pipiens* erythrocytes will swell if exposed to isoproterenol only in the presence of a phosphodiesterase inhibitor. Recently, Tufts, Mense & Randall (1987) have demonstrated that β-adrenergic stimulation had no effect *in vitro* on the pH or water content of erythrocytes from the semi-terrestrial anuran, *Bufo marinus*. The results of the present investigation also indicate that β-adrenergic stimulation has no marked effect on either the cell water content or the pH of erythrocytes from the aquatic air-breathing urodele, *Amphiuma tridactylum*. With regard to β-adrenergic sensitivity, then, *Amphiuma* erythrocytes seem to resemble those of anurans rather than those of teleosts. The functional significance of the difference in β-adrenergic sensitivity between amphibian and teleost erythrocytes is not clear. Both the toad, *Bufo marinus*, and the salamander, *Amphiuma tridactylum*, are air breathers. Therefore, it is possible that the β-adrenergic response may be an adaptation found only among water breathers. However, the difference between amphibian and teleost erythrocytes may be related to differences in the oxygen transport characteristics of the blood. In teleosts, a reduction in the erythrocyte pH lowers the oxygen-carrying capacity of the blood, known as the Root shift (Cameron, 1971; Boutilier, Iwama & Randall, 1986). It would therefore be beneficial for teleosts to regulate erythrocyte pH *via* adrenergic mechanisms during stress in order to maintain the oxygen-carrying capacity of the blood. Amphibian blood does not appear to have a Root shift (Bridges, Pelster & Scheid, 1985) and a reduction in erythrocyte pH does not affect the total oxygen-carrying capacity of the erythrocyte.

In summary, the present study supports the view that the membrane of the *Amphiuma* erythrocyte contains sodium/proton and chloride/bicarbonate exchange mechanisms. Our results indicate that these ion exchange mechanisms are involved in steady-state volume regulation. The secondarily active sodium/proton exchanger may cause a marked intracellular alkalization in the absence of bicarbonate movements. In contrast to the sodium/proton exchanger on the teleost erythrocyte membrane, the sodium/proton exchange mechanisms on the *Amphiuma* erythrocyte membrane does not exhibit a significant β-adrenergic sensitivity.

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