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THE EFFECTS OF HYPERCAPNIA ON INTRACELLULAR AND EXTRACELLULAR ACID-BASE STATUS IN THE TOAD *BUFO MARINUS*

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SUMMARY

Toads (*Bufo marinus*) were exposed to environmental hypercapnia of 5% CO_2 in air, and extracellular and intracellular acid-base parameters were determined 1 and 24 h after the onset of hypercapnia.

The initial drop in pH was compensated by the elevation of extracellular and intracellular bicarbonate. Relating the pH compensation to the pH drop that is expected to occur by increased P_{CO_2} at constant bicarbonate concentration, the pH compensation in the extracellular space was 30% and reached the following values for intracellular body compartments: 65% in skeletal muscle, 77% in heart muscle and 44% in skin. The additional bicarbonate was partly produced by blood and intracellular non-bicarbonate buffers; the major portion of the remainder was related to the excretion of ammonia into the environmental water.

The hypercapnia-induced changes of pH were considerably smaller in all tissue cells than in the extracellular space. Thus *Bufo marinus* exhibits the relative preference of intracellular over extracellular acid-base regulation that has been observed in other vertebrates.

INTRODUCTION

Amphibians are not transitional forms in the strictest evolutionary sense. Rather, they represent a large group of animals that have the ability to tolerate diverse habitats and successfully function in a variety of niches from the aquatic to the terrestrial. Respiratory, circulatory and acid-base regulatory systems respond to hypercapnia, hypoxia, desiccation and extensive exercise in a number of species with compensatory processes to minimize the particular stress (Boutilier *et al.* 1979*a*, *b*, *c*; Boutilier & Toews, 1977; Boutilier, McDonald & Toews, 1980; Toews & Macintyre, 1978; Macintyre & Toews, 1976).

In the toad *Bufo marinus*, environmental hypercapnia causes an initial lowering of extracellular pH (pH_e) which is followed by a compensatory phase where plasma [HCO_3^-] is increased by about 30% and pH recovers by about 17% of the expected

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change in pH at unchanged bicarbonate concentration (Boutilier *et al.* 1979*a*). This is only a very limited compensation in comparison with that observed in fishes where generally more than 80% of the initial pH shift is compensated (Janssen & Randall, 1975; Eddy *et al.* 1977; Heisler, Weitz & Weitz, 1976*a*). The amphibia *Amphiuma* and *Siren*, (Heisler *et al.* 1980) and the facultative air-breathing fish, *Synbranchus* (Heisler *et al.* 1978), however, compensate for hypercapnic disturbance of pH_e to an even smaller extent than does *Bufo*, although intracellular pH (pH_i) was very well protected at least in *Siren* and *Synbranchus* (Heisler *et al.* 1978, 1980; Heisler, 1980). These results and the observation that in dogfish the intracellular compensation of hypercapnic disturbances is even more complete than the 80% extracellular compensation (Heisler, 1980) raise the question of whether, in general, intracellular pH regulation has priority over extracellular pH adjustment.

Following an experimental format of hypercapnic exposure similar to that of Boutilier *et al.* (1979*a*), the present study was designed to test this possibility in *Bufo marinus*. Intracellular and extracellular pH of animals exposed to elevated inspired P_{CO_2} for 1 or 24 h respectively are compared with that of a control group.

MATERIALS AND METHODS

The experiments were conducted on 28 toads (*Bufo marinus*, weight 150–400 g) purchased from a commercial supplier (Rand McNally Corp., Oshkosh, Wisconsin) and transported to Germany by airfreight. The animals were kept for at least 5 days (up to 30 days) in large thermostatically controlled aquaria (25 ± 1 °C) with access to both fresh water and dry areas before experiments. Animals chosen randomly from the holding tanks were chronically cannulated in the right femoral artery (using techniques described previously by Boutilier *et al.* 1979*a*) for blood sampling and were allowed 24 h for recovery from anaesthesia.

Throughout the experiments the animals were kept in 10 l widemouthed plastic chambers containing 500 ml of tap water with 0·3 mM-Na^+, 0·02 mM-K^+ and about 0·3 mM-HCO_3^-. The chambers were kept in thermostatted boxes (25 ± 0·5 °C) and were flushed with a continuous current (650 ml/min) of air or CO_2 in air by a tube which extended to the bottom of the enclosure. Exiting gas mixtures and the polyethylene catheter were passed through additional holes in the lid.

