The Journal of Experimental Biology 210, 2290-2299
Published by The Company of Biologists 2007
doi:10.1242/jeb.002824

Oxygen-sensitive regulatory volume increase and Na transport in red blood cells from the cane toad, *Bufo marinus*

Karina Kristensen[1], Pia Koldkjær[2,*], Michael Berenbrink[2] and Tobias Wang[1]

[1]*Department of Zoophysiology, Aarhus University, Denmark and* [2]*School of Biological Sciences, The Biosciences Building, Crown Street, Liverpool L69 7ZB, UK*

*Author for correspondence (e-mail: piak@liv.ac.uk)

Accepted 24 April 2007

Summary

The red blood cells (RBCs) of cane toad, *Bufo marinus*, are only partially saturated with oxygen in most of the circulation due to cardiac shunts that cause desaturation of arterial blood. The present study examines the oxygen dependency of RBC ouabain-insensitive unidirectional Na transport, using ^{22}Na, in control cells and in cells exposed to hyperosmotic shrinkage or the β-adrenergic agonist isoproterenol. Deoxygenation *per se* induced a slow, but significant Na influx, which was paralleled by a slow increase in RBC volume. Hyperosmotic shrinkage by a calculated 25% activated a robust Na influx that in the first 30 min had a strong P_{O_2} dependency with maximal activation at low P_{O_2} values and a P_{50} of ~5.5 kPa. This activation was completely abolished by the Na/H exchanger (NHE) inhibitor EIPA (10^{-4} mol l^{-1}). Hyperosmotic shrinkage is particularly interesting in *B. marinus* as it withstands considerable elevation in extracellular osmolarity following dehydration. Parallel studies showed that deoxygenated *B. marinus* RBCs had a much faster regulatory volume increase (RVI) response than air-equilibrated RBCs, reflecting the difference in magnitude of Na influxes at the two P_{O_2} values. The extent of RVI (~60%) after 90 min, however, was similar under the two conditions, reflecting a more prolonged elevation of the shrinkage-induced Na influx in air-equilibrated RBCs. There were no significant differences in the ability to perform RVI between whole blood cells at a P_{CO_2} of 1 and 3 kPa or washed RBCs, and 10^{-4} mol l^{-1} amiloride reduced the RVI under all conditions, whereas 10^{-5} mol l^{-1} bumetanide had no effect. Isoproterenol (10^{-5} mol l^{-1}) induced a significant and prolonged increase in an EIPA-sensitive and bumetanide-insensitive Na influx at low P_{O_2} under iso-osmotic conditions, whilst there was no stimulation by isoproterenol for up to 45 min in air-equilibrated RBCs. The prolonged β-adrenergic activation of the Na influx at low P_{O_2} is distinctly different from the rapid and transient stimulation in teleost RBCs, suggesting significant differences in the signal transduction pathways leading to transporter activation between vertebrate groups.

Key words: *Bufo marinus*, oxygen-dependent ion transport, erythrocyte, NHE.

Introduction

Red blood cells (RBCs) in nearly all vertebrate classes ranging from agnathans to mammals restore volume upon hyperosmotic shrinkage by stimulation of Na uptake through the Na/H exchanger (NHE) or the Na/K/2Cl co-transporter (NKCC) (Cossins and Gibson, 1997; Gibson et al., 2000). The regulatory volume increase (RVI) in RBCs from mammals, most teleosts and amphibians is mediated by increased NHE activity, resulting in a net gain of inorganic osmolytes followed by osmotically obliged water (Cala, 1980; Parker et al., 1991; Orlov et al., 1994; Romero et al., 1996; Gusev et al., 1997; Weaver et al., 1999) whereas bird RBCs activate the NKCC (Kregenow, 1981; Muzyamba et al., 1999). In addition to shrinkage, the activity of the NHE and NKCC and other RBC ion transporters is modulated by oxygen, catecholamines or acidification [NHE (Motais et al., 1987; Salama and Nikinmaa, 1988; Nielsen, 1997; Virkki et al., 1998) and NKCC (Palfrey and Greengard, 1981; Muzyamba et al., 1999; Flatman, 2005; Berenbrink et al., 2006)]. Thus, in various vertebrates, the NHE, NKCC and/or RVI response are inhibited in air-equilibrated cells compared with cells at low oxygen partial pressure (P_{O_2}) (Gibson et al., 2000; Brauner et al., 2002; Jensen et al., 2002; Drew et al., 2004).

Plasma osmolality increases naturally in animals during dehydration, physical exercise and upon movement from freshwater to seawater (Shoemaker, 1964; Maxime et al., 1991; Madsen et al., 1996; McKenna et al., 1997; Hyndman et al., 2003; Peterson and Greenshields, 2001). Because of their water-permeable skin, amphibians are prone to dehydration, but the dependence on water differs among species. Terrestrial anurans, such as *Bufo*, tend to be rather tolerant to dehydration (Thorson, 1955), and the cane toad, *Bufo marinus*, tolerates

severe dehydration with 30% loss of body mass (Shoemaker, 1964; Shoemaker, 1965). However, little is known about the RVI response from terrestrial amphibians. Furthermore, the cardiac shunts of the amphibian circulatory system cause desaturation of arterial blood (Johansen and Ditadi, 1966; Wang et al., 1998) and it is of interest, therefore, to examine how RBC volume regulation is affected by oxygen in these animals.

Catecholamines are potent activators of the NKCC and NHE in many vertebrate RBCs (Palfrey et al., 1981; Motais et al., 1987; Nikinmaa, 1992; Weaver et al., 1999; Pedersen and Cala, 2004; Berenbrink et al., 2005) with maximal stimulation at low P_{O_2} levels (Motais et al., 1987; Salama and Nikinmaa, 1988). The influence of adrenergic stimulation on isotonic volume responses and ion fluxes has been examined in some amphibian RBCs, but responses were only significant in the presence of phosphodiesterase inhibitors; thus, the physiological significance of the responses remains unknown (Rudolph and Greengard, 1980; Tufts et al., 1987a; Tufts et al., 1987b; Kaloyianni et al., 1997).

The present work examines the P_{O_2} dependency of the Na transport mechanism induced by hyperosmotic shrinkage or β-adrenergic stimulation in washed *B. marinus* RBCs using ouabain-insensitive unidirectional ^{22}Na influx. In parallel experiments, the influence of oxygenation on the ability to restore cell volume after hyperosmotic shrinkage is studied in both whole blood cells and washed RBCs. The Na influx and RVI mechanisms are characterized pharmacologically.

Materials and methods

Animals

The experiments were performed on 26 cane toads, *Bufo marinus* L., of undetermined sex and a body mass of between 149 and 360 g (214±9 g; mean ± s.e.m.). They were purchased from a commercial supplier (Exotic Tropicals, St Michael, Barbados) and kept in containers (100×50×75 cm; L×W×H) with access to water and dry areas at approximately 25°C. The animals were kept in the holding facilities for at least two weeks before use and were fed mealworms and liver pieces several times a week.

Blood sampling

Animals were anaesthetized by immersion in a solution (1 g l^{-1}) of benzocaine (ethyl *p*-amino benzoate). When all reflexes had disappeared, an incision was made in the hind limb and a blood sample (3–4 ml) was obtained immediately after inserting a heparinised cannula (PE-50) into the femoral artery. The cannula was removed and the incision was closed using sutures, whereupon the animal was placed under running tapwater until it restored spontaneous ventilation. Andersen and Wang reported no change in plasma lactate concentration immediately after similar surgical procedure in *B. marinus* (Andersen and Wang, 2002) and this sampling procedure should therefore not affect whole blood *in vitro* studies.

Unidirectional Na influx studies on washed RBCs

A sub-sample of the blood was centrifuged at 5°C for 3 min at 1700 ***g*** (Sigma-3MK, Osterode, Germany) to remove plasma and buffy coat. The cells were then washed three times in 10 volumes of ice-cold isotonic saline and kept oxygenated by contact with air at 5°C overnight for use in the experiments on washed blood cells. The standard isotonic saline contained: 105 mmol l^{-1} NaCl, 6 mmol l^{-1} KCl, 1 mmol l^{-1} $MgSO_4$, 5 mmol l^{-1} $CaCl_2.2H_2O$, 5 mmol l^{-1} D-glucose and 10 mmol l^{-1} Hepes. It was matched to the plasma osmolality of *B. marinus* of 240 mOsm kg^{-1} and adjusted to pH 7.83 at 25°C (Andersen and Wang, 2003).

