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COMPENSATION OF PROGRESSIVE HYPERCAPNIA IN THE TOAD (*BUFO MARINUS*) AND THE BULLFROG (*RANA CATESBEIANA*)

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Summary

Toads (*Bufo marinus* L.) and bullfrogs (*Rana catesbeiana* Shaw) were subjected to a series of 24 h step increases in aerial CO_2 (2, 4, 6 and 8 %) to assess the degree of extracellular pH compensation at each CO_2 level and to ascertain the importance of cutaneous ion transport in this process. Elevation of plasma $[HCO_3^-]$ occurs during the 24 h period, with the bullfrogs showing a greater ability to compensate at each step. There was no indication that a $[HCO_3^-]$ threshold of $30\,mmol\,l^{-1}$ existed in either species, although bullfrogs appeared to have a greater compensatory potential when exposed to the higher levels of CO_2. The results of the ion flux experiments suggest that neither the terrestrial *Bufo* nor the semi-aquatic *Rana* use their skin to any great extent for acid–base balance during hypercapnia.

Introduction

The physiological responses of fishes and amphibians to hypercapnia are becoming quite clear: apart from a few exceptions, fish protect the pH in the extracellular compartment through compensatory increases in extracellular $[HCO_3^-]$ to a greater degree than amphibians (for reviews see Toews and Boutilier, 1986; Heisler, 1986). The mechanisms used to effect the net $[HCO_3^-]$ increase are varied but involve mainly exchanges of acid–base equivalent ions at the gill surface with some involvement of the kidneys (in fish), whereas skin (Stiffler *et al.* 1987), bladder (Tufts and Toews, 1985) and possibly kidney (Yucha and Stoner, 1987) exchanges are more prevalent in amphibians. Mobilization of $[HCO_3^-]$ reserves (from $CaCO_3$ crystals in endolymphatic sacs) also appears to be important in the amphibians studied (Tufts and Toews, 1985).

One important feature of previous experiments on the effects of hypercapnic exposure in amphibians is that the level of ambient CO_2 has usually been quite

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high (5% in most toad experiments, for example). The subsequent elevation in plasma [HCO_3^-] after 24 h would have been sufficient to compensate plasma pH fully, had the CO_2 level been lower (Boutilier *et al.* 1979*a*; Toews and Heisler, 1982; Boutilier and Heisler, 1988). The only evidence that might suggest an ability to compensate pH during a mild hypercapnia is that provided by Boutilier *et al.* (1979*b*), who showed that complete compensation resulted after *Bufo marinus* had elevated internal CO_2 levels as a result of burrowing for several days.

We began the present experiments to answer several questions. First, what is the degree of compensation in amphibians if the level of hypercapnia is low? Second, is the compensatory response at higher CO_2 levels modified if the initial stages of exposure are more gradual, a type of progressive hypercapnia? Third, we wanted to compare two different types of amphibians, the semi-aquatic *Rana catesbeiana* and the more terrestrial *Bufo marinus*. Because the totally aquatic, larval urodele *Ambystoma tigrinum* (Stiffler *et al.* 1987) relies on its cutaneous ion transport mechanisms for compensatory responses to hypercapnia, we wanted to determine what cutaneous responses these two anuran species make to this acid–base disturbance. Of particular interest are possible differences in the responses of the skin of the terrestrial *Bufo marinus* and the semi-aquatic *Rana catesbeiana*.

Materials and methods

Animals and preparation

Adult *Rana catesbeiana* and *Bufo marinus* were obtained from a commercial supplier (Charles D. Sullivan Co. Inc. Nashville, TN, USA). Toads were kept in large terraria containing large rocks and water, such that animals could choose to be in either air or water. The bullfrogs were kept in large aquaria with water at a depth of 15 cm. Water was changed every 48 h and animals of either sex were chosen at random for the experiments.

