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INTRACELLULAR pH IN THE TOAD *BUFO MARINUS* FOLLOWING HYPERCAPNIA

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Summary

We investigated the effects of hypercapnia on intracellular acid–base regulation in brain and liver of the toad *Bufo marinus* L. After 1 h at 5 % CO_2, arterial P_{CO_2} increased significantly, from 1.6±0.04 to 5.7±0.23 kPa, while brain and liver intracellular pH (pHi) decreased significantly. Reductions in pHi of both tissues were partially compensated by increased levels of bicarbonate. Surprisingly, however, compensation was lower than expected in brain and higher than expected in liver. We suggest that compensation in brain may be limited by secondary effects of bicarbonate loading in this tissue.

Introduction

During hypercapnia, amphibians and reptiles regulate intracellular and extracellular pH relatively poorly compared to aquatic species (Boutilier *et al.* 1979; Toews and Heisler, 1982; Boutilier and Heisler, 1988). Furthermore, it has been suggested that pH regulation in these vertebrates may be limited by high resting levels of bicarbonate (Boutilier and Heisler, 1988), dictated by the physical characteristics of gas exchange in air. Thus, the transition from water to land, with an attendant increase in arterial P_{CO_2}, may have placed constraints on the value of bicarbonate as a buffer: this raises questions about how lower terrestrial vertebrates regulate pH. Unfortunately, little information is available to enable us to formulate general patterns. For example, almost nothing is known about non-bicarbonate buffers in lower vertebrates. Information about pHi in some tissues, e.g. brain, is limited to aquatic vertebrates (Wood *et al.* 1990).

The present study examines pHi in brain and liver during short-term hypercapnia. In addition, we measured *in vitro* non-bicarbonate buffer curves to provide a better picture of the degree to which *in vivo* changes may represent compensation.

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Bufo marinus was selected for this initial study because of the wealth of information about acid–base regulation in this species (Boutilier *et al.* 1979; Toews and Heisler, 1982; Boutilier and Heisler, 1988).

Materials and methods

Bufo marinus L., average body mass 210.9±12.5 g (±S.E. range 167–306 g, N=40), were obtained from Blue Spruce Biological Supply (Castle Rock, CO). Toads were housed in acrylic aquaria (2 m×0.35 m×0.3 m) containing 20 % Holtfreter solution, prepared with deionized water. One end of each container was elevated to provide toads with wet and dry areas. Containers were washed daily with chlorinated tap water followed by deionized water. Toads were fed crickets daily. Temperature of air and water averaged 25±1.0 °C.

Randomly selected individuals were anesthetized with a 0.75 % aminobenzoic acid ethyl ester (MS-222) solution, pH adjusted to 7.7 with $NaHCO_3$. The right femoral artery was then cannulated (PE-50, Clay Adams, Parsippany NJ). Catheters were filled with heparinized amphibian Ringer's solution, and secured with nylon sutures and tissue glue (Nexaband, CRX Medical, Raleigh NC).

After a minimum of 24 h recovery, toads were placed in a 3.5 l plastic chamber containing 200 ml of a 0.3 mmol l^{-1} sodium bicarbonate solution. Gas lines and cannulae were passed through ports in the top of the chamber. The chamber was covered with opaque cloth to eliminate visual contact. In this way, blood samples were withdrawn from unanesthetized and undisturbed animals. Water and air temperatures averaged 25±0.5 °C.

Intracellular pH was determined using the transmembrane distribution of [^{14}C]DMO (5,5-dimethyloxazolidine-2,4-dione-2-[^{14}C]; New England Nuclear) with [^{3}H]inulin (Amersham) to mark extracellular space (Waddell and Butler, 1959; Nestler, 1990). Inulin purity was 97 % as checked with thin layer chromatography [solvent n-butanol : acetone : 95 % ethanol : H_2O (5:5:2:1)]. Although DMO yields an average pHi, the values obtained with this method are similar to those measured with microelectrodes (Roos and Boron 1981). In addition, we (G. K. Snyder and J. I. Shapiro, unpublished observations) have recently determined pHi of limb skeletal muscle and brain using ^{31}P-magnetic resonance spectroscopy with the techniques of Shapiro *et al.* (1989) and found that the values for both tissues were within ±0.02 pH units of, and not significantly different from, average values obtained with [^{14}C]DMO. An equilibration time of 12 h for the radiotracers was established in a preliminary study.