For determination of the intracellular pH by use of the DMO-distribution technique (Waddell & Butler, 1959), [^{14}C]DMO (5·5-dimethyl-2·4-oxazolidinedione) and [^{3}H]inulin were injected into the femoral artery 2 h prior to the final phase of the experiments.

Three experimental series were conducted:

(A) *Controls*

Animals were kept for 24 h in the experimental chambers flushed with air.

(B) 1 *h – hypercapnia*

Animals were kept for 24 h in the chambers in air and then exposed to 5% CO_2 in air for 1 h.

(C) 24 *h – hypercapnia*

Animals remained for 24 h in air, and thereafter were exposed for 24 h to 5% CO_2 in air.

The three experimental protocols were rotated in succession, 1–2 experiments being performed each day in an attempt to eliminate any seasonal or holding factors as an experimental bias.

In all experimental series, three blood samples (250 μl each) for determination of pH_e, P_{a,CO_2} and P_{a,O_2} were taken during a period of 15–20 min immediately before time zero which was defined as the time of switching over from air to CO_2/air in the animal chambers. For series A a final sample was then taken, centrifuged and plasma removed before the experiment was terminated; the toads were then double pithed and tissue samples removed immediately for intracellular pH determination (controls). In series B and C the animals were exposed to 1 or 24 h of hypercapnia respectively, following time zero, then the blood acid base parameters were determined again and the final sampling procedure for plasma and tissue samples was conducted. After each experiment a water sample was taken from the experimental chamber for ammonia analysis.

For the determination of intracellular pH, all individual muscle bands associated with the left femur (10 samples), the left gastrocnemius muscle (2 samples), the pectoral muscles (5 samples), the ventricle (2 samples) and a large piece of abdominal skin (4 samples) were quickly removed, weighed, dried and prepared for liquid scintillation counting by oxidation as described earlier (Heisler *et al.* 1976*b*). Arterial blood pH, P_{CO_2} and P_{O_2} were determined with a set of thermostatted microelectrodes (Radiometer BMS 3) calibrated with precision phosphate buffers and appropriate calibration gases, which were provided by gas mixing pumps (Wösthoff, Bochum, FRG). Bicarbonate concentrations were calculated by application of the Henderson–Hasselbalch equation using values for pK' (6·05) and α_{CO_2} (0·033 mmol/mmHg.l) reported for *Bufo marinus* at 25 °C by Boutilier *et al.* (1979*a*). The ammonia concentration of the environmental water was measured potentiometrically using ammonia electrodes calibrated with ammonium-chloride standard solutions.

RESULTS

Mean arterial pH measured in the femoral artery of *Bufo marinus* fell from 7·82 to 7·40 after 1 h exposure to 5% CO_2 in the environmental air and only slightly recovered to 7·44 after 24 h exposure (Table 1, Fig. 1). Plasma bicarbonate rose from 21·4 to 26·3 (1 h) and 32·7 mM (24 h), arterial P_{CO_2} from 11 to 35 and 41 mmHg, over the same time course. All changes were found to be significant ($P < 0.05$). Arterial P_{O_2} increased significantly from a normocapnic value of 80 mmHg to 94 after 1 h in CO_2 but thereafter decreased to 78 mmHg (24 h), not significantly different from normocapnic levels (Table 1).

Initial statistical analysis showed that there were no significant differences in pH_i between gastrocnemius, femoral or pectoral muscle groups. These groups were therefore considered as one skeletal muscle type. The pH_i in these muscles fell from