After overnight storage at 5°C, the RBCs were washed twice in the isotonic saline and adjusted to a haematocrit (Hct) of 10–20%. For each experiment, 800 μl of RBC suspension was equilibrated with humidified air in a rotating Eschweiler tonometer (obtained from the Dept of Chemistry, Aarhus University, Denmark) at 25°C for 45 min before ouabain was added to a final concentration of 10^{-4} mol l^{-1}. Hct was determined in duplicate and, after an additional 10 min (time zero in the experiment), aliquots of RBC suspension were diluted 10 times by transfer to test tubes containing air-equilibrated isotonic saline, ^{22}Na (~16.7 kBq ml^{-1}), ouabain (10^{-4} mol l^{-1}) and other transport modifiers (specified below). When studying effects of a change in P_{O_2}, the tonometer and test tubes were gassed with the experimental gas mixture for 10 min before transfer of RBCs. The blood cells were subjected to P_{O_2} values ranging from 0 (pure N_2) to 20.5 kPa (air) under isotonic conditions or a calculated 25% shrinkage with or without 10^{-4} mol l^{-1} EIPA [5-(*N*-ethyl-*N*-isopropyl)amiloride]. In shrunken RBCs incubated under nitrogen, a dose–response curve for EIPA was constructed using final concentrations between 10^{-7} and 10^{-5} mol l^{-1}. The effect of 10^{-5} mol l^{-1} isoproterenol with or without 10^{-4} mol l^{-1} EIPA (to inhibit the NHE) or 10^{-5} mol l^{-1} bumetanide (to inhibit the NKCC) was studied in cells incubated in isotonic saline under humidified nitrogen. Finally, the effect of extracellular acidification to pH 7.28 by addition of appropriate volumes of a pH 7.2 saline and of 10^{-5} mol l^{-1} isoproterenol was studied in cells suspended in isotonic saline under air.

Triplicate samples (200 μl) were taken from the test tubes and centrifuged at 6700 ***g*** (Sigma-113) for 10 s before removing the supernatant by aspiration. The RBCs were immediately washed three times in ice-cold isotonic $MgCl_2$ solution (84 mmol l^{-1} $MgCl_2$ and 20 mmol l^{-1} Hepes; adjusted to pH 7.83 at 25°C) before addition of 500 μl of 0.05% Triton X-100 solution and 500 μl of 5% trichloroacetic acid (TCA) to the pellet to lyse and deproteinate the cells. At the end of each experiment, a sample (20 μl) was treated with Triton X-100 and TCA to count the total ^{22}Na activity, which was assumed to correspond to the extracellular ^{22}Na activity at the beginning of the uptake experiment. All samples were centrifuged for 2 min (6700 ***g***), and 800 μl of supernatant was transferred to 5 ml plastic vials or insert vials. Radioactivity was measured directly using a gamma-counter (auto-gamma

5650; Packard Instruments, Greve, Denmark) or after addition of 3–4 ml scintillation cocktail (Pico-Flour 40; Perkin-Elmer, Caversham, England) using a beta-counter (Tri-Carb 2100 TR; Packard). The Na influx (mmol Na l^{-1}RBCs h^{-1}) was calculated as:

$$J_{Na+} = \text{c.p.m. equiv.} \times \Delta\text{c.p.m.} \times 60 / (V_{RBC} \times \Delta t) , \quad (1)$$

where c.p.m. equiv. denotes mmol Na equivalent to 1 c.p.m., Δc.p.m. denotes the difference in triplicate ^{22}Na c.p.m. between two time points, V_{RBC} is the volume of RBCs (litres), determined from the Hct samples taken after 45 min of pre-equilibration, and Δt is the time in minutes between samplings.

Cell volume regulation

Whole blood

A sub-sample of ~2 ml freshly drawn blood was kept on ice for 1–2 h until used for whole-blood RVI studies. Approximately 0.5 ml (or 0.7 ml if pH was measured; see below) was incubated in each of four rotating Eschweiler tonometers. The tonometers were immersed in a water bath kept at 25°C and supplied with a humidified gas mixture of 1% or 3% CO_2, balanced with air, through a Wösthoff gas mixing pump (Bochum, Germany) to achieve physiologically relevant P_{CO_2} and pH values for resting and active animals, respectively (Andersen and Wang, 2003). After a 30 min equilibration period, samples were taken for duplicate pH, Hct and haemoglobin concentration ([Hb]) determinations and, five minutes later (time zero in the experiment), the blood cells were subjected to the following manipulations: (1) osmotic shrinkage alone by addition of appropriate volumes of a 2.3 Osm kg^{-1} stock solution (sucrose-containing standard saline) to increase plasma osmolarity from ~240 to ~320 mOsm l^{-1} (calculated to yield 25% cell shrinkage) or with (2) simultaneous addition of amiloride to a final concentration of 10^{-4} mol l^{-1} to inhibit the NHE or (3) simultaneous addition of bumetanide to a final concentration of 10^{-5} mol l^{-1} to inhibit the NKCC. In addition, parallel control experiments with no osmotic disturbance were performed to determine the influence of incubation alone on the RBCs.

Duplicate Hct determinations were made at time zero as well as 15, 30, 60, 90 and 120 min after treatment, and duplicate samples for [Hb] determinations were taken at 0, 60 and 120 min. Haematocrit was determined after centrifuging heparinized micro-haematocrit tubes at 16 000 ***g*** for 3 min in a haemofuge (Heraeus Sepatech, Usingen, Germany). After conversion of Hb to cyanomethaemoglobin by Drabkin's reagent {11.9 mmol l^{-1} $NaHCO_3$, 0.61 mmol l^{-1} $K_3[Fe(CN)_6]$, 0.77 mmol l^{-1} KCN and 0.05% v/v Triton X-100}, [Hb] was determined at a wavelength of 540 nm (Ultrospec 2; LKB Biochrom, Cambridge, UK), applying a millimolar extinction coefficient of 11.0. The [Hb] was constant after time zero, so an average value was calculated for the time 0, 60 and 120 min measurements and used to calculate mean cellular haemoglobin concentration (MCHC) as [Hb]/Hct at all sampling points after treatment. MCHC was used as an indicator of red cell volume changes. pH was measured with a Radiometer pH electrode (BMS2, Copenhagen, Denmark) at 25°C.

Washed red blood cells

Washed RBCs, as above, were washed twice in the isotonic saline and adjusted to an Hct value of 20–25% (22.2±0.9%, N=16). The RBC suspension was then transferred to a rotating Eschweiler tonometer kept in a water bath at 25°C and equilibrated to humidified air. After 20 min, ouabain was added to a final concentration of 10^{-4} mol l^{-1}, and 5 min later samples were taken for duplicate determinations of Hct and [Hb]. After an additional 5 min (time zero in the experiment), the suspension was distributed into open test tubes and subjected to the same manipulations and sampling procedures as described above for whole blood. The test tubes were kept at 25°C throughout the experiment and gently swirled regularly to ensure oxygenation and avoid sedimentation. In addition, identical volume recovery experiments on deoxygenated washed RBCs were performed. To achieve deoxygenated conditions, humidified N_2 replaced air in the tonometer 10 min before the suspension was transferred to test tubes (at time zero in the experiment), which were also gassed with humidified N_2.

Data analysis and statistics

Differences in the mean MCHC and Na influx between treatments were analysed by a two-way analysis of variance (ANOVA) test for repeated measures. Differences in MCHC between washed cells and whole blood cells were analysed by a three-way ANOVA test for repeated measures. ANOVA tests were followed by a multiple comparison Bonferroni test. Data were transformed according to $x'=x^{\frac{1}{3}}+(x+1)^{\frac{1}{3}}$ if needed to fulfil the requirement of normal distribution (Zar, 1984). All values shown are means ± 1 s.e.m., and statistical significance was accepted at $P<0.05$. The P_{O_2} at which the shrinkage-stimulated Na influx was half-maximal, P_{50}, was estimated by curve fitting, using:

$$y = \left[1 - \left(\frac{x}{x+a}\right) \times 6\right] + c , \quad (2)$$

where y is Na influx, x is P_{O_2}, a is P_{50}, b is maximal oxygen-dependent Na influx and c is oxygen-independent Na influx.

