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VENTILATORY RESPONSES TO CARBOXYHAEMOGLOBINAEMIA AND HYPOXIC HYPOXIA IN *BUFO PARACNEMIS*

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Summary

The modality of the O_2 drive to breathe was evaluated in unanaesthetized *Bufo paracnemis*. Carbon monoxide (CO) hypoxia was applied to reduce Ca_{O_2} (arterial O_2 content). Arterial P_{O_2} (Pa_{O_2}) was reduced through inspiration of hypoxic gas mixtures (hypoxic hypoxia). Measurements included pulmonary ventilation, Ca_{O_2}, Pa_{O_2}, pHa, blood pressure and heart rate. Application of fractional concentrations of CO equivalent to 0.001 reduced Ca_{O_2} from 11.6±1.2 to 8.6±0.7 vol% (mean ± S.E.M., N=6) without any effect on heart rate, blood pressure or ventilation. Inspiration of fractionl concentrations of CO equivalent to 0.01 reduced Ca_{O_2} to 5.1±0.7 vol%, which was accompanied by increased ventilation. This response, however, correlated with a decreased arterial pH. The effect of a fractional concentration of CO of 0.01 on ventilation was abolished by compensation of pH through bicarbonate infusion. Consequently, carboxyhaemoglobinaemia *per se* failed to increase ventilation. By contrast, reductions of Pa_{O_2} clearly stimulated ventilation, which indicates that O_2 partial pressure provides the principal O_2 stimulus to breathe in *Bufo paracnemis*.

Key words: *Bufo paracnemis*, control of ventilation, O_2 receptor, blood oxygen-carrying capacity, hypoxia, carboxyhaemoglobinaemia, blood pressure.

Introduction

Most early studies on pulmonary ventilation in amphibians focused on the mechanics of pulmonary ventilation: the mechanism behind the positive pressure inflation of the lungs (DeJongh and Gans, 1969; Jones, 1982). In addition, the periodic pattern of ventilation was described as breath-holding alternating with bursts of pulmonary ventilation, which in some anuran amphibians are initiated by stepwise pulmonary deflation followed by lung reinflation (Boutilier, 1984; Shelton and Boutilier, 1982).

Recently, studies have evaluated the chemical drive to breathe and the receptors involved. The ventilatory responses to hypoxia and change of temperature were assessed in *Bufo paracnemis* (Kruhøffer *et al.* 1987), and other studies characterized the arterial O_2 receptors (Ishii *et al.* 1985; Van Vliet and West, 1992). In addition, central acid–base receptors have been documented in *Bufo marinus* (Smatresk and Smits, 1991) and *Bufo paracnemis* (Branco *et al.* 1992, 1993). The central chemosensitivity of *Bufo marinus* depends on alterations of pH and/or CO_2 levels (Smatresk and Smits, 1991). The O_2 stimulus to breathe has also been evaluated in *Bufo paracnemis* (Wang *et al.* 1994), with the conclusion that pulmonary ventilation is modulated by changes of arterial P_{O_2} and not by Ca_{O_2} of the blood. The experimental approach used in that study (bleeding with Ringer substitution) can, however, be criticized since cardiovascular alterations occurred. To avoid this problem, the present study applies reductions of Ca_{O_2} by formation of carboxyhaemoglobin instead of bleeding. The animals were exposed to CO hypoxia and/or to reductions of inspired P_{O_2}. The effects on blood variables were measured, and the ventilatory responses recorded together with changes in arterial blood pressure and heart rate.

Materials and methods

Toads, *Bufo paracnemis* (Lutz) (mass 160–510 g, N=27), were collected at the Campus of the University of São Paulo in Ribeirão Preto from November to January (summer season). The toads were maintained in containers with free access to water and a basking area (temperature 25–27 °C). Food was withheld during the 4 days preceding surgery.

Surgical procedure and anaesthesia

Arterial cannulation was performed as described in detail by Boutilier *et al.* (1979). A PE 50 catheter was occlusively inserted into the right femoral artery, after which skin incisions were closed and the catheter secured. This procedure was performed within 15 min, during which ether anaesthesia was applied. For initial anaesthesia, the toad was placed in a closed

box saturated with ether vapour. During surgery, ether was evaporated from cotton pads placed under the belly. The first indications of recovery were observed within a few minutes after removal of ether, and complete recovery was apparent after a few hours. Experiments were initiated 24 h after surgery.