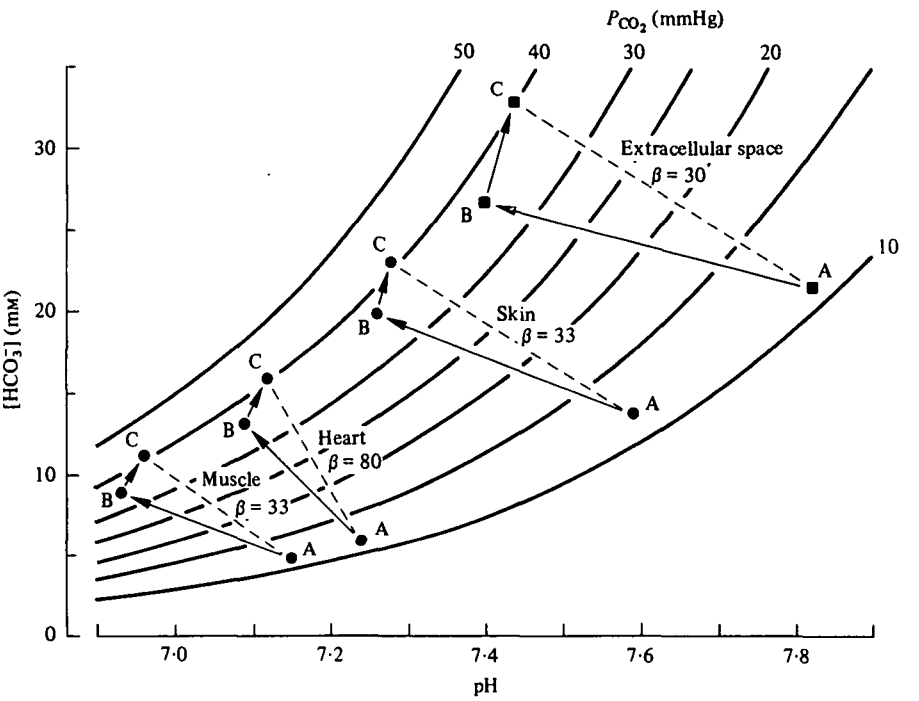

Fig. 1. Arterial and intracellular tissue pH, P_{CO_2} and bicarbonate of *Bufo marinus* in normocapnia (A) and after 1 h (B) or 24 h (C) of hypercapnia plotted on a pH/$[HCO_3^-]$ diagram of the Henderson–Hasselbalch equation. Intracellular apparent buffer values and buffer lines for 24 h hypercapnia (dashed) are given for each tissue as well as the apparent extracellular buffer value.

Table 1. *Arterial pH, P_{CO_2} and $[HCO_3^-]$, and intracellular pH and $[HCO_3^-]$ in skeletal muscle, heart muscle and skin of* Bufo marinus *in normocapnia and in 5% CO_2 for 1 h and 24 h (means ± S.E.)*

	Normocapnia	1 h hypercapnia	24 h hypercapnia
Arterial plasma			
pH_a	7·82 ± 0·03	7·40 ± 0·02	7·44 ± 0·01
P_{a,CO_2}	11·1 ± 0·09	35·4 ± 0·3	40·6 ± 1·1
$[HCO_3^-]_{Pl}$	21·4 ± 1·1	26·3 ± 1·2	32·7 ± 0·6
P_{a,O_2}	80 ± 2·8	94 ± 3·4	78 ± 4·5
Skeletal muscle			
pH_i	7·15 ± 0·01	6·93 ± 0·01	6·96 ± 0·01
$[HCO_3^-]_i$	4·8 ± 0·1	9·1 ± 0·1	11·1 ± 0·2
Heart muscle			
pH_i	7·24 ± 0·02	7·09 ± 0·02	7·12 ± 0·02
$[HCO_3^-]_i$	5·8 ± 0·3	13·1 ± 0·7	15·5 ± 0·6
Skin			
pH_i	7·59 ± 0·02	7·26 ± 0·02	7·28 ± 0·02
$[HCO_3^-]_i$	13·7 ± 1·1	19·7 ± 1·0	23·1 ± 1·0

Table 2. *Fractional water content (F_{H_2O}) and the fractional extracellular volume (Q_e) for skeletal and heart muscle, and skin (mean ± S.E., n = number of determinations)*

Series	F_{H_2O}	Q_e	n
	Skeletal muscle		
Control	0·805 ± 0·003	0·179 ± 0·004	152
1 h hypercapnia	0·803 ± 0·001	0·168 ± 0·003	134
24 h hypercapnia	0·805 ± 0·003	0·177 ± 0·003	152
	Heart muscle		
Control	0·828 ± 0·004	0·272 ± 0·014	17
1 h hypercapnia	0·811 ± 0·012	0·281 ± 0·010	16
24 h hypercapnia	0·821 ± 0·007	0·315 ± 0·023	16
	Skin		
Control	0·723 ± 0·004	0·586 ± 0·009	35
1 h hypercapnia	0·721 ± 0·004	0·604 ± 0·009	31
24 h hypercapnia	0·732 ± 0·007	0·625 ± 0·009	36

7·15 to 6·93 after 1 h CO_2 exposure and slightly recovered to 6·96 after 24 h. This compensatory increase in pH_i with increasing hypercapnic exposure time was significant and was caused by an additional increase in intracellular bicarbonate concentration between 1 h and 24 h of CO_2 exposure (Fig. 1).

