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The effects of arginine vasotocin and catecholamines on nitrogen excretion and the cardio-respiratory physiology of the gulf toadfish, *Opsanus beta*

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Abstract Simultaneous measurements of cardio-respiratory variables, oxygen uptake and whole body urea/ammonia/tritiated water effluxes were performed on cannulated gulf toadfish, *Opsanus beta*, before and after intra-arterial injection of the vasoactive agents, adrenaline, isoproterenol and arginine vasotocin. These experiments were conducted to test the hypothesis that the phenomenon of pulsatile urea excretion might reflect sudden changes in the general diffusive properties of the gill for solute transfer. Injection of isoproterenol (final nominal circulating level = 10^{-6} mol l^{-1}), was used as a tool to maximise the diffusive and perfusive conditions for branchial solute transfer. This protocol caused a pronounced reduction in arterial blood pressure, an elevation of cardiac frequency and associated increases in whole body urea and tritiated water effluxes; ammonia excretion and oxygen uptake were unaffected. Injection of adrenaline (final nominal circulating level = 10^{-6} mol l^{-1}), caused a significant increase in arterial blood pressure and a tachycardia, yet nitrogen excretion and oxygen uptake were unaffected. Injection of arginine vasotocin, caused a dose-dependent (final nominal circulating levels = 10^{-11}–10^{-9} mol l^{-1}) increase in arterial blood pressure without affecting cardiac or ventilation frequency. At the two higher concentrations, arginine vasotocin caused large and transient increases in urea excretion without significantly affecting ammonia, water or oxygen fluxes. These results suggest that increased gill diffusive or perfusive conductance, while capable of augmenting urea efflux, cannot fully explain the sudden and massive increases in urea transfer associated with pulsatile urea excretion in toadfish. It is suggested that pulsatile urea excretion in this species may reflect a specific enhancement of urea excretion under the control of the neurohypophyseal hormone, arginine vasotocin.

Key words Urea · Ammonia · Gill · Arginine vasotocin · Adrenaline

Abbreviations AVT arginine vasotocin · H_f cardiac frequency · J_{amm} ammonia excretion · J_{urea} urea excretion · MO_2 oxygen uptake · RUT renal urea transporter · UT urea transporter · V_f breathing frequency

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Introduction

The marine toadfish, *Opsanus beta*, does not conform to the standard pattern of nitrogenous waste excretion that is observed in the vast majority of teleost fish (see reviews by Mommsen and Walsh 1992; Wright 1995). Unlike the usually ammoniotelic teleosts, the toadfish is ureogenic owing to an active hepatic ornithine-urea cycle (Mommsen and Walsh 1989; Anderson and Walsh 1995); see reviews by Anderson 1995; Walsh 1997). Consequently, under appropriate laboratory conditions such as crowding or confinement, toadfish display fac-

ultative ureotelism, whereby the bulk of nitrogen excretion is in the form of urea (Walsh et al. 1990, 1994). Recently, Wood et al. (1995, 1997) demonstrated that ureotelism in laboratory toadfish is characterised by pulsatile excretion, in which more than 90% of daily urea efflux occurs during a single pulse of less than 3 h duration.

The underlying mechanisms of pulsatile branchial urea excretion in the toadfish have not been established. Although the diffusive permeability of the branchial epithelium to urea is low (P. Pärt, personal communication), it is nevertheless conceivable that the movement of urea across the gill could be accelerated by any factor known to enhance the rate of solute transfer across the gill (see Gilmour 1997). Such factors include hyperventilation, increased cardiac output and enhanced branchial diffusive conductance (primarily related to gill surface area and diffusion distance). Thus, pulsatile urea excretion could reflect sudden and large transitory changes in the diffusive and/or convective properties of the gill for solute transfer.

Walsh (1997) and Wood et al. (1998) have proposed that urea excretion in toadfish may involve a specific urea transport mechanism analogous to the vasopressin-sensitive renal urea transporter (RUT) of mammals (You et al. 1993). If so, an alternate explanation for pulsatile urea excretion might be a specific modulation of the urea transport mechanism.

In the present study, we have investigated two possible explanations for pulsatile urea excretion in the toadfish. First, we tested the idea that a sudden generalised increase in gill conductance may be the underlying mechanism of pulsatile urea excretion. This was accomplished by using intra-vascular injections of the naturally occurring catecholamine, adrenaline, and the synthetic β-adrenoceptor agonist, isoproterenol, as tools to modify gill diffusive and perfusive conductances while assessing the impact on whole body nitrogen excretion. Second, we evaluated the effects of the neurohypophyseal hormone, arginine vasotocin (AVT), on the cardiovascular system and whole body nitrogen excretion. These experiments were designed to test the idea that, by analogy with the RUT of mammals, the permeability of the toadfish gill to urea could be specifically modified independently of any generalised increases in the conditions for solute transfer.