Prior to surgery, animals were anaesthetized in a $1\,g\,l^{-1}$ solution of MS222 (buffered to about pH 7 with $NaHCO_3$). For the compensation experiments, both toads and frogs were occlusively cannulated in the femoral artery with indwelling polyethylene catheters (Boutilier *et al.* 1979*a*). Since these experiments were usually of 8 days duration, both legs were cannulated to ensure a blood supply for analysis should one catheter become clogged. We are convinced that blood flow patterns were not drastically altered in either species as full leg mobility was maintained and there was no discoloration of the skin on the legs. After surgery the animals were allowed to recover in flowing fresh water and blood sampling procedures were not begun for at least 24 h.

Compensation experiments

An animal was placed in a 4 l wide-mouthed glass chamber which was painted black to avoid undue disturbance caused by movements in the laboratory. Water was provided in the chambers (volume equal to body weight) and was changed every evening and also every morning 2–3 h prior to any blood sampling. Air

gas mixtures were delivered either by Wösthoff gas-mixing pumps (Wösthoff, Bochum, FRG) or by Cameron gas-mixing flow meters (Cameron Instrument Co., Port Aransas, TX, USA) at a rate of 600 ml min^{-1}. Two holes in the large rubber bung closing the chambers provided air entry and exit ports as well as exit ports for the 0.75 m long catheters from the animals. All experiments were performed at room temperature (25±1°C) and analyses made at 25±0.1°C.

In vivo *experiments*

On the first experimental day a blood sample was drawn from the femoral artery (0.6 ml) and a 75 μl portion used immediately for a pH measurement. A further sample was injected into a haematocrit centrifuge tube (about 75 μl), sealed at both ends with wax and centrifuged for 1 min. The haematocrit was read and then 20 μl of the plasma was anaerobically withdrawn with a Hamilton gas-tight syringe fitted with a Chaney adaptor for total CO_2 analysis using a Capnicon 3a CO_2 analyser (Cameron Instrument Co.). The remaining 400 μl of blood was analysed for P_{O_2} and P_{CO_2} using a Radiometer BMS3 system and associated electrodes. Electrodes were connected to either a Cameron O_2 meter (P_{O_2}) or a Radiometer PHM84 pH meter (P_{CO_2}). After the P_{O_2} and P_{CO_2} determinations, the 400 μl of blood was reinjected into the animal followed by a 100 μl bolus of heparinized (125 i.u. ml^{-1}) amphibian saline. After the initial blood sampling (normal samples) the pumps were changed to deliver 2 % CO_2 and a similar blood sampling protocol followed after 1 h. On the next morning a 24 h sample was taken and the pumps switched to 4 % CO_2. The procedures were continued daily for 6 and 8 % CO_2. After the 24 h 8 % sample had been taken, the pumps were switched back to air and a recovery sample taken 24 h later. The precise delivery P_{CO_2} values from the pumps were; 2 % = 1.91 kPa; 4 % = 3.75 kPa; 6 % = 5.57 kPa and 8 % = 7.14 kPa.

In vitro *experiments*

The solubility coefficients of CO_2 (αCO_2) and first dissociation constants of carbonic acid (pK′) were determined on five animals of each species using techniques described by Van Slyke *et al.* (1928) and Boutilier *et al.* (1979*a*). The αP_{CO_2} for *Bufo* was 0.3203 (mmol l^{-1} kPa^{-1}) and 0.3053 for *Rana*. The pK′ for *Bufo* was 6.131 and 6.137 for *Rana*. We equilibrated blood for the pK′ determinations at 2, 4, 6 and 8 % CO_2 (exact P_{CO_2} values listed above) but the CO_2 levels caused no significant changes in pK′.

