The Journal of Experimental Biology 200, 2237–2248 (1997)
Printed in Great Britain © The Company of Biologists Limited 1997
JEB0855

DEVELOPMENTAL CHANGES IN CHEMORECEPTIVE CONTROL OF GILL VENTILATION IN LARVAL BULLFROGS (*RANA CATESBEIANA*)

II. SITES OF O_2-SENSITIVE CHEMORECEPTORS

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Accepted 9 June 1997

Summary

The time course of reflex changes in gill ventilation variables caused by a step-wise change in P_{O_2} of inspired water and by the introduction of NaCN into inspired water was measured in two populations of unanesthetized larval bullfrogs (*Rana catesbeiana*) – one with intact gill arches and a second with bilateral ablation of the first gill arch. Developmental stages TK V–VII, IX–XI and XVII–XIX were examined. Inspiring hypoxic water or a pulse of NaCN significantly increased gill ventilation within 7 s in control larvae. Ablation of the first gill arches in larval *R. catesbeiana* eliminated the initial, quick response of gill ventilation to changes in the P_{O_2} of inspired water and to the presence of NaCN in all three developmental groups. These data suggest that O_2-sensitive chemoreceptors are located on the first gill arch and are responsible for the initial rapid ventilatory response. However, a slow response (>15 s) to changes in P_{O_2} of inspired water persisted even after removal of the first gill arch. This response is far slower than the minimum blood circulation time (approximately 5–8 s as measured in all three groups), indicating that a second population of receptors is not directly monitoring arterial or venous blood. Instead, this second population of receptors is likely to be located 'behind' a significant diffusion barrier, possibly monitoring cerebrospinal fluid.

Key words: chemoreceptors, gill ventilation, hypoxia, bullfrog, larva, *Rana catesbeiana*.

Introduction

The regulation of respiratory processes in all vertebrates depends on information fed back from peripheral chemoreceptors and mechanoreceptors to the respiratory center. Such information reflexively provokes a change of ventilation and also ensures appropriate matching of ventilation and perfusion. In teleost fish, peripheral O_2-sensitive chemoreceptors are typically located on the first gill arch (e.g. Milsom and Brill, 1986; Smatresk, 1990; Burleson and Milsom, 1993). In amphibians, the sites of peripheral receptors that control respiration and circulation include the carotid labyrinth, the pulmocutaneous artery and the aortic trunk (see review by West and Van Vliet, 1992). The carotid labyrinth is the morphological homolog of the mammalian carotid body and is considered important as a chemoreceptor in the control of ventilation because of its increased neural output in response to decreases in ambient P_{O_2} or inspired sodium cyanide *in vitro* (Boutilier, 1990; West and Van Vliet, 1992).

Despite our extensive and growing understanding of cardio-respiratory performance in larval amphibians (see Burggren and Just, 1992; Burggren and Fritsche, 1997), we still know little about the chemoreceptive control of this process. West and Burggren (1983) presented evidence for an interoceptor responding to P_{O_2} which did not differentiate between a signal derived from water ventilating the gills or gas ventilating the lungs. More recent studies have been made of putative central chemoreceptors in larval and adult bullfrogs (e.g. Kinkead *et al.* 1994; Gdovin *et al.* 1996), but the existence and role of amphibian branchial receptors has not been explored. In larval amphibians, the close association between the first gill arch and the carotid labyrinth suggests a chemoreceptive role for the gills (Malvin, 1989). The first gill arch (=embryonic arch III) of larval anurans is innervated by cranial nerve IX (glossopharyngeal) (Rugh, 1951), which is the same pattern of innervation as the mammalian carotid body and the carotid labyrinth of adult amphibians (see West and Van Vliet, 1992). The remaining gill arches are innervated by cranial nerve X (Rugh, 1951). These observations, coupled with the findings of our companion study on bullfrog larvae (Jia and Burggren, 1997) showing extremely rapid ventilatory responses to changes in P_{O_2} of inspired water and to the presence of an inspired pulse of NaCN, suggest that the first gill arch may be

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a site for O_2-sensitive chemoreceptors in the larval bullfrog. This study tests the hypothesis by examining ventilatory responses before and after ablation of the first branchial arch, as well as by injecting pulses of NaCN into the central circulation.

Materials and methods

Animals

Larval bullfrogs *Rana catesbeiana* were collected from ponds in western Massachusetts, USA, and maintained in holding tanks. All larvae were weighed and staged according to the criteria of Taylor and Kollros (1946) before each experiment. As in our companion study (Jia and Burggren, 1997), three different developmental groups were examined: stage V–VII, mean body mass 5.33±0.21 g (N=27); stage IX–XI, mean body mass 6.66±0.24 g (N=22); stage XVII–XIX, mean body mass 7.34±0.38 g (N=21) (mean values ± S.E.M.).

Gill ablation experiments

Larvae in each developmental group were anesthetized with a 1:5000 solution of MS-222 in water (buffered to pH 7.3) and then subjected either to sham surgery (the 'control') or to bilateral surgical ablation of the first branchial arch, which after metamorphosis becomes incorporated into the root of the internal carotid artery. In surgically modified larvae, the left and right branchial chambers were opened ventro-laterally. A ligature of 6-0 gauge surgical silk was tied around the proximal and distal ends of both first gill arches. The portion of arch between the ligatures was then excised. The incisions in the branchial chamber wall were then sutured using interrupted sutures of 6-0 gauge silk. At the end of the experiments, larvae were killed and dissected to confirm complete removal of the first gill arch.

Control larvae received sham surgery only. The branchial chambers were opened and the first gill arches exposed as described above. However, the arches of the sham-operated larvae were not touched, and the branchial chamber was then closed with interrupted sutures.

Both sham and surgically modified groups of larvae were allowed to recover overnight in a small aquarium containing aerated water (20–23 °C). The following day, larvae were very briefly anesthetized in MS-222 (1:5000, pH 7.2) for insertion of a PE-50 cannula into the buccal cavity through a naris for measurement of gill ventilation frequency, f_G, buccal pressure, P_B, branchial stroke volume, V_S, and total branchial water flow, $\dot{V}_W$ (for details of all experimental methodology, see Jia and Burggren, 1997). After cannulation and recovery from anesthesia, larvae were transferred to an experimental chamber filled with normoxic water (P_{O_2}=20 kPa). This chamber allowed practically instantaneous changes to the water inspired by unanesthetized larva. Responses to inspired hyperoxic and hypoxic water and to NaCN injections into the inspired water stream were assessed as described in detail in our companion paper. Basically, each larva was allowed a brief 20–30 min acclimation period of breathing in normoxic water. The inspired water source was then immediately switched to control water (normoxic, with food color) for 20 s, then to colored hyperoxic water (P_{O_2}>78 kPa). Food coloring, which allowed precise timing of the entry of the water source into the buccal cavity, was used in a normoxic water control to determine whether coloring itself had any effect on gill ventilation. The results showed that there was no effect. The time courses of the response of f_G, P_B, V_S and $\dot{V}_W$ to hyperoxia were recorded for 40–50 s (but only statistically analyzed during the first 20 s). After this period, the inspired water was changed back to normoxia for 10 min. This procedure was repeated three times at each P_{O_2} level. The time interval between each cycle was 20 min. Hypoxic water was then applied following the protocols described above for hyperoxia.