Ventilation and blood pressure measurements

Pulmonary ventilation was measured directly using a pneumotachographic method described by Glass *et al.* (1978). Briefly, a light-weight face mask was constructed for each toad. Initially, an alginate resin was used to obtain a negative impression of the head, and then a positive plaster cast was made for moulding the masks (Bioplast, Scheu-Dental, Iserlohn, Germany). The masks were placed on the toads and provided an airtight fit between the nostrils and a Fleisch tube. A direct relationship exists between laminar air flow and pressure difference across this tube (Fleisch, 1925; Grenvik *et al.* 1966). The latter was monitored by a highly sensitive differential air pressure transducer (Statham, 12123) connected to a Narco four-channel recorder (Narco Bio-systems, Inc., Houston, Texas, USA; type 7179; model 4-A). Calibration was performed according to recommended routines by passing known volumes of gas through the pneumotachograph (for details, see Hobbes, 1967).

The arterial cannula was connected to a Statham pressure transducer (P23Dd) kept at the same level as the animal's heart. A mercury column was used to calibrate the signals of this transducer before and after the experiment.

Blood gas analysis

Immediately after withdrawal, arterial blood samples were analyzed for P_{O_2} and pH using a FAC 204A O_2 analyzer (FAC Instruments, São Carlos, Brazil) and a Micronal B374 pH meter (São Paulo, Brazil). Electrodes were kept at the experimental temperature. Oxygen electrodes were calibrated with pure N_2 and humidified atmospheric air. The pH electrode was adjusted using high-precision buffers: S1500 and S1510; Radiometer, Copenhagen, Denmark (see Siggaard-Andersen, 1976, for exact composition and calibration values at different temperatures). Arterial O_2 content, C_{O_2}, was measured according to the micromethod of Tucker (1967). Blood P_{CO_2} was estimated using the Astrup technique (Astrup, 1956). This involves equilibration of blood samples with gas mixtures of known P_{CO_2}, after which the logP_{CO_2}/pH relationship is plotted for each animal. The P_{CO_2} of the blood samples can then be interpolated from this relationship. Standard calibration gases (AGA, Brazil) with fractional CO_2 concentrations of 0.01, 0.03 and 0.06 were used for equilibration.

Experimental protocol

The fully recovered toad was equipped with a face mask and left undisturbed for 2 or 3 h in a chamber (volume 2 l) supplied with a continuous flux of humidified air (5 l min^{-1}). After this control period, hypoxic gas mixtures (fractional concentrations, F_{O_2}, of 0.10 and 0.05) were prepared by mixing pure nitrogen with air. Relative flow rates (air and N_2) were controlled by means of precision needle valves. After humidification, the resulting mixture entered the chamber. Its concentration was continuously monitored using a Beckman OM-11 oxygen analyzer. All exposures to test gases were applied for 45 min, at the end of which ventilation was recorded and arterial blood withdrawn. Subsequently, known quantities of pure CO were mixed into the animal chamber to provide initial fractional concentrations of 0.001 or 0.01. To achieve this, 2 or 20 ml of air, respectively, was withdrawn from the 2 l animal chamber and replaced with pure CO. The resulting CO exposure was maintained for 30 min. After this treatment, the chamber was flushed with fresh air for 45 min. Then the series of hypoxic exposures was repeated along with measurements of ventilation and analysis of blood samples. In some experiments, inspiration of FCO=0.01 was accompanied by bicarbonate infusion to keep pH values constant. This was accomplished by infusion of $NaHCO_3$ at 1 mmol kg^{-1} body mass into the dorsal lymph sac. This quantity was previously determined using stepwise $NaHCO_3$ infusion, accompanied by blood sampling. For more details concerning compensation of pH, see Siggaard-Andersen (1976).