Ionic flux experiments

On an additional 16 animals (eight of each species), net and unidirectional fluxes of Na^+ and Cl^- were measured according to the methods described by Kirschner (1970). Both bullfrogs and toads were placed in 250 ml of tap water containing 1–3 μCi l^{-1} of either ^{36}Cl or ^{22}Na (New England Nuclear, Boston, MA, USA). The chambers described above for the blood sampling experiments were used. Bath samples were taken at 0, 4, 8, 12 and 24 h for three consecutive days with the bath contents replaced each day. The first 24 h period served as a control period, the

chambers receiving only air; during the second 24 h period the animals were subjected to hypercapnia by passing 6% CO_2 through their chambers with gas delivered from a Cameron gas-mixing flow meter. The final day served as a recovery period with air only gassing the chambers. Radioactive Na^+ was counted using a Beckman 4000 gamma counter and radioactive Cl^- was counted on a Packard Minaxi 4000 liquid scintillation counter. Sodium concentrations were analysed using a Perkin-Elmer flame photometer (model 51). Chloride concentration was analysed with a Buchler chloridometer.

Influx (J_{in}) was calculated from:

$$J_{in} = -(dQ^*_{out}/dt)/(X_{out}),$$

where dQ^*_{out} is the total radioactivity in the bath, dt is the time increment, J_{in} is the influx, and X_{out} is the specific activity of the bath contents. Net flux was calculated as:

$$J_{net} = dQ_{out}/dt,$$

where dQ_{out} is the total Na^+ counts in the bath and the other symbols are as above. Efflux (J_{out}) was calculated as:

$$J_{out} = J_{net} - J_{in}.$$

Results

Compensation experiments

The combined responses of 10 *Bufo marinus* to step increases in CO_2 of 2, 4, 6 and 8 % are shown in Fig. 1. The degree of pH compensation (as defined by Siesjo, 1971) is maximal at 4 % CO_2 (43 % compensation) and drops to 6 % compensation at 8% CO_2 (Table 1). Although the final $[HCO_3^-]$ levels accumulated in the plasma after 4 days of progressive hypercapnia reached 40.8 mmol l^{-1}, a level which could have brought about a much higher degree of compensation at the lower exposure levels, only 26 % compensation was found at 2 % CO_2 after 24 h. Using the $[HCO_3^-]$ and pH values for normal animals and for the 24 h exposure points the effective or achieved buffer value (β_{eff}) of 32.3 mequiv pH $unit^{-1}$ was calculated (least squares, $r=-0.97$). After 24 h of recovery in air, pH values returned to normal but plasma $[HCO_3^-]$ had not yet returned to control values, a response similar to that found by Boutilier *et al.* (1979*a*).

Table 1. *Level of compensation attained by* Bufo marinus *and* Rana catesbeiana *after 24 h of exposure to 2, 4, 6 and 8 % environmental* CO_2

	Bufo marinus		*Rana catesbeiana*	
	% CO_2	% Compensation	% CO_2	% Compensation
	2	26	2	40
	4	43	4	14
	6	24	6	34
	8	6	8	55

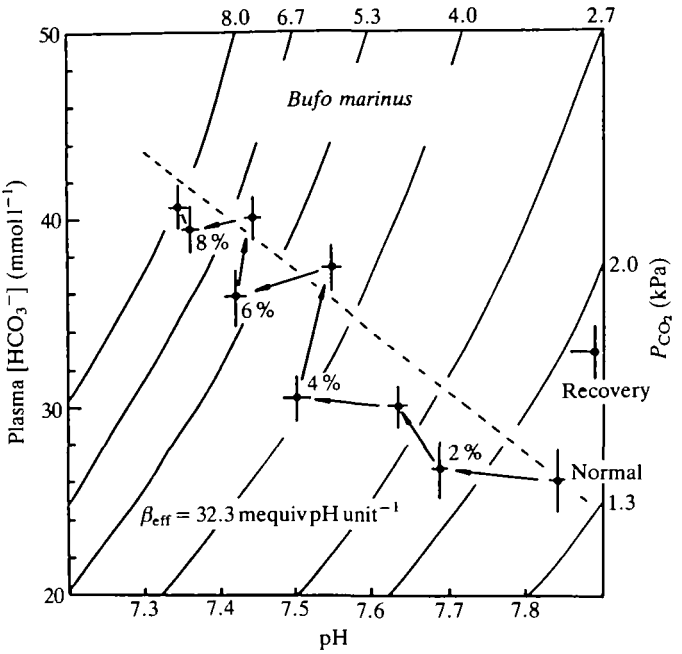

Fig. 1. pH–HCO_3^- diagram showing the responses of 10 *Bufo marinus* to progressive hypercapnia increases of 2, 4, 6 and 8 % environmental CO_2. Means ±S.E. of the 1 h and 24 h pH and $[HCO_3^-]$ values are given plus normal and recovery values. The β_{eff} or achieved buffer line (broken line) was calculated using normal and 24 h values.