Intravascular injection of acetylcholine and NaCN

Larval *R. catesbeiana* were anesthetized by immersion in a 1:5000 solution of MS-222 in water (buffered to pH 7.3), then immobilized ventral surface up in an experimental chamber containing approximately 1 cm of normoxic water. The branchial chambers of the larva were continuously ventilated with a 1:10 000 MS-222 solution *via* a cannula placed in the mouth. Larvae remained lightly anesthetized throughout this experimental series.

Blood circulation time

Blood circulation time in these larvae was determined by measuring the delay between the time of injection of a body-mass-dependent pulse of 10^{-2} mol l^{-1} acetylcholine (ACh) into the central arterial circulation and the onset of a cardiac slowing caused by ACh reaching the sinus venosus in the venous return. The ACh (with 0.5 % heparin, pH 7.3) was injected into the conus immediately distal to the pylangial valves separating the ventricle and the conus (see Pelster and Burggren, 1991). The pulse of ACh then flowed through the arteries, gills, systemic capillary beds and veins before returning to the heart. Anurans have a very simple and limited coronary circulation that perfuses only the outer connective tissue of the heart and not the myocardium *per se*, as it does in birds and mammals (Burggren *et al.* 1997). We assumed, therefore, that the delay between ACh injection and the first sign of cardiac slowing was a measure of 'minimum blood circulation time' and did not reflect direct blood flow *via* the coronary arteries to the cardiac pacemaker. The sensitivity of the heart to ACh appears very early in the development of larval bullfrogs, at least as early as stage III (Burggren and Doyle, 1986; Kimmel, 1992). Therefore, this technique could be used in all stages of larvae used in this study.

To permit intravascular injection, the heart of the anesthetized, inverted larva was exposed by a small mid-ventral incision in the body wall. The opening formed by this incision was rinsed periodically with saline to keep exposed tissue from desiccating. A 10 µl Hamilton syringe filled with the ACh solution was mounted in a micromanipulator. The sharp tip of a drawn-out glass capillary connected to the Hamilton syringe was inserted into the proximal region of the

conus of the larva with the aid of a binocular microscope. A pair of 40 gauge copper wire electrodes was placed on the surface of the heart and connected to a Biocom impedance converter. Heart rate (f_H) was calculated by passing the signal from the impedance converter through a biotachometer (Narco 7302) to a chart recorder (Narco Physiograph DMP-4B).

After recording f_H prior to any experimental treatment, either a saline pulse (as a control to eliminate the possible blood volume loading effect of injection) or an ACh pulse was injected into the conus. For larvae whose body mass was less than 9 g, 0.4 µl of saline or ACh solution was injected. For larvae whose body mass was more than 9 g, 1 µl of saline or ACh was injected. The response of f_H to injected saline or ACh was then recorded.

NaCN injection

Injections of NaCN (0.5 % solution in saline) were made into the central circulation of anesthetized larva. Initially, a water-filled PE-50 cannula was placed into the buccal cavity through one narial opening for measurement of buccal pressure (P_B). Gill ventilation frequency was determined from the pressure signal using a biotachometer (see Jia and Burggren, 1997). The conus arteriosus of the larva was exposed by a small incision in the mid-ventral body wall. The same injection apparatus, protocol and body-mass-dependent dosages described above for ACh injection were then used to inject NaCN.

Data analysis

Values of f_G, P_B, V_S and $\dot{V}_W$ and response times to changes in P_{O_2} of inspired water and to external and internal exposure to NaCN were determined and analyzed as described in Jia and Burggren (1997). Two-way analysis of variance (ANOVA) was also used to test the differences in measured variables between different treatments (surgery or sham surgery) and among the three developmental groups under normoxic conditions. Response times to externally and internally applied NaCN, as well as the response time of heart rate (f_H) to injected acetylcholine (ACh) (that is, the minimum blood circulation time) were analyzed by two-way ANOVA. A completely randomized split-plot design ANOVA was used to examine the difference between control and surgically modified larvae within each developmental group (Damon and Harvey, 1987). Differences in heart rate before injection of ACh in the three developmental stages of larvae were tested by one-way ANOVA. Significant differences revealed by ANOVA were further evaluated using the Student–Neuman–Keuls multiple-range test. $P<0.05$ was chosen as the minimum level of significance for all analyses. All values presented are means ±1 S.E.M.

Results

Effects of gill arch 1 ablation

Normoxia

Representative traces of gill ventilation in an unanesthetized stage X larva breathing normoxic water before and after bilateral ablation of gill arch 1 are shown in Fig. 1. Overall, surgical removal of the first pair of gill arches significantly ($P<0.05$) decreased mean $\dot{V}_W$ and V_S by approximately 40–60 % in all three developmental groups examined (Table 1). Bilateral ablation of gill arch 1 significantly ($P<0.05$) increased f_G in young larvae (V–VII) from 96 beats min^{-1} before gill arch ablation to 116 beats min^{-1} afterwards. However, f_G in unanesthetized larval groups IX–XI and XVII–XIX breathing normoxic water was not as greatly affected, though the changes were significant.

Hyperoxia

f_G in unanesthetized control larvae was significantly

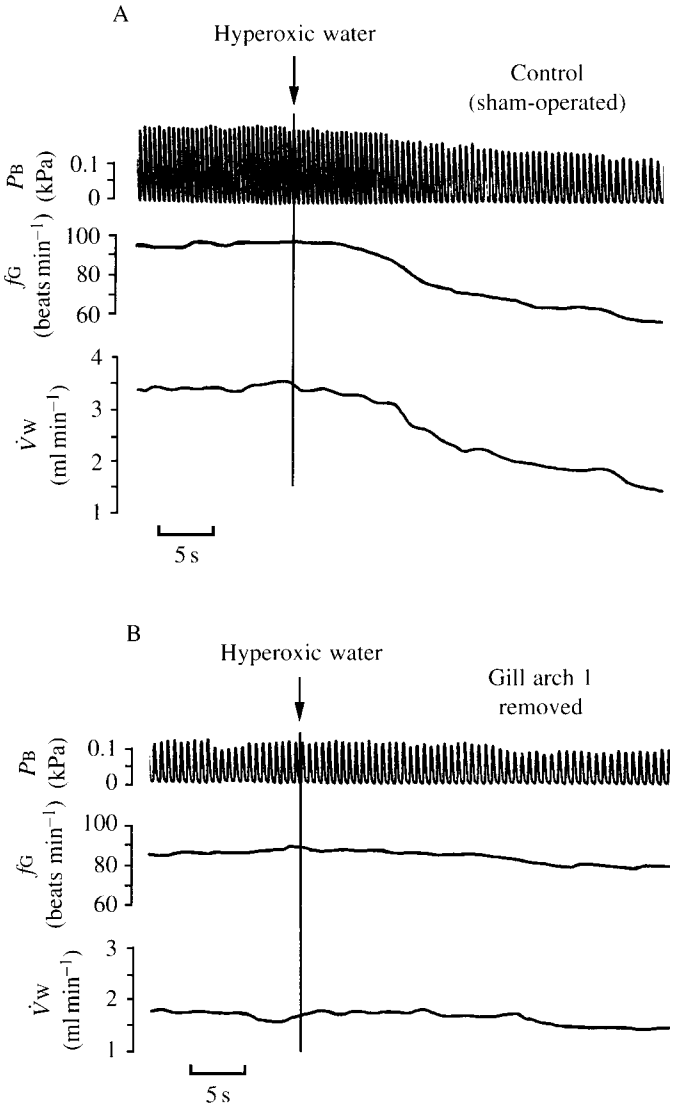

Fig. 1. Representative recordings of gill ventilation frequency (f_G), buccal pressure (P_B) and total branchial water flow ($\dot{V}_W$) in an unanesthetized stage X larval bullfrog in response to a step-wise change in inspired water from normoxic to hyperoxic (arrow). (A) The response under control (sham-operated) conditions; (B) the response in the same larva after recovery from bilateral ablation of gill arch 1. Time marker, 5 s.