Data analysis

Pulmonary ventilation was distinguished from buccal movements by a biphasic flow profile during expiration (Jones, 1982) and ventilation was calculated excluding buccal movements. Ventilation, blood pressure and heart rate were calculated on the basis of 10 min recording periods. Tidal volume was obtained from the integrated area of the expired flow signal. Effects of hypoxic hypoxia and CO treatment were evaluated using analysis of variance (ANOVA) and the difference between means was assessed by Tuckey's test. A P value of less than 0.05 was considered significant.

Results

Table 1 shows the data for the effects of hypoxic hypoxia and/or CO hypoxia on C_{O_2} of the arterial blood. Hypoxic hypoxia down to F_{O_2}=0.05 reduced C_{O_2} of the blood by about 50 %. CO hypoxia decreased C_{O_2} by the same amount when FCO=0.01 was inspired. By combining the treatments of hypoxic hypoxia (inspired F_{O_2}=0.05) and CO hypoxia (FCO=0.01), C_{O_2} declined to 30 % of the normoxic control value.

Table 2 shows the effects of treatments on blood pressure, heart rate and blood gases. None of the experimental conditions had any significant effect on mean arterial pressure. Heart rate rose during hypoxic hypoxia, but was not affected by CO hypoxia. Hypoxic hypoxia (F_{O_2}=0.05) reduced $P_{a_{O_2}}$ to 30 % of the normoxic control value. Carboxyhaemoglobinaemia did not affect $P_{a_{O_2}}$. Arterial pH increased during hypoxic hypoxia as a result of hyperventilation (Fig. 1). Treatment with FCO=0.01 caused a reduction of pHa. In some experiments, this reduction was

Table 1. *Effects of hypoxic hypoxia and/or CO hypoxia on oxygen content of the arterial blood of* Bufo paracnemis

	Oxygen content (vol%)			
FO_2	Control	*F*CO=0.001	*F*CO=0.01	*F*CO= 0.01+HCO_3^-
0.21	11.6±1.2	8.6±0.7†	5.1±0.7†	5.6±1.2†
0.10	8.7±0.8*	6.5±0.5*,†	6.5±0.5†	4.7±0.5†
0.05	6.2±0.7*	5.1±0.7*,†	3.6±0.7*,†	3.5±0.7*,†

Values are mean ± S.E.M. (*N*=6).
*A significant effect of hypoxic hypoxia relative to normoxic control group.
†A significant effect of CO hypoxia.

compensated by bicarbonate infusion. Arterial P_{CO_2} was not influenced by inspiration of *F*CO=0.01.

Fig. 1 shows the effects of hypoxic and CO hypoxia on pulmonary ventilation. Hypoxic hypoxia was accompanied by marked increases of ventilation. Inspiration of CO at low levels (*F*CO=0.001) did not affect ventilation which, however, increased slightly at the higher level (*F*CO=0.01). This increase did not occur when arterial pH was controlled through bicarbonate infusion.

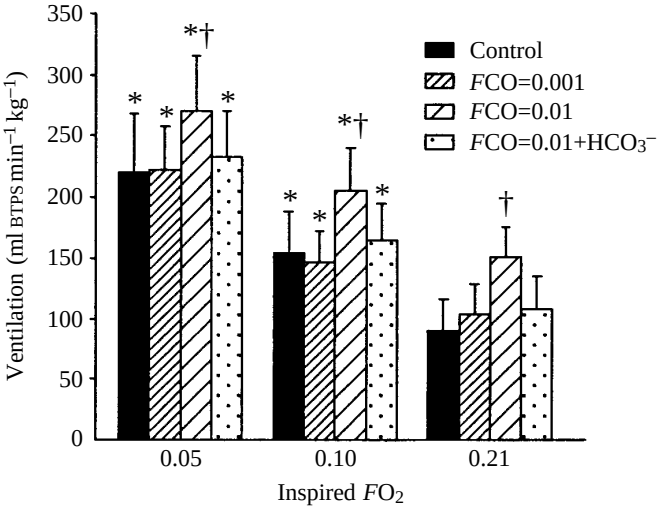

Fig. 1. The effects of hypoxic hypoxia and CO hypoxia on pulmonary ventilation. Hypoxic hypoxia was accompanied by marked increases of ventilation. Inspiration of CO at low levels (*F*CO=0.001) did not affect ventilation. However, ventilation increased slightly at the higher level (*F*CO=0.01). This increase disappeared when arterial pH was controlled through bicarbonate infusion. Mean values + S.E.M. (*N*=6). * denotes a significant effect of hypoxic hypoxia relative to normoxic control values; † denotes a significant effect of CO hypoxia.