Toads were injected with 300 μl of a solution containing 15 μCi of DMO, 45 μCi of inulin, 6.9 μmol of 'cold' DMO and 1.25 μmol of 'cold' inulin into the femoral artery 12 h after they had been placed in the chamber. The chambers were flushed with room air (20.95 % O_2: 0.04 % CO_2) at 500 ml min^{-1} for 11 h. Air inflow for the 'hypercapnic' animals (N=9) was then switched to 20.95 % O_2: 5 % CO_2 and perfusion continued for an additional hour. Air inflow for 'control' animals (N=7) was continued as room air for the final hour.

After gas perfusion, blood (150–300 μl) was drawn from the cannula to determine pH and P_{CO_2} (Radiometer BMS3 Mk2 Blood Micro System, at 25 °C). An additional 150 μl sample was centrifuged, blood hematocrit recorded, and the plasma used for radioactive counts. Animals were then injected with pentobarbitol–KCl and immediately double pithed to prevent struggling. Heart, liver and skeletal muscle (left thigh muscles) were removed within 30–60 s. Removal of the brain required another 1–2 min. During this time, radiotracer washout was prevented by stopping the circulation prior to tissue sampling. All tissues were blotted, weighed and lyophilized to constant mass (24–36 h). Perchloric acid extracts were prepared for liquid scintillation counting.

Tissue pHi was calculated using the equations of Wood and Cameron (1985). Liver accumulated inulin and, thus, artificially high (50–80 %) extracellular water (ECW) values were obtained. Therefore, an ECW value of 15 % was used for liver (Nestler, 1990). An opposite trend, low ECW (2–6 %), was noted in brain, caused by the low permeability of the blood–brain barrier (Zubrod and Rall, 1959). Since values for toad brain ECW are not available, we measured ECW of brains from eight normocapnic toads using the methods of Bradbury *et al.* (1968). Brains were removed, the meninges teased away to allow free distribution of inulin, and the brain incubated for 1 h in 5 ml of a pH 7.25 solution containing 2.0 μCi of [^{3}H]inulin. The incubation solution consisted of 90 mmol l^{-1} NaCl, 25 mmol l^{-1} $NaHCO_3$, 2.0 mmol l^{-1} KCl, 3.0 mmol l^{-1} KH_2PO_4, 0.9 mmol l^{-1} $CaCl_2$, 1.2 mmol l^{-1} $MgCl_2$, 1.8 mmol l^{-1} Na_2SO_4 and 13.8 mmol l^{-1} glucose. During incubation, the solution was bubbled with 95 % O_2: 5 % CO_2.

Bicarbonate concentrations were calculated with the Henderson–Hasselbalch equation. Values used for pK′ (6.05) and CO_2 solubility (0.248 mmol kPa^{-1}) were those reported for *Bufo marinus* at 25 °C (Boutilier *et al.* 1979).

Non-bicarbonate buffering capacity was determined using the methods of Castellini and Somero (1981). Toads (N=7) were cannulated and housed as described above for the control animals. After air-perfusion, a blood sample was taken and the animal was killed. Tissues were rapidly (30–60 s) removed, frozen in liquid nitrogen and stored at −85 °C. Frozen tissue samples were homogenized 1:20 (w/v) in 0.6 % NaCl and titrated at 25 °C with 0.2 or 0.02 mol l^{-1} NaOH, between pH 6 and 7. Changes in pH, monitored with a Radiometer PHM 64 pH meter and GK 2401C combination electrode calibrated with precision buffers, were used to calculate intracellular non-bicarbonate buffer capacity with the equations of Heisler and Piiper (1971).

Statistical analyses were completed using SPSS-X. Differences in values between control and hypercapnic animals were examined using two-tailed t-tests and analysis of covariance (ANCOVA) with a significance level of 0.05. All data are expressed as mean±S.E.

Results

Extracellular water volumes were statistically similar for all tissues. However,

Table 1. *Total tissue water (TTW) and extracellular water (ECW) of tissues from control (exposed to 20.95% O_2, 0.04% CO_2, remainder N_2 for 1 h) and hypercapnic (exposed to 20.95% O_2, 5.0% CO_2, remainder N_2 for 1 h)* Bufo marinus

	Control (N=7)	Hypercapnic (N=9)
Brain		
TTW	82.9±0.8	82.0±0.7
ECW	22.6±3.1	–
Heart		
TTW	80.6±0.8	80.5±0.1
ECW	19.4±1.4	17.3±1.1
Muscle		
TTW	79.6±0.7	80.8±0.5
ECW	20.6±2.9	20.7±1.5
Liver		
TTW	69.2±1.1	73.2±1.3

ECW of brain was determined using a separate group of eight toads (see Materials and methods).

Control and hypercapnic values are not significantly different (P>0.05, ANCOVA) for any variable.

Data are means±S.E.

TTW and ECW are in units of ml H_2O 100 g^{-1} wet tissue.

liver total water content was significantly lower than that of other tissues. One hour of hypercapnia did not significantly alter total tissue water content or extracellular water volume for any tissues (Table 1).