Results

P_{O_2} dependency of shrinkage-induced Na influx and RVI

Hyperosmotic shrinkage of RBCs suspended in physiological saline significantly increased a ouabain-insensitive Na influx at all P_{O_2} values (Fig. 1). Within 5–15 min, there was a strong P_{O_2} dependency of the shrinkage-induced Na influx, which increased significantly at a P_{O_2} of 4.1 kPa compared with at 8.2 and 20.5 kPa and reached a maximal value of ~50 mmol Na l^{-1} RBCs h^{-1} in deoxygenated cells (0 kPa P_{O_2}) (Fig. 1A). The kinetics of the shrinkage-induced Na influx also depended on P_{O_2}. The Na influx in deoxygenated cells remained high throughout the initial 30 min

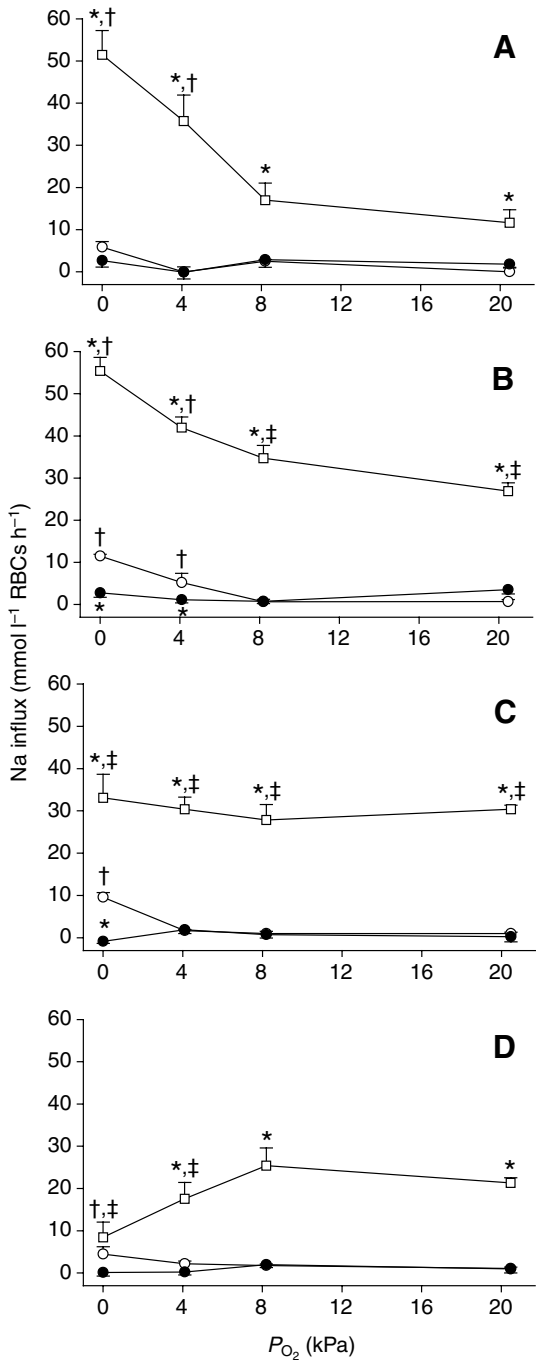

Fig. 1. Na influx in washed red blood cells (RBCs) equilibrated to a range of oxygen partial pressure (P_{O_2}) values in the presence of 10^{-4} mol l^{-1} ouabain. The influx was measured in unstimulated cells to get basal influx (○) and in cells stimulated by a calculated 25% osmotic shrinkage alone (□) or in the presence of 10^{-4} mol l^{-1} EIPA (●). The cell suspensions were exposed to the experimental gas mixture 10 min before shrinkage (time zero in the figures). (A) 5–15 min, (B) 15–30 min, (C) 30–45 min and (D) 45–60 min. * indicates statistical difference from basal influx within the same P_{O_2} and time interval; † indicates statistical difference from air-equilibrated RBCs within the same treatment and time interval; ‡ indicates statistical difference from the same treatment and P_{O_2} level in the 5–15 min time interval. N=4, P<0.05.

(Fig. 1A,B), whereupon it returned to basal levels between 45 and 60 min (Fig. 1D). However, while the Na influx at 4.1 kPa P_{O_2} also remained high (36–42 mmol Na l^{-1} RBCs h^{-1}) during the first 30 min, the influx declined but remained significantly above basal levels throughout the experiment (Fig. 1). At higher P_{O_2} values, there appeared to be a 15-min lag before maximal Na influx values of ~30 mmol Na l^{-1} RBCs h^{-1} were reached between 15 and 45 min (Fig. 1B,C), whereupon the influx declined but remained significantly elevated at 20–25 mmol Na l^{-1} RBCs h^{-1} (Fig. 1D).

The P_{O_2} dependency of the shrinkage-induced Na influx was maximal during the first 5–15 min (Fig. 1A), decreased between 15 and 30 min (Fig. 1B), and between 30 and 45 min there was no significant P_{O_2} dependency of the shrinkage-induced Na influx (Fig. 1C). In the 45–60 min interval, the P_{O_2} dependency even reversed, with maximal shrinkage-induced Na influxes at 8.2 and 20.5 kPa P_{O_2} (Fig. 1D). In the 5–15 min interval, a P_{50} value of 5.5±1.0 kPa (N=4) was determined for shrinkage-induced Na influx.

The shrinkage-induced Na influx was completely abolished by 10^{-4} mol l^{-1} EIPA, a selective inhibitor of the NHE, at all P_{O_2} values (Fig. 1). A dose–response curve for EIPA is shown in Fig. 2, where the effect of EIPA was studied in deoxygenated RBCs exposed to a calculated 25% shrinkage. An IC_{50} of ~10^{-6} mol l^{-1} EIPA can be estimated from the dose–response curve.

The shrinkage-induced Na uptake was associated with RVI in washed RBCs kept under conditions identical to those in Fig. 1 and exposed to 0 or 20.5 kPa P_{O_2} (Fig. 3A). The RVI response was faster in deoxygenated compared with oxygenated RBCs, with MCHC recovery corresponding to 50% of the initial change attained after ~23 and 70 min, respectively, under the two conditions (Fig. 3B). The faster RVI response of deoxygenated

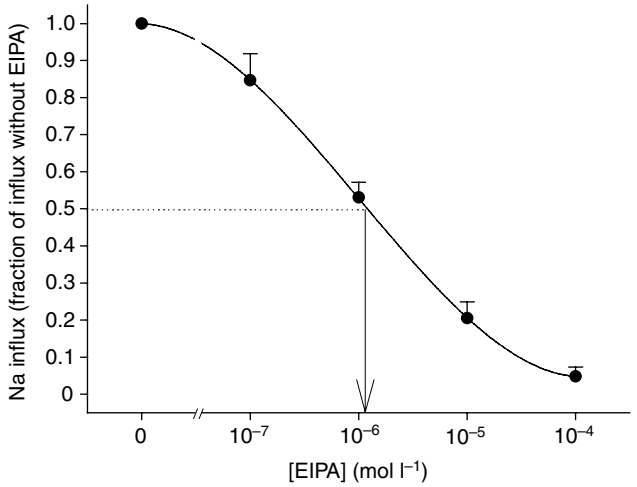

Fig. 2. Effect of EIPA on fractional Na influx induced by 25% shrinkage of N_2-equilibrated cells. The Na influx was measured between 15 and 30 min, where maximal shrinkage-induced influxes were found. A curve was fitted using non-linear regression (SigmaPlot 8.0). N=4.