Discussion

Studies on the carotid bodies of mammals indicate that O_2 partial pressure is the specific stimulus modulator for the O_2 receptor (Mills and Edwards, 1968; Garland *et al.* 1994). The transducer mechanism may involve cytochrome a_3^{2+} (Larihi, 1994). Participation of O_2-sensitive K^+ channels is also suggested (López-Barneo *et al.* 1993; López-López *et al.* 1989). A complete and consistent picture is, however, not yet available in spite of considerable recent progress (see Lahiri, 1994). The final stimulus modulator may be P_{O_2}, but receptor function depends on blood flow and oxygen delivery to the receptor site. If blood flow is relatively low, then the output from the receptor becomes dependent on Ca_{O_2}, which has been documented for the aortic O_2 receptors of the cat (Lahiri *et al.*

Table 2. *Effects of reduction in* Ca_{O_2} *and hypoxic hypoxia on mean arterial pressure, heart rate and blood gases in* Bufo paracnemis

Group	FO_2	Mean aterial pressure (kPa)	Heart rate (min^{-1})	Pa_{O_2} (kPa)	pHa	Pa_{CO_2} (kPa)
Control	0.21	5.3±0.6	37.1±3.1	9.3±0.4	7.81±0.02	1.3±0.1
	0.10	5.6±0.8	41.9±3.2*	5.4±0.5*	7.91±0.01*	1.0±0.1*
	0.05	6.0±1.0	52.7±3.6*	3.4±0.3*	7.96±0.02*	0.8±0.1*
*F*CO=0.001	0.21	5.2±0.8	39.8±4.0	9.2±0.7	7.81±0.04	–
	0.10	5.5±0.5	39.6±4.1	5.8±0.6*	7.92±0.04*	–
	0.05	6.1±0.7	47.2±4.4*	3.9±0.7*	7.97±0.04*	–
*F*CO=0.01	0.21	5.3±0.4	38.3±4.8	9.9±0.5	7.65±0.03†	1.4±0.1
	0.10	5.7±0.6	39.5±5.1	5.0±0.6*	7.79±0.06*,†	0.8±0.4*
	0.05	6.1±0.7	51.5±5.8*	3.9±0.6*	7.83±0.04*,†	0.7±0.1*
*F*CO=0.01+HCO_3^-	0.21	5.4±0.9	36.8±3.8	9.1±0.7	7.85±0.04	–
	0.10	5.8±0.7	42.0±3.7*	5.2±0.6*	7.88±0.06	–
	0.05	6.0±0.9	47.0±5.4*	3.5±0.5*	7.92±0.04*	–

Values are mean + S.E.M. (*N*=6).
*A significant effect of hypoxic hypoxia relative to the normoxic control group.
†A significant effect of CO hypoxia.

1981). Conversely, carotid O_2 receptors are heavily perfused and their output strongly depends on O_2 partial pressure; they are largely unaffected by carboxyhaemoglobinaemia (Lahiri *et al.* 1981). This implies that the final O_2 modulator (P_{O_2}) is masked unless the receptor site is heavily perfused and, thus, perfusion characteristics determine the apparent arterial O_2 stimulus (P_{O_2} or [O_2]).

In vertebrates, the apparent O_2 stimulus to breathe has been most widely studied in mammals and, according to the traditional view, their O_2 drive to breathe depends on arterial O_2 partial pressure rather than on $C_{a}O_2$ (cf. Comroe, 1974). In a recent study, Garland *et al.* (1994) reported a considerable ventilatory response to carboxyhaemoglobinaemia in squirrels and rats. The ventilatory responses to hypoxic hypoxia seem to exceed those to CO hypoxia in both species. Increases of ventilation in response to CO hypoxia have also been reported for cats and goats (Santiago and Edelman, 1976; Gautier and Bonora, 1983; Gautier *et al.* 1990). It is not yet possible to relate these responses to the relative contributions from carotid and aortic receptor groups.