The responses of eight *Rana catesbeiana* to hypercapnic exposure were similar to those of the toads, although the final level of plasma $[HCO_3^-]$ accumulated was higher (46.6 mmol l^{-1}), and levels of compensation were generally higher (Fig. 2; Table 1). The β_{eff} for the bullfrogs of 40 (least squares, $r=-0.96$) was substantially higher than that of the toads. During recovery, the pH returned to normal but plasma $[HCO_3^-]$ remained elevated. For any experimental animal 10 blood samples were withdrawn, with the majority of the blood reinfused following measurement. There were no significant changes in haematocrits during the 6-day period, indicating that sampling was not depleting the circulating red blood cell levels.

It has been quite well documented that after 24 h of CO_2 exposure, amphibians are in an equilibrium situation and a longer time does not result in any further changes in $[HCO_3^-]$ or pH (Boutilier *et al.* 1979*a*; Toews and Heisler, 1982; Boutilier and Heisler, 1988). Samples prior to this time (the 1 h samples, Figs 1, 2) represent an unsteady state, and the $[HCO_3^-]$ decrease associated with the higher CO_2 exposures indicates a metabolic and/or respiratory acidosis, perhaps as a result of increased respiratory or physical activity.

Ion fluxes

Neither species altered the rate of Na^+ influx during hypercapnia (Table 2).

Both, however, increased the rate of net flux of Na^+ by decreasing the rate of efflux. The results of the chloride flux experiments presented a slightly different picture (Table 3). *Bufo marinus* showed a significant decrease in Cl^- influx whereas *Rana catesbeiana* did not. *Rana catesbeiana*, in contrast, significantly reduced net loss and efflux of Cl^- whereas *Bufo marinus* did not.

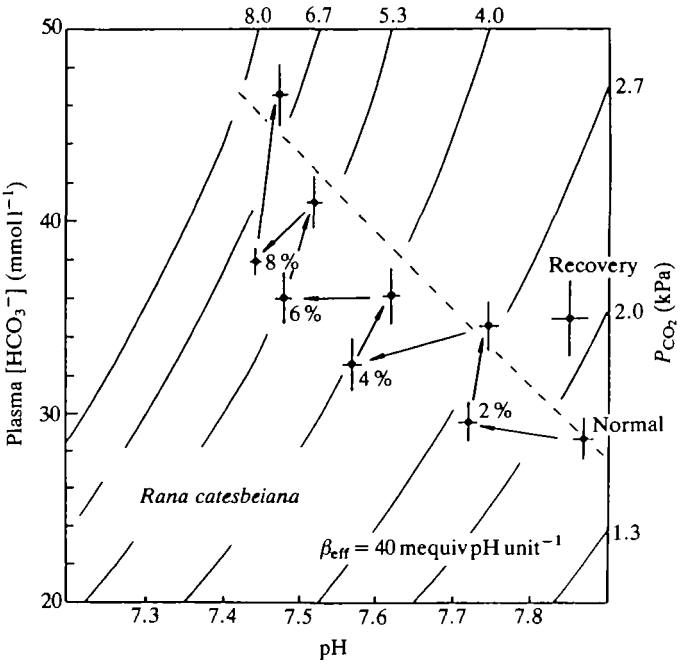

Fig. 2. pH–HCO_3^- diagram showing the responses of 10 *Rana catesbeiana* to progressive hypercapnia increases of 2, 4, 6 and 8 % environmental CO_2. Means ±S.E. of the 1 h and 24 h pH and [HCO_3^-] values are given plus normal and recovery values. The β_{eff} buffer line (broken line) was calculated using normal and 24 h values.