RBCs (Fig. 3B) reflects that the Na influx was already maximal in the initial two time intervals under those conditions (Fig. 1A,B), whereas maximal Na influx values were not attained

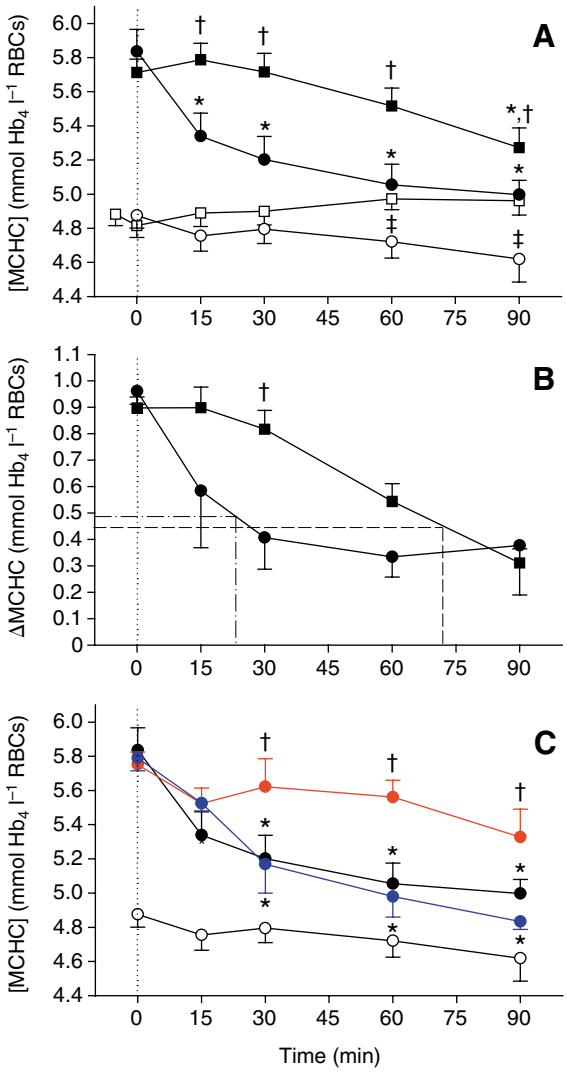

Fig. 3. Changes in mean cellular haemoglobin concentration (MCHC) as a function of time in washed red blood cells (RBCs) in the presence of 10^{-4} mol l^{-1} ouabain. The dotted line indicates osmotic shrinkage of the RBCs following addition of sucrose (calculated to yield 25% shrinkage). (A) Oxygenated control (□) and shrunken (■) as well as deoxygenated control (○) and shrunken (●) RBCs. (B) Difference in MCHC between shrunken and control cells under oxygenated (■) or deoxygenated (●) conditions. The dashed and the dash-dot-dashed lines indicate 50% recovery from initial change in MCHC upon shrinkage in oxygenated and deoxygenated RBCs, respectively. (C) Deoxygenated control and shrunken RBCs with and without transport inhibitors. Control cells (○), shrunken cells (●), shrunken cells treated with 10^{-4} mol l^{-1} amiloride (●) or with 10^{-5} mol l^{-1} bumetanide (●). * indicates mean values that are statistically different from the respective time zero value; † indicates mean values that are statistically different from shrinkage under deoxygenated conditions; ‡ indicates values for deoxygenated control RBCs that are statistically different from values for oxygenated control RBCs. N=5, P<0.05.

until after 15–45 min in oxygenated cells (Fig. 1B,C), delaying the RVI response (Fig. 3B). Maximal volume recovery of deoxygenated cells was attained after ~30 min (Fig. 3B), after which the Na influx was reduced and reached basal levels after 45–60 min (Fig. 1C,D). The volume recovery was slower in oxygenated cells, but after 90 min the extent of volume recovery (~60%) was similar to that attained in deoxygenated cells (Fig. 3B), reflecting the more sustained Na influx in cells kept at 20.5 kPa P_{O_2} (Fig. 1B–D). The RVI response was substantially reduced by the NHE inhibitor amiloride (10^{-4} mol l^{-1}), whereas the NKCC inhibitor bumetanide (10^{-5} mol l^{-1}) had no effect (Fig. 3C).

The above experiments were carried out on RBCs suspended in a physiological saline to allow comparisons with the Na influx data. However, RVI experiments on whole blood were also carried out under similar conditions of pH and temperature. Fig. 4 depicts the time courses in MCHC of whole blood at 19.9–20.3 kPa P_{O_2} after different treatments at 1% CO_2 (P_{CO_2} ~1 kPa) and 3% CO_2 (P_{CO_2} ~3 kPa), corresponding to pH 7.86±0.05 (N=7) and 7.55±0.03 (N=4), respectively, as measured immediately before shrinkage. These are typical P_{CO_2} and pH values for resting and active or burrowing animals, respectively (Boutilier et al., 1979). MCHC of untreated control cells at both P_{CO_2} values did not change significantly during the experiment (Fig. 4A,B). At pH 7.86, the RVI response after a calculated 25% shrinkage of the cells was statistically significant from 30 min onwards, and after 120 min volume recovery had attained 89% (Fig. 4A). Amiloride (10^{-4} mol l^{-1}) significantly reduced RVI, whereas bumetanide (10^{-5} mol l^{-1}) did not have any effect on the extent of RVI (Fig. 4A). Whole blood, at pH and P_{CO_2} values more typical for an active or burrowing animal, showed an overall lowering of the MCHC from ~4.7 to ~3.9 mmol Hb_4 l^{-1} RBCs and, thereby, had a new increased steady-state RBC volume (Fig. 4B). The time course and extent of volume recovery after shrinkage were similar to those at 1% CO_2 but, due to larger differences in MCHC values between individual experiments, there was no difference between MCHC values of control and shrunken cells from 60 min onwards (Fig. 4B).

A three-way ANOVA comparing the whole-blood experiments (Fig. 4A) to parallel RVI experiments on oxygenated washed RBCs (Fig. 4C) showed that the extent and inhibitor sensitivity of the RVI were not different under similar conditions of temperature and pH. Haemolysis was not observed in whole blood up to 120 min of experimentation, whereas haemolysis started to develop after 90 min in washed cell suspensions. Therefore, data at 120 min for washed cells are not included.

Effects of deoxygenation per se and extracellular acidification on Na influx

In the 15–30 min interval, reduction of P_{O_2} to 4.1 and 0 kPa induced a 7- and 16-fold increase in the ouabain-insensitive basal flux, respectively, relative to that measured in fully oxygenated cells (Fig. 1B). In cells exposed to a P_{O_2} of 0 kPa, a 10-fold increase in basal flux relative to that of oxygenated

cells was still present in the 30–45 min interval (Fig. 1C). Deoxygenation *per se* increased cell volume, as illustrated by significantly lowered MCHC values compared with oxygenated RBCs from 60 min onwards (Fig. 3A), corresponding well with the increased Na influx in deoxygenated RBCs in the 15–45 min interval (Fig. 1B,C).