Table 1. *Gill ventilation variables in normoxic larval* Rana catesbeiana *after recovery from either sham operation or bilateral ablation of gill arch 1*

Gill ventilation variable	Stage V–VII		Stage IX–XI		Stage XVII–XIX	
	Sham-operated	Gill arch 1 ablated	Sham-operated	Gill arch 1 ablated	Sham-operated	Gill arch 1 ablated
f_G (beats min^{-1})	96±3	116±4*	98±6	91±3*	88±3	96±4*
P_B (kPa)	0.09±0.01	0.09±0.01	0.11±0.01	0.11±0.01	0.09±0.01	0.08±0.01
V_S (ml cycle^{-1})	0.025±0.005	0.01±0.004*	0.026±0.001	0.016±0.001*	0.028±0.001	0.012±0.002*
$\dot{V}_W$ (ml min^{-1})	2.4±0.3	1.13±0.3*	2.6±0.3	1.4±0.2*	2.5±0.4	1.15±0.1*

Mean values ± 1 S.E.M. are presented; N=6 for all values for sham-operated larvae, while N=8 for all larva with bilateral ablation of gill arch 1. An asterisk indicates a significant difference (P<0.05) from values in sham-operated larvae.
f_G, gill ventilation frequency; P_B, buccal pressure; V_S, branchial stroke volume; $\dot{V}_W$, total branchial water flow.

(P<0.05) decreased by inspiring hyperoxic water in all three developmental groups (as illustrated for stages X and V–VII in Figs 1 and 2). However, the extent and time course of the effect of inspired hyperoxic water on gill ventilation in larvae lacking the first pair of gill arches differed between developmental groups. In stage V–VII larvae, ablation of the first pair of gill arches completely eliminated any short-term (<20 s) hyperoxic response in f_G, P_B, $\dot{V}_W$ and V_S (Fig. 2). In stage IX–XI larvae, ablation of the first gill arches approximately doubled the response time to 17 s for f_G and to 16 s for P_B (Fig. 3). The responses of $\dot{V}_W$ and V_S to inspiration of hyperoxic water were eliminated, as in the youngest larval group. In the oldest larvae (stage XVII–XIX), the ablation of the first gill arches eliminated any significant response in f_G, P_B and V_S, but a much delayed (beyond 20 s) response in $\dot{V}_W$ persisted (Fig. 4).

Hypoxia

Inspiring hypoxic water caused a significant (P<0.05) increase in gill ventilation in sham-operated larvae at all three

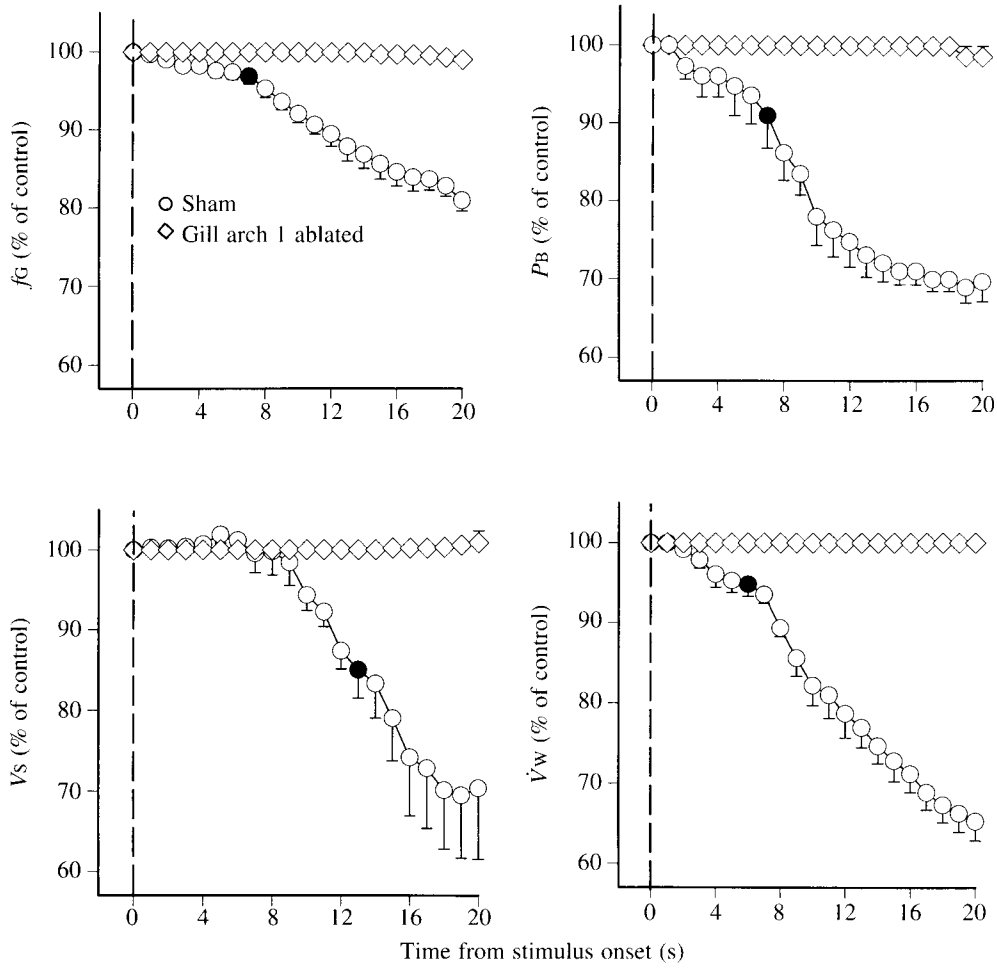

Fig. 2. Responses of gill ventilation variables in stage V–VII larval *Rana catesbeiana* to a step-wise change in inspired water from normoxic to hyperoxic water (broken vertical line at time 0 s). Open circles, sham-operated, gill arches intact, N=6. Open diamonds, gill arch 1 ablated, N=8. Mean values (±1 S.E.M.) are presented. In many points for the larvae with gill arches ablated, the error bars in this figure (and subsequent figures) are smaller than the size of the plotted symbol. The onset of a significant change from time 0 is indicated for each data set by a single filled symbol. The lack of a filled symbol indicates no significant change within the 20 s period of statistical analysis.