Table 2. *Net and unidirectional fluxes of Na^+ before, during and after hypercapnia in* Bufo marinus *and* Rana catesbeiana

Flux	Control	Hypercapnia	Recovery
Rana catesbeiana			
Influx	8.72±1.83	7.35±1.80	8.45±0.96
Efflux	−12.36±1.94	−5.75±1.50*	−8.81±1.60
Net flux	−3.6±0.47	1.51±0.75*	−1.58±1.91
Bufo marinus			
Influx	15.13±1.89	13.12±1.65	15.16±2.82
Efflux	−15.65±2.75	−8.92±2.48	−16.50±4.21
Net flux	0.13±2.40	5.21±1.85*	−1.34±1.83

Units are μequiv 100 g^{-1} h^{-1}; values are mean±S.E.M., N=8 for both species.
* $P<0.05$ (unpaired t-test *vs* control).
Negative values indicate movement of ions out of the animal.

Table 3. *Net and unidirectional Cl^- fluxes before, during and after hypercapnia in* Bufo marinus *and* Rana catesbeiana

Flux	Control	Hypercapnia	Recovery
Rana catesbeiana			
Influx	3.19±1.00	2.45±0.40	4.16±0.90
Efflux	−9.82±3.28	−5.40±2.05*	−5.48±0.82
Net flux	−6.65±2.74	−2.99±1.81*	−1.35±0.60
Bufo marinus			
Influx	11.40±2.10	6.40±1.00*	6.60±0.70
Efflux	−8.83±2.52	−7.24±2.24	−11.74±2.90
Net flux	2.55±0.84	−0.86±2.09	−4.78±2.41

Units are μequiv $100\,g^{-1}\,h^{-1}$; values are mean±S.E.M., N=8 for both species.
* $P<0.05$ (*vs* control).
Negative values indicate movement of ions out of the animal.

Discussion

Bufo marinus and *Rana catesbeiana* respond to step increases in CO_2 levels by slowly equilibrating arterial P_{CO_2} to levels higher than those of the environment. The equilibration is incomplete after 1 h and, as a result, 24 h exposure levels are always higher. Boutilier and Heisler (1988) have sampled toads more frequently during a 5 % CO_2 exposure and found that during the initial stages (1–2 h) there was a marked reduction in the difference between the arterial and inspired P_{CO_2}. They attribute this to a CO_2-stimulated ventilatory increase, because they found a significant increase in plasma P_{O_2}.

Elevations in plasma $[HCO_3^-]$, in response to progressive hypercapnia, followed a series of stepwise increases, with bullfrogs showing a much greater ability to exchange acid–base equivalent ions, such that plasma $[HCO_3^-]$ was higher than in the toads at the final P_{CO_2} exposure level. There is every indication that the bullfrogs could tolerate even higher CO_2 levels and would have the compensatory potential to respond. The compensatory response in the toad, in contrast, appears to be near maximum at the 8 % level. Certainly, both species elevated plasma $[HCO_3^-]$ to levels in excess of the 30 mmol l^{-1} value suggested previously as a maximum $[HCO_3^-]$ threshold (Heisler, 1986; Toews and Boutilier, 1986; Boutilier and Heisler, 1988).

The continued elevation in plasma $[HCO_3^-]$ levels in *Bufo* and *Rana* in response to progressive increases in CO_2 is similar to that described recently by Cameron and Iwama (1987) for the channel catfish and blue crabs. They suggest that at each exposure level the fish and crabs reach a 'set-point' where pH is regulated in a fashion similar to that during normocapnia. We feel that in the amphibian species we have studied there are $[HCO_3^-]$ reabsorptive thresholds established at every CO_2 exposure level, giving a pH which results from the reabsorption and/or excretion of acid–base equivalent ions across all ion exchange surfaces. Indeed, such processes as blood oxygenation and degree of vascular shunting could also

play a role in dictating a pH set-point for a particular CO_2 exposure level. Nonetheless, while the compensatory potential exists in both *Bufo* and *Rana* (ability to elevate plasma $[HCO_3^-]$ to levels of 40 mmol l^{-1} or higher), complete compensation does not take place at even the lower CO_2 exposure levels (2 or 4 % CO_2).