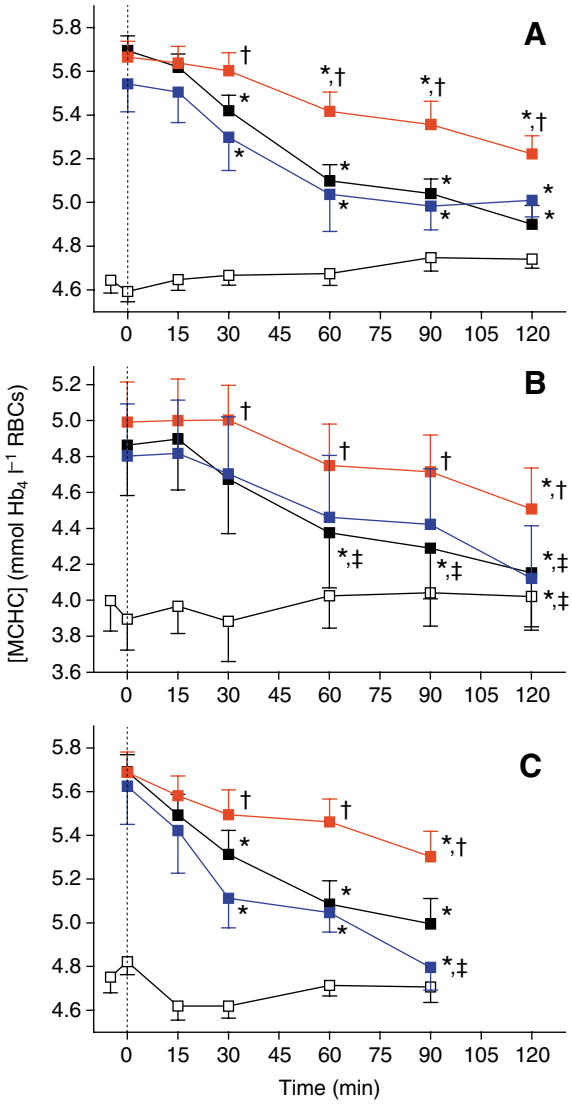

Fig. 4. Changes in mean cellular haemoglobin concentration (MCHC) as a function of time in air-equilibrated whole blood at 1% CO_2 (A) or 3% CO_2 (B) or air-equilibrated washed red blood cells (RBCs) in the presence of 10^{-4} mol l^{-1} ouabain (C). The dotted lines indicate osmotic shrinkage by addition of sucrose (calculated to yield 25% shrinkage). Control cells (□; *N*=9, 4 and 11 in A, B and C, respectively), shrunken cells (■; *N*=9, 4 and 11 in A, B and C, respectively), shrunken cells treated with 10^{-4} mol l^{-1} amiloride (■; *N*=9, 4 and 11 for A, B and C, respectively) or with 10^{-5} mol l^{-1} bumetanide (■; *N*=4, 4 and 6 in A, B and C, respectively). * indicates mean values that are statistically different from the respective control value at time zero; † indicates mean values that are statistically different from shrinkage alone; ‡ indicates values that are not statistically different from corresponding values in control blood samples. $P<0.05$.

Extracellular acidification (to pH 7.28) of oxygenated RBCs suspended in isotonic saline did not change the Na influx compared with basal values. Thus, basal Na influxes in oxygenated RBCs in the 5–15, 15–30 and 30–45 min intervals were 3.9±2.1, 0.4±1.3 and 2.2±1.2 mmol l^{-1} RBCs h^{-1} (*N*=6) whereas the Na influxes in acidified RBCs were 1.5±2.9, 3.5±2.8 and 1.3±1.0 mmol l^{-1} RBCs h^{-1} (*N*=6).

Oxygen dependence of isoproterenol-induced Na influx

The β-adrenergic agonist isoproterenol significantly increased the ouabain-insensitive Na influx in deoxygenated RBCs from ~6 and ~11 mmol Na l^{-1} RBCs h^{-1} to ~25 and ~35 mmol Na l^{-1} RBCs h^{-1}, respectively, within the first two time intervals (Fig. 5A). The effect of isoproterenol on Na influx was statistically significant for up to 45 min, whereupon the influx was reduced to basal levels (Fig. 5A). Bumetanide did not significantly reduce the isoproterenol-stimulated Na influx at any time, whereas EIPA completely blocked the effects of isoproterenol on Na influx (Fig. 5A). In air-equilibrated RBCs, on the other hand, isoproterenol

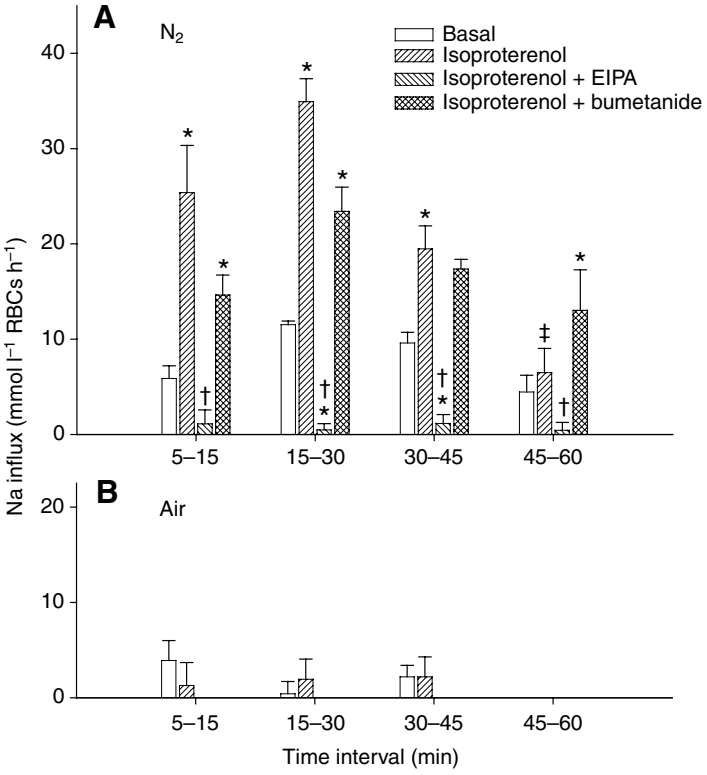

Fig. 5. Na influx as a function of time in washed red blood cells (RBCs) in the presence of 10^{-4} mol l^{-1} ouabain in (A) deoxygenated cells (equilibrated to humidified N_2 for 10 min prior to treatments) or (B) air-equilibrated cells. The Na influx was measured in unstimulated cells to get the basal influx and in cells stimulated by 10^{-5} mol l^{-1} isoproterenol at time zero either alone or in the presence of transport inhibitors. * indicates statistical difference from basal influx within same time interval; † indicates statistical difference from influx in isoproterenol stimulated cells within same time interval; ‡ indicates statistical difference from same treatment in the 5–15 min time interval. $P<0.05$. (A) *N*=4 and (B) *N*=6.

had no effect on Na influx within 45 min of stimulation (Fig. 5B).

Discussion

P_{O_2} dependency of shrinkage-induced Na influx and RVI

Oxygenation-dependent RBC membrane transporters have now been described in nearly all vertebrate classes, ranging from lamprey to mammals (Motais et al., 1987; Nielsen et al., 1992; Gibson et al., 1998; Virkki et al., 1998; Muzyamba et al., 1999; Berenbrink et al., 2000; Berenbrink et al., 2006; Flatman, 2005) (reviewed by Gibson et al., 2000). This also applies to the transporters involved in volume regulation (Jensen, 1995; Nielsen, 1997; Gibson et al., 2000; Brauner et al., 2002; Drew et al., 2004). In *Bufo marinus*, there is a strong P_{O_2} dependency of the shrinkage-induced Na influx, with maximal stimulation at low P_{O_2} during the initial 30 min (Fig. 1). The shrinkage-induced Na influx was completely abolished by the specific NHE inhibitor EIPA (10^{-4} mol l^{-1}), showing that the Na uptake was entirely due to activation of the NHE (Fig. 1). The P_{50} value of 5.5 kPa for half-maximal shrinkage-induced Na influx in the first time interval is similar to the *in vitro* Hb P_{50} value of 5.3 kPa that can be estimated for *B. marinus* at a similar pH and temperature [based on data in table 1 of Andersen et al. (Andersen et al., 2001)]. Therefore, with matching oxygen affinities of NHE activation and Hb, it is conceivable that Hb is involved in oxygen sensing and modulation of NHE activation in *B. marinus* RBCs. This role of Hb has been suggested for modulation of NHE and KCC (K/Cl co-transporter) activity in rainbow trout RBCs (Motais et al., 1987; Nielsen et al., 1992). However, other studies on rainbow trout and crucian carp RBCs indicate that the P_{50} of KCC activation is significantly different from the P_{50} of Hb, implying that the effect of oxygen is not regulated by bulk Hb oxygen saturation (Berenbrink et al., 2000; Berenbrink et al., 2006). In rainbow trout, the effect of oxygen on transporter activity seems to be mediated through hydroxyl radicals, which stimulate KCC activity, whilst they inhibit the βNHE at high P_{O_2} (Bogdanova and Nikinmaa, 2001; Nikinmaa et al., 2003). The oxygen dependency of NKCC activity in RBCs of some tetrapods such as turkey and ferret and in crucian carp, however, is consistent with modulation by the Hb oxygenation state (Muzyamba et al., 1999; Flatman, 2005; Berenbrink et al., 2006).