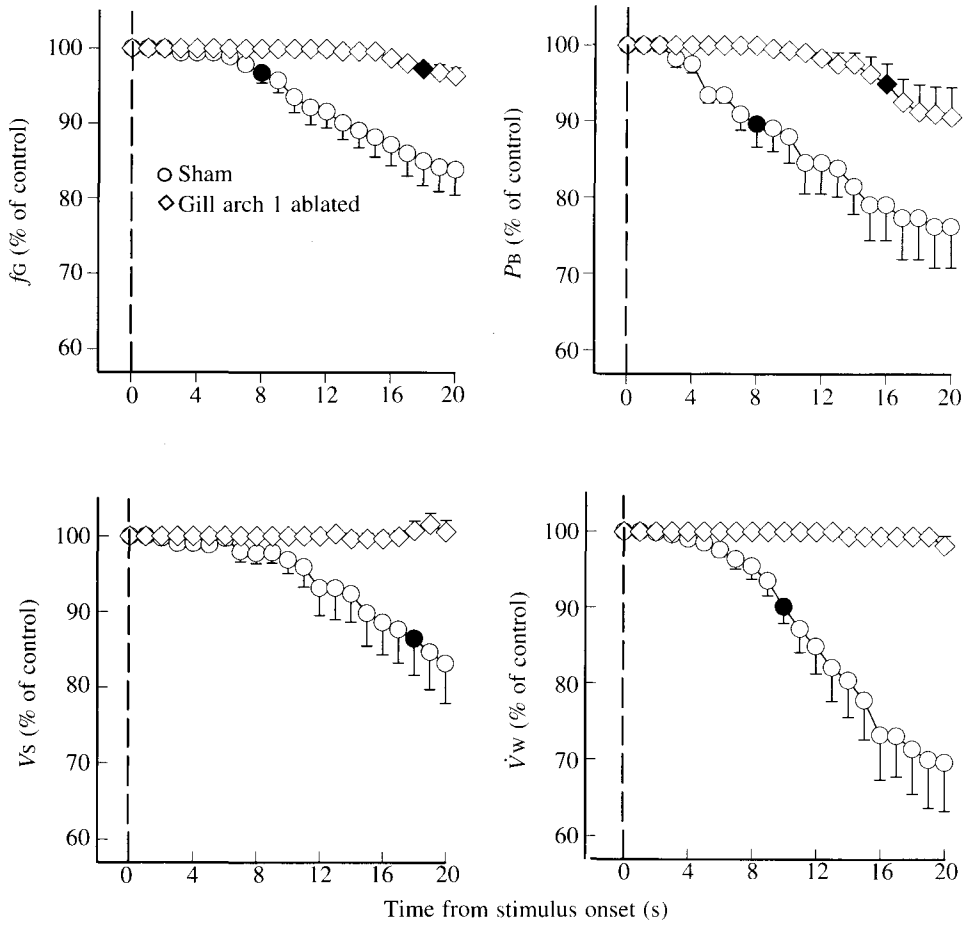

Fig. 3. Responses of gill ventilation variables in stage IX–XI larval *Rana catesbeiana* to a step-wise change in inspired water from normoxic to hyperoxic (broken vertical line at time 0 s). Open circles, sham-operated, gill arches intact, N=6. Open diamonds, gill arch 1 ablated, N=9. See legend to Fig. 2 for additional details of data presentation.

developmental stages (e.g. Fig. 5). However, as in the case of a step-wise change to hyperoxic water, in larvae lacking gill arches 1, the ventilatory effects of inspiring hypoxic water differed as a function of development. In the youngest larvae (stage V–VII), ablation of the first gill arch eliminated the typically profound response in gill ventilation to hypoxic water that occurred in sham-operated larvae within 20 s (Fig. 6). In larval stage IX–XI, ablation of the first gill arches significantly delayed the response time in f_G and P_B to more than 20 s and eliminated the short-term response in $\dot{V}_W$ and V_S (Fig. 7). The oldest larvae showed no significant response of f_G or V_S within 20 s after both sham operation and gill arch ablation. However, ablation of the first gill arches eliminated the previously significant response in $\dot{V}_W$ in sham-operated larvae and very slightly delayed the response time in P_B (Fig. 8).

Externally applied NaCN

The response times of f_G to a pulse of NaCN solution added to the inspired water in unanesthetized larvae are presented for both sham-operated and surgically altered populations in Table 2. A typical response of a stage VI larva is shown in Fig. 9. The response time of f_G to externally applied NaCN in control larvae was 1.3 s in stage V–VII, 1.5 s in stage IX–XI and 3.3 s in stage XVII–XIX. There were no significant differences between the first two groups, but the response of the oldest group was significantly delayed. In larvae lacking gill arch 1, the response time of f_G was significantly delayed to 19 s for stage V–VII, 11 s for stage IX–XI and 7.7 s for stage XVII–XIX. These response times were also significantly different from each other.

Gill ventilation responses to internally injected NaCN

A NaCN pulse injected into the conus of a young

Table 2. *Time to significant gill ventilation frequency response to an inspired pulse of NaCN in unanesthetized sham-operated larvae and unanesthetized larvae with bilateral gill ablation of arch 1*

	Stage V–VII		Stage IX–XI		Stage XVII–XIX	
	Sham	Arch 1 ablated	Sham	Arch 1 ablated	Sham	Arch 1 ablated
Time to significant response (s)	1.3±0.5 (6)	19.0±3.4 (8)	1.5±0.3 (6)	11.0±1.9 (9)	3.3±0.5 (6)	7.7±1.3 (7)

Mean values ± S.E.M. (N) are presented.
All times for sham-operated larvae are significantly shorter (P<0.05 or smaller) than for larvae with gill arches 1 ablated.

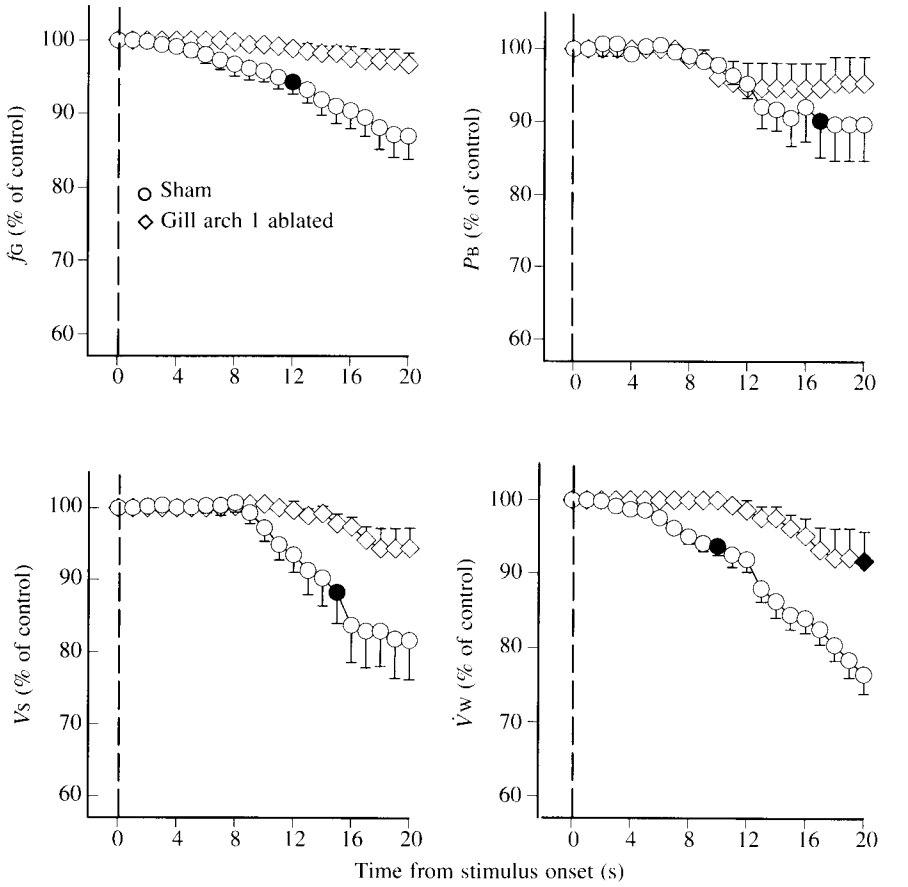

Fig. 4. Responses of gill ventilation variables in stage XVII–XIX larval *Rana catesbeiana* to a step-wise change in inspired water from normoxic to hyperoxic (broken vertical line at time 0 s). Open circles, sham-operated, gill arches intact, N=6. Open diamonds, gill arch 1 ablated, N=7. See legend to Fig. 2 for additional details of data presentation.