Over the same time-course, heart muscle pH_i changed only from 7·24 to 7·09 (1 h) and then to 7·12 (24 h). Bicarbonate in heart muscle increased during CO_2 exposure to a much higher extent (2·7 fold) than in skeletal muscle (2·3 fold) and skin (1·7 fold) (Table 1). Consequently the change of pH_i was larger in skin (−0·31) than in skeletal muscle (−0·19) and least in heart muscle (−0·12).

The ammonia concentration in the ambient water (500 ml) after the 24 h control period was 1·07 ± 0·33 mM (mean ± S.E.), rose to 1·25 ± 0·31 mM after 24 h at room air and subsequent 1 h CO_2-exposure, and was 3·95 ± 0·44 mM after 24 h at room air and subsequent 24 h at 5 % CO_2 in air. The amount of ammonia excreted in addition to the control release is then about 0·3 mmol/l body-water content for the first hour and about 4·1 mmol/l body water content for 24 h of hypercapnic exposure (for an average animal weight of 275 g and a fractional water content of 0·8).

The values for tissue fractional water content (F_{H_2O}) and fractional extracellular volume (Q_e), obtained by the inulin distribution technique are presented in Table 2. There was no significant effect of hypercapnia on either measurement.

DISCUSSION

The elevation of P_{a,CO_2} in *Bufo* by increasing inspired P_{CO_2} is a relatively slow process as illustrated by the arterial P_{CO_2}, which is still slightly below the inspired value after 1 h of CO_2 exposure. Rough calculation on the basis of observed [HCO_3^-] changes in the extracellular space and tissues yields an increase of about 4·5 mmol/kg animal in the total CO_2 pool (after 1 h of CO_2); only half of this can be attributed to CO_2 production by metabolism. The rest must be attributed to net CO_2 uptake during the first hour: therefore, our present results suggest that steady state conditions for P_{CO_2} in *Bufo* cannot be expected before about 1·5–2 h after environmental P_{CO_2}

elevation. This lengthy disequilibrium exists even though the elevation of P_{a,O_2} during the initial hypercapnic period would indicate a period of hyperventilation by the animal at least for the first hour.

The patterns of extracellular pH and $[HCO_3^-]$ changes are, in general, similar to those observed by Boutilier *et al.* (1979*a*).

Differences in the slope of the apparent extracellular buffer line, determined as the changes in $[HCO_3^-]$ and pH between control conditions and after 1 h of hypercapnia ($-\Delta HCO_3^-/\Delta pH$ = 14 mequiv/pH.l in the present study, 6·2 mequiv/pH.l in Boutilier *et al.* 1979*a*) probably reflect the efficient compensatory accumulation of bicarbonate even within the first 1 h of CO_2 exposure, rather than reflecting real differences in the chemical buffering capacity of the blood. For additional comparison, the true plasma buffer value for *Bufo* at 25 °C has been shown to be 20·4 (Boutilier *et al.* 1979*a*). Compensation of pH_e after 24 h of CO_2 is about 30% of the expected pH change at constant $[HCO_3^-]$, a value significantly higher than the 17% observed by Boutilier *et al.* (1979*a*). This degree of compensation is still poor in comparison with water-breathing fishes (see Heisler, 1980) and it appears as if compensation was dependent upon another uncontrolled factor in these experiments.