Shrinkage of *B. marinus* RBCs was attended by a pronounced amiloride-sensitive RVI response (Fig. 3C, Fig. 4C) that was much faster under deoxygenated conditions (Fig. 3B), reflecting the time course of activation and the magnitude of the Na influxes under the two levels of P_{O_2} (Fig. 1). The RVI response was, however, not complete under either of the conditions within the 90–120 min studied here. It appears that the fast RVI response in deoxygenated *B. marinus* RBCs is due to immediate and large NHE activation, whereas the slower RVI in oxygenated cells reflects the fact that the Na influx is not maximal until 15–45 min after shrinkage activation (Fig. 1, Fig. 3B). In European flounder, the RVI is progressively faster with decreasing levels of Hb oxygen saturation (Jensen et al., 2002). In light of this, studies carried out at high P_{O_2} values, as for example during air equilibration, may mistakenly underestimate the ability of RBCs to perform RVI under conditions prevailing in the venous circulation or, in animals with cardiac shunts, even in arterial blood.

The NHE also mediates RVI in *Rana ridibunda* RBCs, and a flux of 11.6±1.1 mmol Na l^{-1} RBCs h^{-1} under oxygenated conditions was measured within the first 30 min after a calculated 31% shrinkage (Gusev and Ivanova, 2003). This Na influx is lower than the influx between 0 and 30 min in *B. marinus* RBCs, which at P_{O_2} 20.5 kPa can be estimated to be 19.1±2.0 mmol Na l^{-1} RBCs h^{-1} (N=4) and it suggests that, at least initially, the NHE activity upon shrinkage is larger in the terrestrial toad than in the semi-aquatic frog. These data, however, are too sparse to allow a realistic comparison of the RVI ability of terrestrial and semi-aquatic amphibians because, as shown here, the RVI responses depend on blood P_{O_2} and time frame.

The mechanism of RVI in *B. marinus* RBCs seems to be the same at low and high P_{O_2} as the response was significantly reduced by amiloride but insensitive to bumetanide under both conditions (Fig. 3C and Fig. 4C, respectively). At a concentration of 10^{-4} mol l^{-1}, amiloride did not completely block the RVI response, which was also found in shrunken *Rana temporaria* RBCs (Jørgensen, 1995). Based on the Na influx experiments (Fig. 1), it is likely that 10^{-4} mol l^{-1} EIPA would abolish the RVI completely, but at present we cannot rule out that other, Na-independent, mechanisms may be involved in the RVI response.

Many RVI studies are only carried out on cells suspended in defined, artificial media containing Na^+/K^+-ATPase inhibitors (Cala, 1977; Siebens and Kregenow, 1985; Romero et al., 1996; Weaver et al., 1999). Our study shows that the RVI response was not significantly different in whole blood and washed RBCs from *B. marinus* (Fig. 4). RVI did therefore not depend on washing and storage procedures or activity of the Na^+/K^+-ATPase. Cala also reported that RVI did not depend on the Na^+/K^+-ATPase (Cala, 1977), and Caldwell et al. showed that effects of β-adrenergic stimulation in trout RBCs taken *via* caudal puncture and then washed and stored for up to 96 h were not significantly different from those of whole blood taken *via* cannulae 48 h after surgery (Caldwell et al., 2006). Haemolysis was not detected in *B. marinus* whole blood during the course of the experiment, making it appear less fragile than the washed RBCs. However, the influence of, for instance, stress hormones can be avoided when replacing the plasma and incubating the cells overnight (Bourne and Cossins, 1982).

Effects of deoxygenation per se and extracellular acidification on Na influx

Volume changes due to the Haldane effect and associated shifts in the Donnan equilibrium are very fast (Hladky and Rink, 1977; Borgese et al., 1991) and can therefore not explain the slow volume increase in *B. marinus* RBCs caused by deoxygenation alone (Fig. 3A). The same treatment caused a significant increase in the basal Na influx (Fig. 1B,C), which

could explain the volume increase. The P_{50} for half-maximal deoxygenation-induced Na influx in the 15–30 min time interval is in the same range as the values for Hb and shrinkage-induced Na influx (see above) and, thus, support that Hb oxygenation could also modulate basal NHE activity. The mechanism of the increased deoxygenation-induced Na influx was not addressed specifically, but 10^{-4} mol l^{-1} EIPA lowered the shrinkage-induced Na influx in deoxygenated cells to basal values measured in oxygenated RBCs (Fig. 1B,C), so it likely involved the NHE. Weaver et al. showed that deoxygenation *per se* activates the NHE in European flounder RBCs (Weaver et al., 1999).

Elevation of P_{CO_2} caused swelling of the RBCs (Fig. 4B), but since extracellular acidification in washed RBCs did not influence the Na influx (see above), swelling is probably caused by a change in the Donnan equilibrium across the membrane and not a pH regulatory Na transport. In rainbow trout RBCs, extracellular acidification stimulates the NHE in the acidic pH range, whereas intracellular acidification stimulates the NHE in the alkaline pH range (Borgese et al., 1987). Intracellular acidification also stimulates the NHE in *Amphiuma tridactylum* and *R. temporaria* RBCs (Cala and Maldonado, 1994; Jørgensen, 1995). This regulation of intracellular pH may help maintain the oxygen affinity of haemoglobin. As at 1 kPa P_{CO_2}, bumetanide did not affect RVI at 3 kPa P_{CO_2}, indicating also that, under conditions typical for an active or burrowing animal, the NKCC did not contribute to volume regulation.

Oxygen dependence of isoproterenol-induced Na influx

Stimulation by the β-adrenergic agonist isoproterenol at 0 kPa P_{O_2} elicited a pronounced increase in the EIPA-sensitive and bumetanide-insensitive Na influx (Fig. 5A). At present, we do not know how widespread β-adrenergic NHE activation is within amphibians, but it was not found in deoxygenated *Xenopus laevis* RBCs (Berenbrink et al., 2005). Adrenergic stimulation increased cAMP and caused swelling of deoxygenated RBCs from *R. ridibunda*, but the mechanism(s) involved in the response were not characterized (Kaloyianni and Rasidaki, 1996). In preliminary experiments on *B. marinus* RBCs there was a slower, but significant, increase in the isoproterenol-stimulated Na influx at high P_{O_2} and there also appeared to be a prolonged lag phase in the Na influx in shrunken RBCs (data not shown). The reason for the variable lag time between experiments is currently not understood.

The magnitude of the β-adrenergic response of *B. marinus* RBCs is comparable to that measured in the initial 5 min after stimulation in some teleosts such as chub (36±6 mmol Na l^{-1} RBCs h^{-1}; N=3) and striped bass (40±8 mmol Na l^{-1} RBCs h^{-1}; N=4), but much higher values of more than 200 mmol Na l^{-1} RBCs h^{-1} have been measured in other teleost RBCs (Berenbrink et al., 2005). However, in teleosts, maximal activation of the βNHE is observed within 1–2 min followed by a rapid desensitization (Motais et al., 1992) (P.K. and M.B., personal observations), whereas in *B. marinus* the Na influx remained maximal for up to 30 min and was still elevated significantly above basal level for up to 45 min (Fig. 5A).

Tufts et al. found elevated plasma catecholamine levels after forced activity in *B. marinus* (Tufts et al., 1987a), and it is tempting to speculate that, as in teleosts (Nikinmaa, 1992), the physiological role of βNHE activation in deoxygenated RBCs is to safeguard intracellular pH and oxygen binding under conditions of general acidosis. Tufts et al., however, found no evidence of *in vivo* βNHE activation as cell volume remained unaltered after 30 min of vigorous exercise (Tufts et al., 1987a).