anesthetized larva (stage V–VII) had no effect on f_G, P_B or $\dot{V}_W$ within the 20 s measurement period. However, both middle and late stage larvae responded with increased gill ventilation, although with slower response times than when NaCN was injected into inspired water. The mean ventilatory response time for internally injected NaCN was 11.5±1.9 s in stage IX–XI larvae and 10.5±1.8 s in stage XVII–XIX larvae; these values were not significantly different from each other.

Blood circulation time

Mean f_H in anesthetized larvae (22–24 °C) ranged between 67 and 73 beats min^{-1} (Table 3), with no significant differences between developmental stages. A control injection of saline into the conus had no observable effect on f_H in any larval stage, but f_H began to decrease within 4–11.5 s of ACh injection. The heart typically came to a complete stop within a few beats after the first observation of slowing (Fig. 10). The mean response time (circulation time) for each stage was

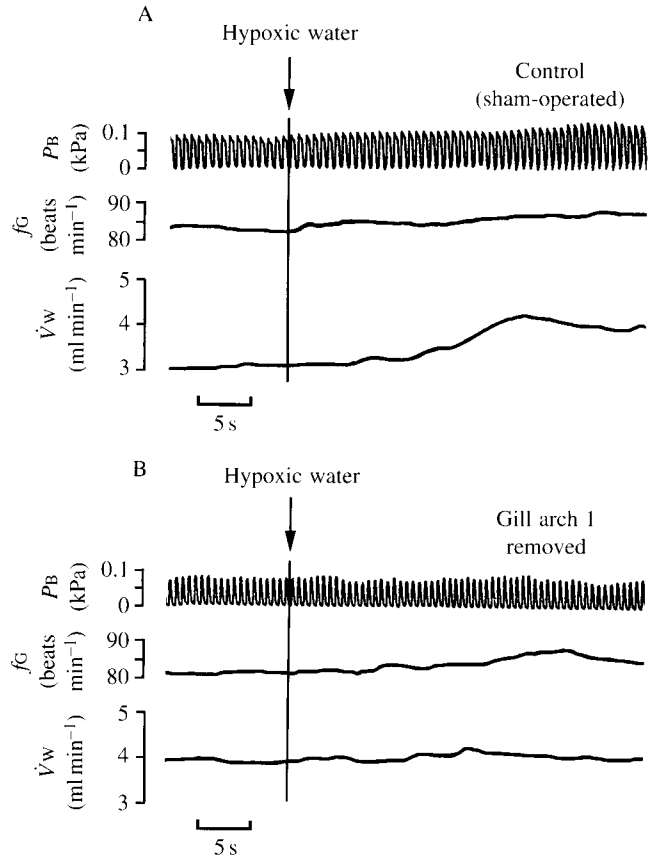

Fig. 5. Representative recordings of buccal pressure (P_B), gill ventilation frequency (f_G) and total branchial water flow ($\dot{V}_W$) in an unanesthetized stage X larval bullfrog in response to a step-wise change in inspired water from normoxic to hypoxic water (arrow). (A) The response under control (sham-operated) conditions. (B) The response in the same larva after recovery from bilateral ablation of gill arch 1. Time marker, 5 s.

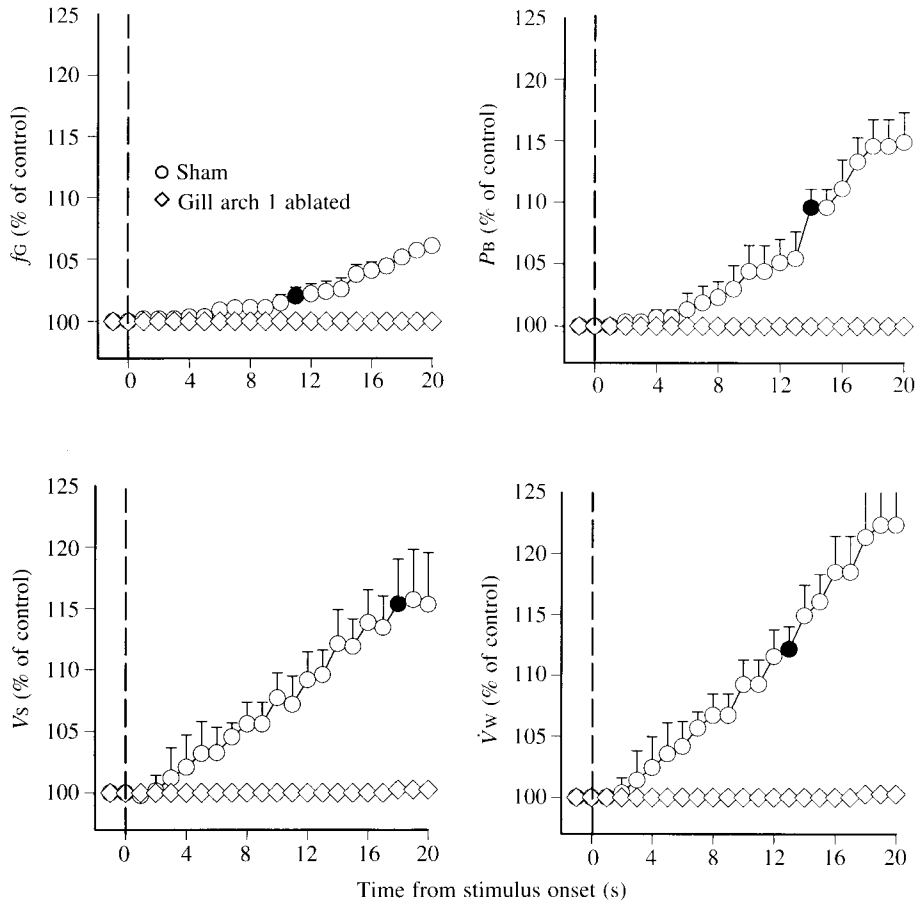

Fig. 6. Responses of gill ventilation variables in stage V–VII larval *Rana catesbeiana* to a step-wise change in inspired water from normoxic to hypoxic (broken vertical line at time 0 s). Open circles, sham-operated, gill arches intact, N=6. Open diamonds, gill arch 1 ablated, N=8. See legend to Fig. 2 for additional details of data presentation.

between 4.9 and 7.6 s, with no significant difference evident between developmental stages (Table 3).