The mean intracellular pH (pH_i) of skeletal muscle in *Bufo* determined in this study (7·15) is in the range of values reported by other authors for *Rana pipiens* (7·12 at 17–20 °C, Kostyuk & Sorokina, 1960), *Rana catesbeiana* (6·9, 25 °C, Malan, Wilson & Reeves, 1976), *Rana catesbeiana in vitro* (7·3, at 23·5–26 °C, Izutsu, 1972). Heart muscle was only determined in *Rana catesbeiana* by Malan *et al.* (1976) and found at 7·27 to be similar to the value reported in this study (7·24, both at 25 °C). No literature data are available for amphibian skin. The values determined in this study are rather high in comparison with muscle values and should be considered with caution because of the inhomogeneous structure of the tissue and the fact that the skin in amphibians is the site of extensive ion exchanges with the environment.

The changes of pH_i in hypercapnia are much smaller than those in the plasma, with the *milieu interieur* of heart muscle being the best protected of these three tissue types studied ($\Delta pH_i/\Delta pH_e$ = 0·5, 0·316 and 0·8 for skeletal muscle, heart muscle and skin respectively after 24 h hypercapnia). This pattern of pH_i in heart muscle being better defended than in skeletal muscle is similar to observations in other vertebrate species (Heisler *et al.* 1978; Heisler *et al.* 1980; Heisler, 1980). The compensatory increase in intracellular bicarbonate concentration is of about the same amount as in the extracellular space but, because of the initially lower bicarbonate concentration, is much more effective and results in about 65% pH compensation in skeletal muscle, 77% in heart muscle and 42% in skin (all of the expected pH changes at a constant bicarbonate concentration). The additional bicarbonate in intracellular and extracellular spaces is partly produced by non-bicarbonate buffering. The observed apparent extracellular buffer value after 24 h CO_2 exposure (29·7 mequiv/pH.l), however, is by a factor of more than 4 higher than that attributable to blood and extracellular non-bicarbonate buffers ($\sim$6·5 mequiv/pH.l, roughly estimated from the *in vitro* non-bicarbonate buffer value of real plasma, and an assumed extracellular volume of 20–25% of the total body water). Intracellular chemical buffer values have not been determined for *Bufo*, but at least in heart muscle

the apparent value (80 mequiv/pH.l), after 24 h in hypercapnia, is also much higher than comparable chemical values in other species (dogfish: 36 mequiv/pH.l, Heisler & Neumann, 1980; rat: 41 mequiv/pH.l, Gonzalez *et al.* 1979). These data strongly suggest that part of the accumulated bicarbonate in the extracellular space and heart muscle cells originates from sources other than buffering of CO_2 by non-bicarbonate buffers. Also in skeletal muscle and skin cells, similar mechanisms can be assumed, as the bicarbonate concentrations in these tissues continue to rise even after 1 h of hypercapnia when buffering processes are clearly complete. It cannot be excluded, however, that muscle and skin cells contribute to extracellular compensation by a relatively large bicarbonate release during the first hour of the experiment, as found in dogfish (Heisler *et al.* 1976*a*), and then partially recover later.

Except from buffering of CO_2, bicarbonate may originate from mobilization of carbonate from the calcium-containing structures of the animal, as shown by Sulze (1942) in frogs exposed to hypercapnia. Probably more important is the production of bicarbonate resulting from excretion of H^+ or NH_4^+, or the direct uptake of bicarbonate from the environment.

In our experiments, ammonia excretion to the environment was significantly elevated in hypercapnia. The amount excreted throughout the hypercapnic exposure time ($\sim$4 mmol/l body water content) in addition to the basal rate contributes considerably to the estimated gain of the total animal bicarbonate pool ($\sim$8 mmol/l body water content). Dumping of NH_4^+ into the small amount of ambient water (500 ml), however, must clearly be limited by the toxic build-up of ammonia. Also, excretion of H^+ or uptake of bicarbonate is limited by the total amount of sodium and bicarbonate in the water (see Methods), and, probably before that, by the concomitant progressive drop in water pH, as found in dogfish (Heisler & Neumann, 1977). How far the above mentioned limitations, which apply for both the present study as well as for the experiments of Boutilier *et al.* (1979*a*), are responsible for the much lower degree of compensation of the extracellular pH in *Bufo* in comparison with several fish species, must be the subject of further studies. Regardless of the limitations of compensatory bicarbonate accumulation, the present results confirm that in *Bufo marinus*, as in a number of other vertebrates, there is a clear preference for intracellular over extracellular pH regulation in hypercapnia.

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