In rainbow trout RBCs, the βNHE is partially inhibited at high P_{O_2} values (Motais et al., 1987; Nikinmaa et al., 2003). We did not find βNHE activation at 20.5 kPa P_{O_2} for up to 45 min in *B. marinus* RBCs (Fig. 5B). Tufts et al. were similarly unable to show *in vitro* β-adrenergic changes in intracellular pH and water content of air-equilibrated *B. marinus* RBCs kept under 5% CO_2 (extracellular pH of ~7.5) (Tufts et al., 1987a) or of air-equilibrated *A. tridactylum* RBCs kept under 4 or 8% CO_2 (extracellular pH of ~7.65 or 7.44, respectively) (Tufts et al., 1987b). Air-equilibrated RBCs from *R. ridibunda* and *R. pipiens*, nevertheless, increase cAMP levels and swell when stimulated by isoproterenol, but these responses have only been demonstrated under non-physiological conditions in the presence of the phosphodiesterase inhibitor EDTA (Rudolph and Greengard, 1980; Kaloyianni and Rasidaki, 1996). In air-equilibrated RBCs, therefore, it seems that βNHE stimulation is lacking within amphibians.

In conclusion, this study demonstrates a pronounced P_{O_2} sensitivity of the shrinkage-induced RVI response and the underlying Na influxes mediated through the NHE in RBCs from the terrestrial anuran *Bufo marinus*. Deoxygenation reveals β-adrenergic activation of the NHE mediated Na influx, which has been missed in earlier studies on air-equilibrated amphibian RBCs. The regulation of the RBC βNHE seems to differ from that in teleosts, where rapid desensitization of the transporter may be the rule, whereas the activation was prolonged for up to 45 min in *B. marinus*. The RVI response in *B. marinus* RBCs may protect the HbO_2-affinity *in vivo* by reducing intracellular concentrations of Hb and organic phosphates and it may thus contribute to the high tolerance to elevated plasma osmolarity following dehydration in this species (Shoemaker, 1964; Shoemaker, 1965). A description of the relationship between RBC volume and HbO_2-affinity in amphibians with different tolerance to dehydration would help understand the role of RVI.

List of abbreviations and symbols

βNHE	β-adrenergically stimulated Na/H exchanger
cAMP	cyclic AMP
DIDS	4,4′-diisothiocyanatostilbene-2,2′-disulphonic acid
EDTA	ethylenediaminetetraacetic acid
Hb	haemoglobin

Hct	haematocrit
Hepes	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
KCC	K/Cl co-transporter
MCHC	mean cellular haemoglobin concentration
NHE	Na/H exchanger
NKCC	Na/K/2Cl co-transporter
P_{CO_2}	carbon dioxide partial pressure
P_{O_2}	oxygen partial pressure
RBC	red blood cell
RVI	regulatory volume increase
TCA	trichloroacetic acid

The authors would like to thank Dr Ole Bækgaard Nielsen for kindly allowing us to use his facilities at Aarhus University, Denmark, for radioisotope work.

References

Andersen, J. B. and Wang, T. (2002). Effects of anaesthesia on blood gases, acid-base status and ions in the toad, *Bufo marinus*. *Comp. Biochem. Physiol*. **131A**, 639-646.

Andersen, J. B. and Wang, T. (2003). Cardio-respiratory effects of forced activity and digestion in toads. *Physiol. Biochem. Zool*. **76**, 459-470.

Andersen, J. B., Jensen, F. B. and Wang, T. (2001). Effects of temperature and oxygen availability on circulating catecholamines in the toad *Bufo marinus*. *Comp. Biochem. Physiol*. **129A**, 473-486.

Berenbrink, M., Völkel, S., Heisler, N. and Nikinmaa, M. (2000). O_2-dependent K^+ fluxes in trout red blood cells: the nature of O_2 sensing revealed by the O_2 affinity, cooperativity and pH dependence of transport. *J. Physiol*. **526**, 69-80.

Berenbrink, M., Koldkjær, P., Kepp, O. and Cossins, A. R. (2005). Evolution of oxygen secretion in fishes and the emergence of a complex physiological system. *Science* **307**, 1752-1757.

Berenbrink, M., Völkel, S., Koldkjær, P., Heisler, N. and Nikinmaa, M. (2006). Two different oxygen sensors regulate oxygen-sensitive K^+ transport in carp red blood cells. *J. Physiol*. **575**, 37-48.

Bogdanova, A. Y. and Nikinmaa, M. (2001). Reactive oxygen species regulate oxygen-sensitive potassium flux in rainbow trout erythrocytes. *J. Gen. Physiol*. **117**, 181-190.

Borgese, F., Garcia-Romeu, F. and Motais, R. (1987). Ion movements and volume changes induced by catecholamines in erythrocytes of rainbow trout: effect of pH. *J. Physiol*. **382**, 145-157.

Borgese, F., Motais, R. and Garcia-Romeu, F. (1991). Regulation of Cl-dependent K transport by oxy-deoxyhemoglobin transitions in trout red cells. *Biochim. Biophys. Acta* **1066**, 252-256.

Bourne, P. K. and Cossins, A. R. (1982). On the instability of K^+ influx in erythrocytes of the rainbow trout, *Salmo gairdneri*, and the role of catecholamine hormones in maintaining *in vivo* influx activity. *J. Exp. Biol.* **101**, 93-104.

Boutilier, R. G., Randall, D. J., Shelton, G. and Toews, D. P. (1979). Acid–base relationships in the blood of the toad, *Bufo marinus*. *J. Exp. Biol.* **82**, 357-365.

Brauner, C. J., Wang, T. and Jensen, F. B. (2002). Influence of hyperosmotic shrinkage and β-adrenergic stimulation on red blood cell volume and oxygen binding properties in rainbow trout and carp. *J. Comp. Physiol. B* **172**, 251-262.

Cala, P. M. (1977). Volume regulation by flounder red blood cells in anisotonic media. *J. Gen. Physiol*. **69**, 537-552.

Cala, P. M. (1980). Volume regulation by *Amphiuma* red blood cells. *J. Gen. Physiol*. **76**, 683-708.

Cala, P. M. and Maldonado, H. M. (1994). pH regulatory Na/H exchange by *Amphiuma* red-blood-cells. *J. Gen. Physiol*. **103**, 1035-1053.

Caldwell, S., Rummer, J. L. and Brauner, C. J. (2006). Blood sampling techniques and storage duration: effects of the presence and magnitude of the red blood cell β-adrenergic response in rainbow trout (*Oncorhynchus mykiss*). *Comp. Biochem. Physiol*. **144A**, 188-195.

Cossins, A. R. and Gibson, J. S. (1997). Volume-sensitive transport systems and volume homeostasis in vertebrate red blood cells. *J. Exp. Biol.* **200**, 343-352.

Drew, C., Ball, V., Robinson, H., Ellory, J. C. and Gibson, J. S. (2004). Oxygen sensitivity of red cell membrane transporters revisited. *Bioelectrochemistry* **62**, 153-158.

Flatman, P. W. (2005). Activation of ferret erythrocyte Na^+-K^+-$2Cl^-$ cotransport by deoxygenation. *J. Physiol*. **563**, 421-431.

Gibson, J. S., Speake, P. F. and Ellory, J. C. (1998). Differential oxygen sensitivity of the K^+-Cl^- cotransporter in normal and sickle human red blood cells. *J. Physiol*. **551**, 225-234.

Gibson, J. S., Cossins, A. R. and Ellory, J. C. (2000). Oxygen-sensitive membrane transporters in vertebrate red cells. *J. Exp. Biol.* **203**, 1395-1407.

Gusev, G. P. and Ivanova, T. I. (2003). Activation of Na^+/H^+ exchange by protein phosphatase inhibitors in red blood cells of the frog *Rana ridibunda*. *J. Comp. Physiol. B* **173**, 429-435.

Gusev, G. P., Lapin, A. V. and Agulakova, N. I. (1997). Volume regulation in red blood cells of the frog *Rana temporaria* after osmotic shrinkage and swelling. *Membr. Cell Biol*. **11**, 305-317.

Hladky, S. B. and Rink, T. J. (1977). pH equilibrium across red cell membrane. In *Membrane Transport in Red Cells* (ed. J. C. Ellory and V. L. Lew), pp. 115-135. London: Academic Press.

Hyndman, C. A., Kieffer, J. D. and Benfey, T. J. (2003). Physiology and survival of triploid brook trout following exhaustive exercise in warm water. *Aquaculture* **221**, 629-643.