Discussion

Peripheral receptors are of major importance in relaying chemical and mechanical information about ongoing ventilation and gas exchange with the blood (see review by Boutilier, 1988; Smatresk, 1988; Mitchell *et al.* 1990; Burggren *et al.* 1997). Among the lower vertebrates, the process of ventilatory regulation by chemoreceptive feedback is best described for fish. Aquatic hypoxia increases and aquatic hyperoxia decreases gill ventilation. O_2-sensitive chemoreceptors mediating these responses are located on the gills in channel catfish (*Ictalurus punctatus*) and specifically on the first gill arch in trout (*Oncorhynchus mykiss*) (Daxboeck and Holeton, 1978; Smith and Jones, 1978; Smith and Davie, 1984; Burleson and Milsom, 1993). The first gill arch of both fish and larval Amphibia are innervated by cranial nerve IX (Rugh, 1951; Nilsson, 1984), which is the same innervation as that of the carotid body in mammals and the carotid labyrinth in adult amphibians. Stimulating the receptors on the first branchial arch of teleosts also causes bradycardia. However, these receptors do not affect either the amplitude or the frequency of breathing (Daxboeck and Holeton, 1978; Burleson and Smatresk, 1990), indicating that there are at least two loci of O_2-sensitive chemoreceptors in teleost fish.

In adult anurans, the carotid labyrinth, which is innervated by the glossopharyngeal nerve, plays an important O_2-chemoreceptive function in the control of breathing. Perfusion of the carotid labyrinth of the adult toad (*Bufo vulgaris*) with O_2-free saline or dilute solutions of cyanide increases action potential frequency in nerve IX, corresponding to a stimulation of breathing (Ishii *et al.* 1966; see reviews by Boutilier, 1988; West and Van Vliet, 1992). In adult bullfrogs, injection of cyanide into the carotid arch increases ventilation (Lillo, 1980). However, in adult

Table 3. *Body mass, heart rate and blood circulation time in anesthetized larval bullfrogs*

	Stage V–VII	Stage IX–XI	Stage XVII–XIX
Body mass (g)	4.1±0.5 (10)	15.8±0.8 (9)	12.4±1.4 (9)
f_H (beats min^{-1})	72.9±1.6 (10)	73.3±1.2 (9)	66.7±3.4 (9)
Blood circulation time (s)	7.1±0.4 (10)	7.6±0.6 (9)	4.9±0.6 (8)

Mean values ± S.E.M. (N) are presented.

Stage of development had no significant effects (P<0.05) on either heart rate (f_H) or blood circulation time.

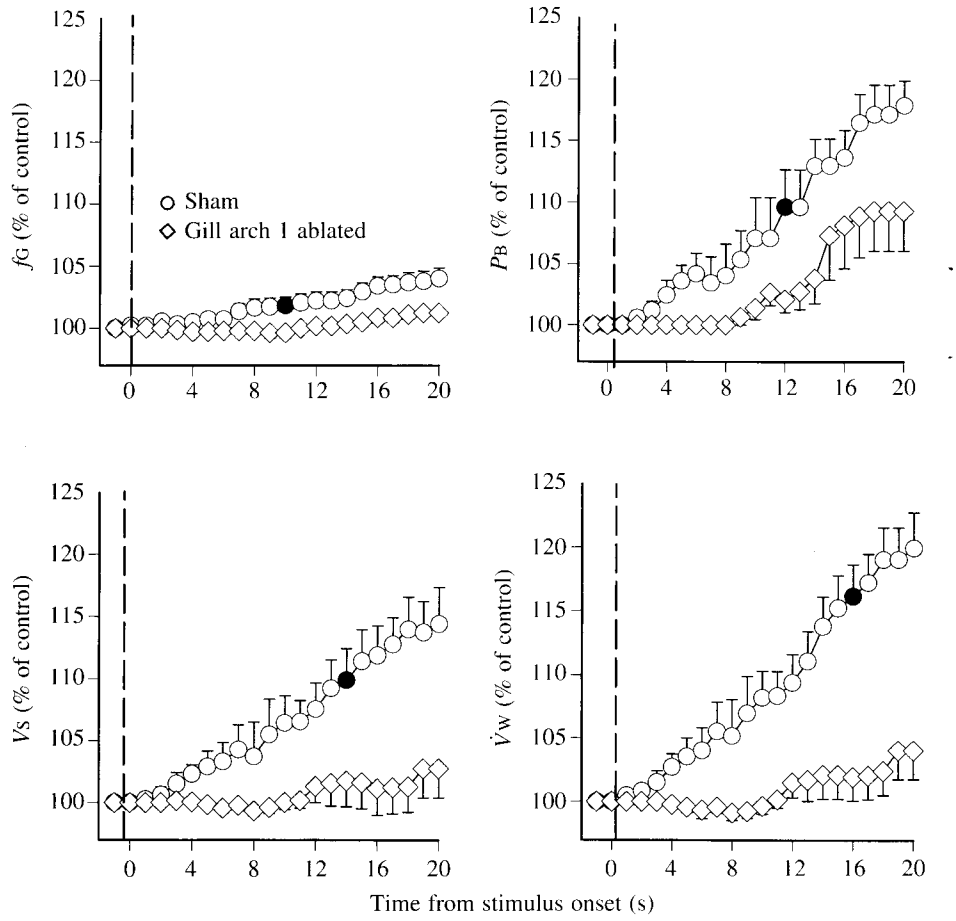

Fig. 7. Responses of gill ventilation variables in stage IX–XI larval *Rana catesbeiana* to a step-wise change in inspired water from normoxic to hypoxic (broken vertical line at time 0 s). Open circles, sham-operated, gill arches intact, N=6. Open diamonds, gill arch 1 ablated, N=9. See legend to Fig. 2 for additional details of data presentation.

Xenopus laevis and *Bufo marinus*, denervation of the carotid labyrinth causes an overall reduction in ventilation but does not abolish the ventilatory sensitivity to hypoxemia (Van Vliet and West, 1986). Thus, the carotid labyrinth has a role, but not the only role, in regulating lung ventilation (see Boutilier, 1988). Indeed, injection of cyanide into the pulmocutaneous artery of the bullfrog stimulates ventilation within 5–8 s, indicating that this arch or its derivatives also supplies a chemoreceptive area, probably in the arch wall (Lillo, 1980). Ishii *et al.* (1985) have shown that there are chemoreceptive regions within the aortic trunk of the toad (*Bufo vulgaris*) that are apparently linked to respiratory control. There is evidence, therefore, for more than one site of O_2-sensitive chemoreception in adult anurans.

Little is known about peripheral chemoreceptors affecting ventilation in larval amphibians. West and Burggren (1983) provided evidence of lung mechanoreceptors and proposed the existence of O_2-sensitive receptors in stage XVII–XIX larval bullfrogs. The present study, however, provides the first direct proof of peripheral O_2-sensitive receptors modulating gill ventilation in larval bullfrogs. Our results, which indicate that the gill ventilation of larval bullfrogs is continuously modulated by afferent inputs from peripheral chemoreceptors, will now be summarized and discussed according to developmental stages.

O_2 chemoreception in early (stage V–VII) larvae

Larvae in stages V–VII show more pronounced changes in gill ventilation in response to inspired hyperoxic or hypoxic water than older stage XVII–XIX larvae (Figs 2–4, 6–8; also Jia and Burggren, 1997). This response is mediated by O_2-sensitive chemoreceptors on the first gill arches, since bilateral ablation of the first gill arch eliminated all significant gill ventilatory responses to changes in the P_{O_2} of inspired water within 20 s. In addition, the extremely rapid response to externally applied NaCN, coupled with the delayed response to NaCN internally injected into the circulation (see below), suggests that these receptors monitor P_{O_2} of the branchial water. Again, and consistent with this observation, removal of the first branchial arch eliminated or greatly delayed the rapid NaCN response.