After 1 h at 5% CO_2, blood P_{CO_2} of *Bufo marinus* increased from 1.6±0.04 to 5.7±0.23 kPa. Although the difference in P_{CO_2} is highly significant, we found no evidence of a CO_2-stimulated hyperventilation, since the difference in P_{CO_2} between arterial blood and inspired gas was roughly the same (1.5 kPa) for normocapnic and hypercapnic toads (e.g. at Boulder elevation P_{CO_2}= 84 kPa×0.05=4.2 kPa). After 1 h of hypercapnia, plasma bicarbonate had increased significantly, from 23.9±1.0 to 27.0±1.8 mequiv l^{-1}, while arterial pH had decreased significantly, from 7.85±0.02 to 7.33±0.01. During this same time, brain pHi decreased from 7.16±0.02 to 6.72±0.05, similar to the decrease in pHi of skeletal muscle and heart (Fig. 1). Control pHi of liver, 7.44±0.14, in contrast, was significantly higher than in other tissues, and decreased the least following hypercapnia, to 7.25±0.05 (Fig. 1).

Tissue bicarbonate concentrations increased significantly following hypercapnia, to a greater extent than would be predicted from passive buffering, indicating compensation for the acidosis induced by hypercapnia. The increase in bicarbonate was greatest in liver (+14.4 mequiv l^{-1}) and least in brain (+2.0 mequiv l^{-1}).

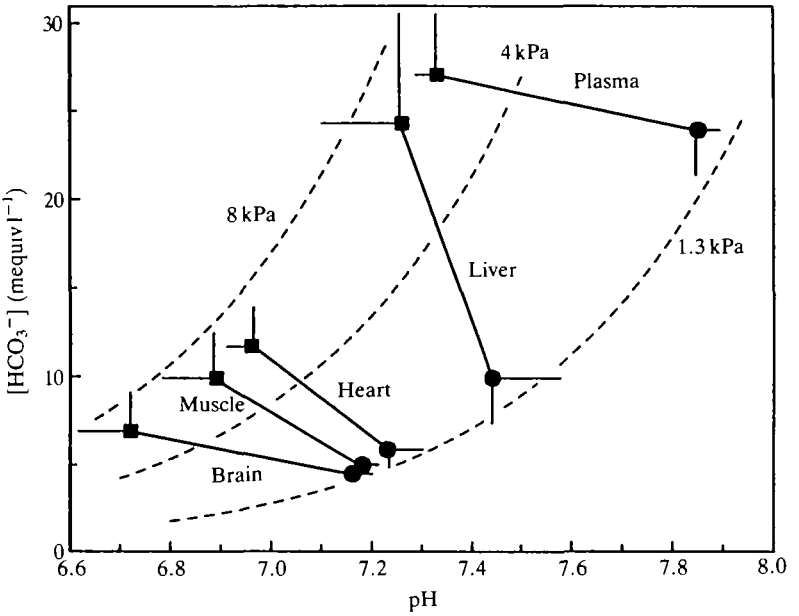

Fig. 1. pH–bicarbonate diagram for plasma, liver, heart, skeletal muscle and brain in *Bufo marinus*. Circles represent data from animals exposed to 20.95 % O_2, 0.04 % CO_2, balance N_2 (N=7). Squares represent data from animals exposed to 5 % CO_2, 20.95 % O_2, balance N_2 (N=9). Slopes of lines connecting points are apparent buffer values (see Table 2). Dashed curves are P_{CO_2} isopleths at 1.33, 4 and 8 kPa. Values are means±S.E.

When expressed as the difference between the observed pHi and the expected pHi at constant bicarbonate concentrations (Toews and Heisler, 1982), compensation in liver, 66 %, is close to values for heart and skeletal muscle, while compensation in brain, 35 %, is only slightly greater than in plasma, 25 %.

Changes in pHi were uniformly less than changes in extracellular pH (pHe). In addition, pHi appeared to be best protected in the liver (pHi/pHe=0.37) and least protected in brain (relative change=0.83). Consequently, the *in vivo* non-bicarbonate buffer values were lowest in brain and highest in liver (Table 2). In addition, the *in vivo* non-bicarbonate buffer value for brain was significantly lower than the non-bicarbonate buffer curve measured *in vitro* (Table 2), indicating a net loss of base equivalents or a net gain of acid equivalents during hypercapnia. For liver, however, the apparent *in vivo* buffer value was significantly higher than the value measured *in vitro*, indicating a net gain of base equivalents or a net loss of acid equivalents in this tissue (Table 2).