Jensen, F. B. (1995). Regulatory volume decrease in carp red blood cells: mechanisms and oxygenation-dependency of volume-activated potassium and amino acid transport. *J. Exp. Biol.* **198**, 155-165.

Jensen, F. B., Lecklin, T., Busk, M., Bury, N. R., Wilson, R. W., Wood, C. M. and Grosell, M. (2002). Physiological impact of salinity increase at organism and red blood cell levels in the European flounder (*Platichthys flesus*). *J. Exp. Mar. Biol. Ecol*. **274**, 159-174.

Johansen, K. and Ditadi, A. S. F. (1966). Double circulation in the giant toad, *Bufo paracnemis*. *Physiol. Zool*. **39**, 140-150.

Jørgensen, N. C. (1995). Amiloride-sensitive Na^+/H^+ exchange stimulated by cellular acidification of shrinkage in red blood cells of the frog, *Rana temporaria*. *J. Comp. Physiol. B* **165**, 450-457.

Kaloyianni, M. and Rasidaki, A. (1996). Adrenergic responses of *R. ridibunda* red cells. *J. Exp. Zool*. **276**, 175-185.

Kaloyianni, M., Giannisis, G., Gavriil, P. and Boukla, A. (1997). Metabolic effects and cellular volume responses induced by noradrenaline in nucleated erythrocytes. *J. Exp. Zool*. **279**, 337-346.

Kregenow, F. M. (1981). Osmoregulatory salt transporting mechanisms: control of cell volume in anisotonic media. *Annu. Rev. Physiol*. **43**, 493-505.

Madsen, S. S., Larsen, B. K. and Jensen, F. B. (1996). Effects of freshwater to seawater trasnfer on osmoregulation, acid-base balance and respiration in river migrating whitefish (*Coregonus lavaretus*). *J. Comp. Physiol. B* **166**, 101-109.

Maxime, V., Pennec, J. P. and Peyraud, C. (1991). Effects of direct transfer from freshwater to seawater on respiratory and circulatory variables and acid-base status in rainbow trout. *J. Comp. Physiol. B* **161**, 557-568.

McKenna, M. J., Heigenhauser, J. F., McKelvie, R. S., MacDougall, J. D. and Jones, N. L. (1997). Sprint training enhances ionic regulation during intense exercise in men. *J. Physiol*. **501**, 687-702.

Motais, R., Garcia-Romeu, F. and Borgese, F. (1987). The control of Na^+/H^+ exchange by molecular oxygen in trout erythrocytes. *J. Gen. Physiol*. **90**, 197-207.

Motais, R., Borgese, F., Fievet, B. and Garcia-Romeu, F. (1992). Regulation of Na^+/H^+ exchange and pH in erythrocytes of fish. *Comp. Biochem. Physiol*. **102A**, 597-602.

Muzyamba, M. C., Cossins, A. R. and Gibson, J. S. (1999). Regulation of Na^+-K^+-$2Cl^-$ cotransport in turkey red cells: the role of oxygen tension and protein phosphorylation. *J. Physiol*. **512**, 421-429.

Nielsen, O. B. (1997). Effects of haemoglobin O_2 saturation on volume regulation in adrenergically stimulated red blood cells from the trout, *Oncorhynchus mykiss*. *J. Comp. Physiol. B* **167**, 159-168.

Nielsen, O. B., Lykkeboe, G. and Cossins, A. R. (1992). Oxygenation-activated K fluxes in trout red blood cells. *Am. J. Physiol*. **263**, C1057-C1064.

Nikinmaa, M. (1992). Membrane transport and control of hemoglobin-oxygen affinity in nucleated erythrocytes. *Physiol. Rev*. **72**, 301-321.

Nikinmaa, M., Bogdanova, A. and Lecklin, T. (2003). Oxygen dependency of the adrenergic Na/H exchange in rainbow trout erythrocytes is diminished by a hydroxyl radical scavenger. *Acta Physiol. Scand*. **178**, 149-154.

Orlov, S. N., Kuznetsov, S. R., Kolosova, I. A. and Makarov, V. L. (1994). Na^+/H^+ and Na^+/Na^+ counter transport in human, rabbit, and rat erythrocytes: evidence for two independent ion transport systems. *Biochemistry* **59**, 467-472.

Palfrey, H. C. and Greengard, P. (1981). Hormone-sensitive ion transport systems in erythrocytes as models for epithelial ion pathways. *Ann. N. Y. Acad. Sci*. **372**, 291-308.

Palfrey, H. C., Feit, P. W. and Greengard, P. (1981). Specific inhibition by 'loop' diuretics of an anion-dependent Na^++K^+ cotransport system in avian erythrocytes. *Ann. N. Y. Acad. Sci*. **341**, 134-138.

Parker, J. C., Colclasure, G. C. and McManus, T. J. (1991). Coordinated regulation of shrinkage-induced Na/H exchange and swelling-induced [K-Cl] cotransport in dog red cells. *J. Gen. Physiol*. **98**, 869-880.

Pedersen, S. F. and Cala, P. M. (2004). Comparative biology of the ubiquitous Na^+/H^+ exchanger, NHE1: lessons from erythrocytes. *J. Exp. Zool*. *A* **301**, 569-578.

Peterson, C. C. and Greenshields, D. (2001). Negative test for cloacal drinking in a semi-aquatic turtle (*Trachemys scripta*), with comments on the functions of cloacal bursae. *J. Exp. Zool*. **290**, 247-254.

Romero, M. G., Guizouarn, H., Pellissier, B., Garcia-Romeu, F. and Motais, R. (1996). The erythrocyte Na^+/H^+ exchanger in eel (*Anguilla anguilla*) and rainbow trout (*Oncorhyncus mykiss*): a comparative study. *J. Exp. Biol.* **136**, 405-416.

Rudolph, S. A. and Greengard, P. (1980). Effects of catecholamines and prostaglandin E_1 on cyclic AMP, cation fluxes, and protein phosphorylation in the frog erythrocyte. *J. Biol. Chem*. **255**, 8534-8540.

Salama, A. and Nikinmaa, M. (1988). The adrenergic responses of carp (*Cyprinus carpio*) red cells: effects of P_{O_2} and pH. *J. Exp. Biol.* **136**, 405-416.

Shoemaker, V. H. (1964). The effects of dehydration on electrolyte concentrations in a toad, *Bufo Marinus*. *Comp. Biochem. Physiol*. **13**, 261-271.

Shoemaker, V. H. (1965). The stimulus for the water-balance response to dehydration in toads. *Comp. Biochem. Physiol.* **15**, 81-88.

Siebens, A. W. and Kregenow, F. M. (1985). Volume-regulatory responses of *Amphiuma* red cells in anisotonic media. *J. Gen. Physiol*. **86**, 527-564.

Thorson, T. B. (1955). The relationship of water economy to terrestrialism in amphibians. *Ecology* **36**, 100-116.

Tufts, B. L., Mense, D. C. and Randall, D. J. (1987a). The effects of forced activity on circulating catecholamines and pH and water content of erythrocytes in the toad. *J. Exp. Biol.* **128**, 411-418.

Tufts, B. L., Nikinmaa, M., Steffensen, J. F. and Randall, D. J. (1987b). Ion exchange mechanisms on the erythrocyte membrane of the aquatic salamander, *Amphiuma tridactylum*. *J. Exp. Biol.* **133**, 329-338.

Virkki, L. V., Salama, A. and Nikinmaa, M. (1998). Regulation of ion transport across lamprey (*Lampetra fluviatilis*) erythrocyte membrane by oxygen. *J. Exp. Biol.* **201**, 1927-1937.

Wang, T., Abe, A. S. and Glass, M. L. (1998). Temperature effects on lung and blood gases in *Bufo paracnemis*: consequences of bimodal gas exchange. *Respir. Physiol*. **113**, 231-238.

Weaver, Y. R., Kiessling, K. and Cossins, A. R. (1999). Responses of the Na^+/H^+ exchanger of European flounder red blood cells to hypertonic, β-adrenergic and acidotic stimuli. *J. Exp. Biol.* **202**, 21-32.

Zar, J. H. (1984). Data transformations. In *Biostatistical Analysis*, pp. 236-243. Upper Saddle River, NJ: Prentice-Hall.