Following bilateral ablation of gill arch 1, the rapid response to an inspired pulse of NaCN was replaced by a much slower (19 s) ventilatory response (Table 2). This slow response was also far longer than the minimum blood circulation time of approximately 7 s for this stage (Table 3). The long delay could be explained by a significant diffusion barrier between the blood and internal chemoreceptor sites, as one might expect for receptors monitoring cerebrospinal fluid or other tissue compartments not receiving direct blood flow. An alternative explanation for the long delay might be a delayed secondary response produced by the release of adrenal catecholamines or

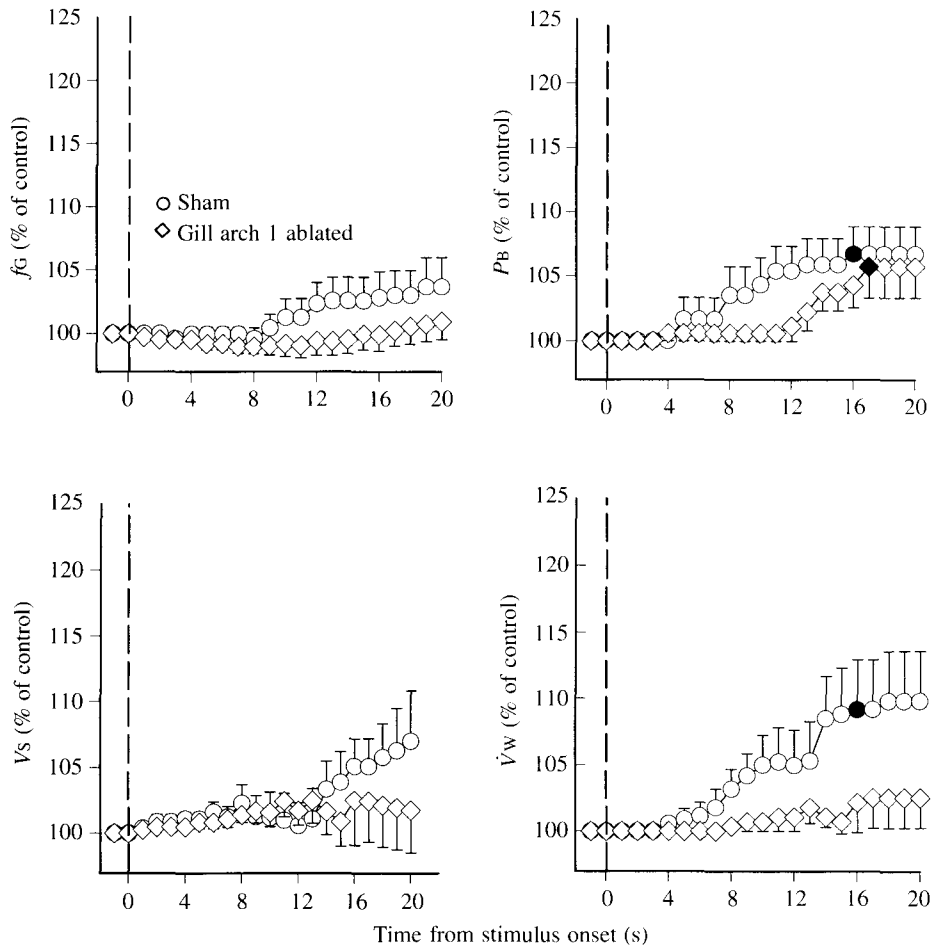

Fig. 8. Responses of gill ventilation variables in stage XVII–XIX larval *Rana catesbeiana* to a step-wise change in inspired water from normoxia to hypoxic (broken vertical line at time 0 s). Open circles, sham-operated, gill arches intact, N=6. Open diamonds, gill arch 1 ablated, N=7. See legend of Fig. 2 for additional details of data presentation.

other hormones in response to injections of NaCN. We think it unlikely, however, that the delay is simply due to compromised NaCN uptake from inspired water following ablation of gill arch 1, since the surface of gill arch 1 in stage XV larval *R. catesbeiana* probably accounts for less than 25 % of the total branchial surface area, based on estimates derived from gill morphometric studies (Burggren and Mwalukoma, 1983). Instead, these observations of a secondary ventilatory response are more likely to be explained by the presence of a second, internally located group of O_2-sensitive chemoreceptors that ultimately influence the respiratory center in the central nervous system, and they also indicate that these receptors do not lie directly in the path of arterial or venous blood.

The finding of a slow ventilatory response approximately 18–20 s after inspiration of NaCN in larvae with bilateral ablation of gill arch 1 also indicates that the input of peripheral O_2-sensitive chemoreceptors located in the first gill arch (accounting for the fast response) normally either inhibits input from more centrally situated chemoreceptors in the central nervous system (CNS) or is preferentially integrated and acted upon by the respiratory center of the autonomic nervous system. A complex interaction between known peripheral and central receptors on respiration has also been reported in the adult toad (*Bufo marinus*) (Smatresk and Smits, 1991).

Finally, it is noteworthy that bilateral ablation of the first gill arch elevated f_G in normoxic water in unanesthetized stage V–VII larvae. One explanation is that there is a tonic input from the first gill arch to the CNS that contributes to the maintenance of normal gill ventilation rates in early bullfrog larvae. Alternatively, ablation of gill arch 1 could lower arterial P_{O_2} and reflexively stimulate f_G *via* internal receptors. The effects on blood oxygenation of bilateral ablation of gill arch 1 in the bullfrog larva are unknown but, as noted above, the surface area of branchial arches 1 in larval bullfrogs is a relatively small percentage of the total gill surface area. In elasmobranchs, at least, ligation of individual gill arches is compensated for by changes in ventilation/perfusion relationships of the other gill arches, such that neither arterial P_{O_2} nor O_2 consumption are affected (Cameron *et al.* 1971).

O_2 chemoreception in middle (stage IX–XII) larvae

As in the stage VI–VII larvae, middle stage larvae showed pronounced and very rapid changes in gill ventilation in response to changes in the P_{O_2} of inspired water or to the presence of NaCN. Ablation of gill arch 1 similarly either eliminated the gill ventilatory response within 20 s or greatly delayed them. As in the younger larvae, these data indicate that there are at least two sites of O_2-sensitive chemoreceptors.