Blood hematocrit after 1 h at 5 % CO_2 increased significantly, from 23.9±1.5 % (N=7) in control toads to 31.6±1.2 % (N=9) in hypercapnic toads. Thus, although no corrections for plasma trapping were made, our results indicate that blood hematocrit may increase by as much as 32 % during hypercapnia.

Table 2. *Non-bicarbonate buffer values determined by titrating tissue homogenates* in vitro *(β) and apparent* in vivo *non-bicarbonate buffer values (β') calculated from normocapnic and hypercapnic (5 % CO_2) tissues in* Bufo marinus

Tissue	β (N=8)	β' (N=7)
Plasma	21.4±1.3	6.0±1.6
Brain	38.6±1.7	4.6±0.7
Heart	39.9±2.3	21.6±1.1
Liver	38.4±0.7	79.9±1.3
Muscle	50.9±2.5	19.2±1.3

All values are in units of mequiv l^{-1} cell H_2O pH $unit^{-1}$.
In all cases β and β' are significantly different (P<0.01, ANCOVA).
Data are means±S.E.

Discussion

Arterial P_{CO_2} and acid–base balance during hypercapnia

Exposing *Bufo marinus* to elevated ambient P_{CO_2} produces an immediate increase in lung ventilation followed by adaptation of the CO_2 ventilatory response (Boutilier *et al.* 1979). We did not observe a change in the difference between blood and ambient P_{CO_2}. Thus, in our toads, hyperventilation was not a major factor following 1 h of acclimation; that is, adaptation of CO_2 sensitivity had occurred by the time blood samples were taken.

Control values of arterial pH, plasma bicarbonate and plasma buffers are all similar to those reported previously (Boutilier *et al.* 1979). In addition, control and hypercapnic pHi in skeletal muscle and heart are in the range of values reported for *Bufo marinus* (Toews and Heisler, 1982), other anuran amphibians (Malan *et al.* 1976) and fish (Wood *et al.* 1990). Brain and liver pHi values in amphibians have not been reported previously. However, our data for these tissues are consistent with pHi of the same tissues in mammals (Nestler, 1990).

Compensation of pHi during hypercapnia

We have examined compensation of hypercapnia by comparing *in vivo* buffer curves to those determined *in vitro*. Previous studies have shown that *in vitro* methods yield buffer values consistent with tissue protein concentrations (Zubrod and Rall, 1959; Wood *et al.* 1990) and similar to buffer values obtained using microelectrode techniques (Aickin and Thomas, 1977). Thus, although we cannot exclude the possibility that titration may overestimate buffer values in some tissues, it is likely that the lower *in vivo* than *in vitro* non-bicarbonate buffer values in all tissues except liver (Table 2) indicate a net accumulation of acid equivalents or a net loss of base equivalents during hypercapnia.

Acid–base balance in brain following hypercapnia

The relatively poor buffering of brain (Fig. 1, Table 2) is surprising, but similar

to findings in fish (Wood *et al.* 1990) and mammals (Nestler, 1990). In contrast, the fact that brain pHi decreased to a greater extent than did pHi of other tissues is not consistent with findings from other vertebrates. In the skate, compensation of brain pHi was more rapid and more precise than in other tissues, in spite of the lower tissue buffer capacity (Wood *et al.* 1990).

The relatively poor buffering of brain cannot be attributed entirely to a lack of non-bicarbonate buffer capacity since the *in vitro* buffer value of brain is similar to those of heart and liver (Table 2). However, it is possible that secondary effects of elevated intracellular bicarbonate limit compensation in brain. Increased intracellular bicarbonate is followed by increased intracellular water. In the skate, this fluid shift appears to occur during rapid (<1 h) compensation, and persists for the first 24 h of hypercapnia (Wood *et al.* 1990). Further, intracellular fluid volume is eventually re-established by a reduction of $[Cl^-]_i$ (Nishimura *et al.* 1989), which may interfere with electrophysiological mechanisms (Yoshizaki *et al.* 1989). Thus, it is possible that potential disruptions of normal brain function following loss of electrolytes may outweigh the benefit of rapid pH compensation.

Acid–base balance in liver following hypercapnia

The responses of liver to hypercapnia have not been studied previously, and the results presented here do not permit conclusions about the need for compensation specific to the liver. However, the presence of high concentrations of carbonic anhydrase in liver and its importance to normal hepatic tissue function are well established (Dodgson and Forster, 1986; Walsh and Milligan, 1989) and consistent with the relatively rapid accumulation of bicarbonate in this tissue. Furthermore, the toad bladder excretes H^+ and NH_4^+ during hypercapnia (Tufts and Toews, 1986), which may be an important sink for the excess protons formed from bicarbonate production in liver.

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