It is quite possible that in amphibians the compensatory response is hormonally mediated and that, after the initial stimulus (elevated CO_2, lowered pH), the response (net elevation in $[HCO_3^-]$) takes place, followed by a period where there is a downregulation of hormone receptors and a stabilization of the animal's acid–base state. A further compensatory increase in $[HCO_3^-]$ might not occur until an additional CO_2 stimulus is presented. This episodic nature of hormone release has been shown by Toews *et al.* (1989) to be the case with arginine vasotocin in the control of renal function in *Bufo marinus*.

An obvious potential site for the exchange of ions involved in pH compensation is the permeable amphibian skin. The skin transports Na^+ and Cl^- in an inward direction in exchange for H^+ (or NH_4^+) and HCO_3^- (or OH^-), respectively (Kirschner, 1983). This requires that changes in internal pH follow non-parallel changes in Na^+ and Cl^- influx. When unequal changes in Na^+ and Cl^- influx occur, the internal acid–base conditions must change. These cutaneous ion exchange mechanisms are involved in the response of larval and aquatic *Ambystoma tigrinum* to hypercapnia, as Na^+ influx increases while Cl^- influx decreases; a response that would increase internal base concentrations in a compensatory fashion (Stiffler *et al.* 1987). The results of the present ion flux experiments suggest that neither the terrestrial *Bufo* nor the semi-aquatic *Rana* increases Na^+ influx across its skin during hypercapnia. *Bufo* did lower Cl^- influx which would tend to conserve HCO_3^- by slowing Cl^-/HCO_3^- exchange. The net effect of this decrease in Cl^- influx would result in the generation of about 5 μequiv 100 g^{-1} h^{-1} of base in the extracellular fluid of the toad. Over a 24 h period *Bufo* is capable of elevating its extracellular $[HCO_3^-]$ by 10 mmol l^{-1} when exposed to 5 % CO_2 (Boutilier *et al.* 1979*a*; Boutilier and Heisler, 1988). Assuming a 25 ml 100 g^{-1} extracellular fluid volume, 250 μequiv of base would have to be generated. The increased rate of cutaneous base generation we have observed in this species would only account for about half of the total increase in $[HCO_3^-]$. *Bufo marinus* also responded to hypercapnia with a significant change in net Na^+ flux, going from neutral Na^+ balance to a net uptake of Na^+. This was presumably caused by a decreased efflux, although the response was too variable to confirm. Decreases in efflux could take place across the skin or *via* urinary excretion. The latter is an especially attractive possibility because of the known importance of the urinary bladder to hypercapnic compensation in *Bufo marinus* (Tufts and Toews, 1985). The significant increase in net Na^+ uptake of 5 μequiv 100 g^{-1} h^{-1} would account for the other 50 % of base generation necessary to elevate $[HCO_3^-]$ by 10 mmol l^{-1}.

Rana catesbeiana responded to hypercapnia with a decreased Na^+ efflux, which resulted in a net increase in Na^+ uptake (net flux). This could result, as with the

toad, from a decrease in either cutaneous or urinary loss of Na^+, and evidence for a renal tubular Na^+/H^+ exchange system has been reported in a closely related species, *Rana esculenta* (Oberleithner *et al.* 1984).

Rana catesbeiana also showed a significant decrease in Cl^- efflux which would indicate an increased HCO_3^- efflux by the Cl^-/HCO_3^- exchanger in the urinary tract of amphibians. There is evidence for this exchange in the amphibian bladder (Weiner, 1980). This does not mean, however, that there could not be a urinary Cl^- transport system which is not involved in HCO_3^- exchange; indeed, evidence for Cl^--independent HCO_3^- reabsorption has been reported for the amphibian proximal tubule (Boron and Boulpaep, 1983). The decreased efflux of Cl^- might be related to osmoregulatory adjustments to the hypercapnia-induced acidosis.

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