Middle stage larvae responded differently from early larvae, however, in that, when intact, middle stage larvae showed a

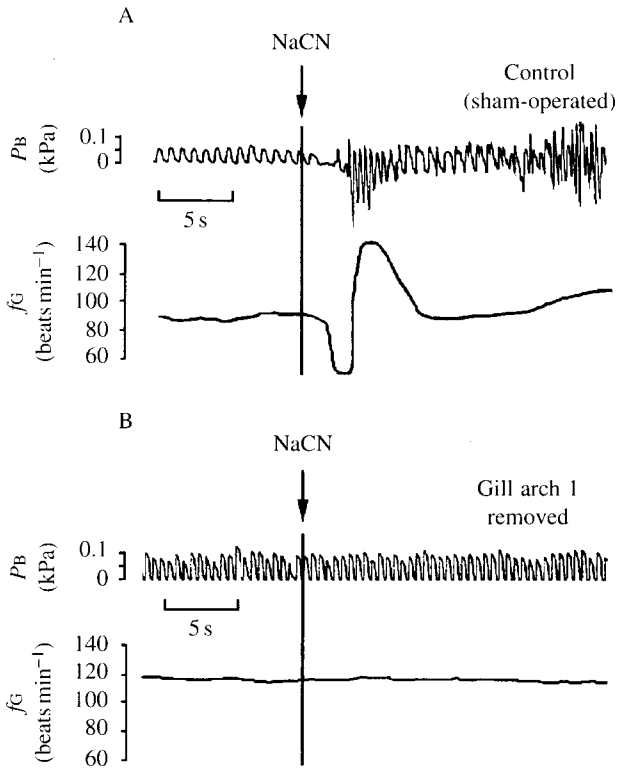

Fig. 9. Representative recordings of buccal pressure (P_B) and gill ventilation frequency (f_G) in an unanesthetized stage VI larva in response to a pulse of 0.5 % NaCN introduced into the inspired water stream (arrow). (A) The response under control (sham-operated) conditions. (B) The response in the same larva after recovery from bilateral ablation of gill arch 1. Time marker, 5 s.

gill ventilatory response to NaCN injected into the conus. This NaCN response, requiring approximately 11–12 s, was significantly slower than the 7–8 s blood circulation time. It is possible that the internally applied NaCN diffused out of the branchial capillaries and stimulated O_2 receptors in the first gill arches. However, the slower response still occurred after bilateral ablation of the first gill arch, indicating that the slow response must be from chemoreceptors at other sites.

Also in contrast to early stage larvae, removal of the first gill arch caused a modest drop in f_G in normoxic water in middle stage larvae, suggesting that there is little tonic input from these receptors to the CNS.

O_2 chemoreception in late (stage XVII–XIX) larvae

In older larvae, the reflex responses of gill ventilation to changes of inspired water P_{O_2} were not as pronounced as in younger larvae, and the response time in intact larvae was also slower than in the two younger groups. Bilateral ablation of the first gill arch in older stage larvae eliminated the quick gill ventilatory responses to step-wise changes of P_{O_2} of inspired water and to externally applied NaCN. These data confirm the existence of O_2-sensitive chemoreceptors on the first gill arch of late stage larvae, even as these arches are beginning to degenerate and be incorporated into the more proximal elements of the future carotid circulation (see Burggren and Just, 1992). As in stage IX–XI larvae, removal of the first gill arches did not significantly affect gill ventilation in normoxic water, indicating that there is little or no tonic afferent activity in these branchial receptors.

Ablation of gill arch 1 still allowed a slower ventilatory response to occur, suggesting a second, remote site of O_2-sensitive chemoreceptors in older larvae, as found in young and middle stage larvae. The response time to externally applied NaCN was also slower in intact older larvae than in the other two groups. This may be because these receptors are intrinsically less sensitive to changes in P_{O_2}. In mammals (sheep), the carotid chemoreceptors are active and responsive to natural stimuli in the fetus, but are quiescent in the lamb on the day of birth when arterial P_{O_2} has risen. The hypoxic sensitivity of chemoreceptors is reset from the fetal to the adult range over the first few days following birth (Blanco *et al.* 1984; Hanson *et al.* 1993). An analogous resetting phenomenon could occur in the bullfrog at around the onset of air-breathing or later on in development towards metamorphic climax. However, a

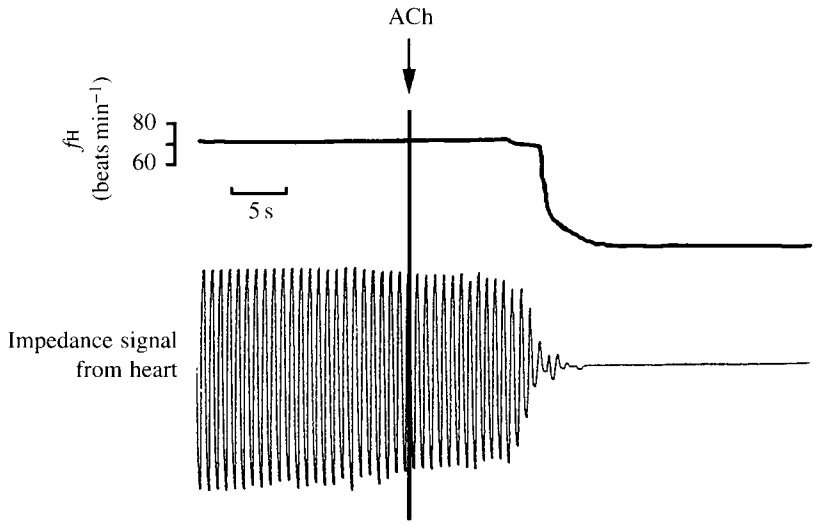

Fig. 10. Representative recordings of calculated f_H and the heart impedance signal and in an unanesthetized stage VI larva. A 0.4 µl pulse of 10^{-2} mol l^{-1} acetylcholine (ACh) was injected at the arrow into the conus immediately proximal to the pylangial valves separating the ventricle from the central arterial circulation. Time marker, 5 s.

reduction in response time to inspired NaCN in late larvae could also be due to the degenerative thickening of the gill arches, which has begun by this developmental stage and which would provide a greater barrier for diffusion of NaCN to the branchial sites of the receptors. Additional data from anurans will be needed to help resolve this issue.

As in middle larval stages IX–XI, NaCN injected into the central arterial circulation transiently increased gill ventilation. The response time was again far slower than the blood circulation time, indicating that these O_2-sensitive elements are not directly facing arterial or venous blood.

Overview of O_2 reception in larval bullfrogs

The data from the present study and our companion study (Jia and Burggren, 1997) confirm the existence of peripheral chemoreceptors located in the first branchial arch of larval bullfrogs. These receptors convey information to the CNS on changes in ambient O_2 levels. Additionally, in early (but not middle or late) larvae, these receptors appear to have tonic activity in normoxia and provide a 'hypoxic drive' for ventilation, since removal of these receptors by ablation of the first pair of gill arches significantly reduces both buccal pumping and the volume of water passing over the gills. Receptors located more centrally, as well as peripheral receptors, appear to be involved in modulating gill ventilation in larval bullfrogs. Studies on modulation of output from central pattern generators in the brain stem of larval bullfrogs (Kinkead *et al.* 1994; Gdovin *et al.* 1996) reveal complex patterns of efferent motor activity associated with both rhythmic gill ventilation and, in older larvae, irregular lung ventilation.

Mechanoreceptors are also involved in regulating gill ventilation in anuran larvae. Mechanoreceptor activity from the lungs of larval bullfrogs conveys information on the state of lung inflation (West and Burggren, 1983). Although the sites of vascular mechanoreceptors have not been specifically identified, there are certainly a myriad of cardiovascular reflex responses that are likely to be mediated, wholly or in part, by such mechanoreceptors (Burggren, 1995; Hou and Burggren, 1996; Fritsche and Burggren, 1996).

Taking into account that middle and later stage larval bullfrogs are breathing with two respiratory media (air and water) using three distinct modes for gas exchange (cutaneous, pulmonary and branchial) and that both peripheral and more centrally located chemoreceptors are involved, then the overall picture of respiratory control in bullfrog larvae is complex indeed. In this respect, the overall complexity of larval anuran respiratory and cardiovascular control appears to be at least as great as that in higher vertebrates and suggests that their emerging use as a model for complex higher vertebrate systems is well justified.

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