Information is scarce for other vertebrate classes. Positive evidence for a dependence of ventilation on $C_{a}O_2$ has been obtained for birds (Tschorn and Fedde, 1974) and teleost fish (Smith and Jones, 1982). Corresponding data are few for reptiles and amphibians. Studying the toad *Bufo paracnemis*, Wang *et al.* (1994) reduced $C_{a}O_2$ by replacing blood with Ringer's solution. Ventilation is unaffected by this treatment, whereas decreases of arterial P_{O_2} stimulate ventilation. This anaemia is, however, accompanied by tachycardia and probably also by viscosity changes, and this complicates interpretation of the results. An increased heart rate may partially restore O_2 delivery to receptor sites (Lahiri *et al.* 1981). In the present study, we reduced C_{aCO_2} by applying CO hypoxia and thus avoided cardiovascular side effects. Moreover, we controlled acid–base status through bicarbonate infusion. Because of these precautions, the reduction of $C_{a}O_2$ was not accompanied by significant stimulation of ventilation. It therefore seems justifiable to conclude that the O_2 drive in *Bufo paracnemis* is, indeed, linked to changes of O_2 partial pressure and is largely independent of $C_{a}O_2$. The possibility that very severe reductions of $C_{a}O_2$ may, eventually, cause ventilatory responses cannot be excluded. Furthermore, it should be noted that recent work suggests the possibility that CO is an atypical neurotransmitter (Snyder, 1992). Such a role for CO may complicate the interpretation of data obtained by application of CO hypoxia (Garland *et al.* 1994). Carbon monoxide has been used as a probe to study *in vitro* the sensor mechanism of mammalian O_2 receptors (Lahiri, 1994). The receptors were excited when very high levels of CO were applied (6.67 kPa=500 mmHg). These findings cannot be applied directly to the present study because of differences in experimental protocols and levels of CO at the receptor site.

An increase of heart rate during hypoxic hypoxia was reported for *Bufo marinus* (Boutilier and Toews, 1977). The increase was, however, marginal, and severe hypoxia caused a pronounced bradycardia relative to normoxic control values. Recently, Wang *et al.* (1994) observed a progressive increase of heart rate with reduction of inspired P_{O_2}, which is consistent with the present results. The present blood gas data agree well with previous studies on *Bufo paracnemis* (Kruhøffer *et al.* 1987; Branco *et al.* 1993; Wang *et al.* 1994) and *Bufo marinus* (Boutilier *et al.* 1987; Wood and Malvin, 1991). The observation that P_{CO_2} was not altered by CO hypoxia indicates that the reduction of pH during treatment with FCO=0.01 was due to a metabolic acidosis. Our ventilation data are also in close agreement with earlier measurements on *Bufo paracnemis* at 25 °C (Kruhøffer *et al.* 1987; Branco *et al.* 1992; Wang *et al.* 1994).

A number of studies report on peripheral O_2 receptors in anuran amphibians. Ishii *et al.* (1966, 1985) located such receptors in the carotid labyrinth as well as in the aortic arch. Recently, Van Vliet and West (1992) managed to record the afferent neural activity from these regions, applying reductions of $C_{a}O_2$ as well as alterations of P_{O_2}. They concluded that the output is related to changes of P_{O_2}, which is consistent with the data on the ventilatory drive (Wang *et al.* 1994; present study). This suggests that the O_2 receptors that dominate the hypoxic response are not situated in O_2-demanding tissue but instead in a well-perfused region.

Anuran amphibians seem to share some essential features of ventilatory control with mammals: in both vertebrate classes, the O_2 drive to breathe depends on peripheral receptors. Moreover, ventilatory acid–base regulation is based on central chemoreceptors, backed up by a peripheral component (Smatresk and Smits, 1991; Branco *et al.* 1992). These common characteristics seem unexpected and are intriguing, considering the large evolutionary distance between the groups, the very different mechanism of pulmonary ventilation employed by the two groups and the high dependence on cutaneous respiration in amphibians.

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