Materials and methods

Animals, holding conditions and surgical procedures

Sexually mature gulf toadfish were captured by trawl by local fishermen in Biscayne Bay, Florida. At the University of Miami, fish were maintained initially in glass aquaria supplied with flowing Biscayne Bay seawater (29–34 ppt, temperature = 25 ± 1 °C). Each aquarium contained a sand/gravel substrate (5–10 cm depth) and several polyvinyl chloride tubes that acted as individual shelters. Typically, between three and four fish inhabited a single 45-l aquarium corresponding to a density of approximately 7–10 g fish l^{-1}. On days 1 and 3 after arrival, the fish were bathed in a mixture of Malachite Green and formalin (Wood et al. 1995) as a prophylactic treatment against the ciliate *Cryptocaryon irritans*. Fish were maintained under these conditions for at least 1 week and were fed ad libitum with live shrimp on alternate days.

To induce ureotelism, fish, weighing between 43 and 230 g (mean mass = 106.7 ± 9.0 g; $n = 25$), were subjected to a standardised crowding protocol (Walsh et al. 1994; Hopkins et al. 1995; Wood et al. 1995) 48–72 h prior to experimentation. Briefly, this procedure involved placing from three to six fish and their tube shelter in small plastic tubs (about 6 l volume) to achieve densities exceeding 80 g fish l^{-1}. The tubs were aerated continually and supplied with flowing seawater. The fish were not fed during the period of crowding.

Surgery

Fish were anaesthetised in a solution of MS 222 (0.67 g l^{-1}) that was adjusted to approximately pH 8.0 using NaOH. Upon cessation of breathing, fish were transferred to an operating table where anaesthesia was sustained for the duration of surgery (usually less than 1 h) by wrapping the fish in paper towels soaked with anaesthetic solution. The gills were not irrigated during surgery.

In all fish, the caudal artery was cannulated with polyethylene tubing (Clay Adams PE 50) after exposing the vessel with a lateral incision along the caudal peduncle. Prior to suturing the wound, the exposed muscle was dusted with oxytetracycline. The cannula was secured to the tail using several ligatures. In a few fish, the caudal vein was cannulated additionally using a similar technique. In two fish, the ventral aorta was cannulated percutaneously by puncturing the soft tissue of the tongue and inserting a guide wire and cannula into the vessel. The ventral aorta cannula was led out of the lower jaw through a polyethylene sleeve (PE 160).

To collect expired water and to assess ventilation (see below), cannulae (PE 160) were inserted into each gill pouch and secured with ligatures. Inspired water was collected by inserting a cannula (PE 160) into the buccal cavity. In some fish, ventilation was assessed by measuring impedance changes associated with movements of the gill pouches. In these cases, small diameter shielded wires (28–30 A.W.G.) were sutured to each gill pouch cover.

Following surgery, fish were transferred to individual respirometers that were supplied with flowing seawater (approximately 100 ml min^{-1}). Spontaneous breathing recommenced within 1–2 min of being placed in the respirometer. The fish were allowed to recover for 24 h prior to experimentation.

Experimental protocol

The basic experimental protocol involved monitoring cardio-respiratory variables (arterial blood pressure, cardiac frequency, ventilation frequency and amplitude and oxygen uptake), nitrogen excretion (ammonia and urea) and tritiated water efflux before and after injecting isoproterenol, adrenaline or AVT. In all experiments, data were collected during a 5-min pre-injection period followed by a 35-min post-injection period.

The synthetic selective β-adrenoceptor agonist, isoproterenol, or the naturally occurring non-selective adrenoceptor agonist, adrenaline, were used as tools to increase the functional surface area of the gill (Oduleye and Evans, 1982; Oduleye et al. 1982) and thus increase branchial diffusive conductance for solute transfer. Fish were injected with 3×10^{-7} mols kg^{-1} of L-isoproterenol bitartrate or L-adrenaline bitartrate dissolved in 150 mmol l^{-1} NaCl to yield final nominal circulating levels in the blood of 1×10^{-6} mol l^{-1}. This calculation assumed rapid equilibration of the injected drug with the extracellular fluid volume (estimated as 300 ml kg^{-1}). By analogy with the vasopressin/vasotocin-sensitive water channels or urea transporters of the mammalian kidney/amphibian bladder (e.g. Nielsen et al. 1996), injection of the naturally occurring neurohypophyseal peptide AVT was used to determine whether urea and/or tritiated water efflux could be specifically stimulated. Fish were injected with 3×10^{-12}–3×10^{-10} mols kg^{-1} of AVT (Sigma) dissolved in physiological

saline to yield final nominal circulating levels in the blood of 10^{-11} – 10^{-9} mol l^{-1}. These levels were chosen based on prior measurements of circulating AVT in fish (Perrott et al. 1991; Pierson et al. 1995; Warne and Balment 1995).

Cardio-respiratory variables

Arterial blood pressure was measured by attaching the saline-filled caudal artery cannula to a pressure transducer (Model 1050BP; UFI) connected to a data acquisition system (Harvard Biopac) and computer and calibrated against a static column of water. Cardiac frequency (H_f) was determined automatically using a rate function feature of the data acquisition system. In two fish, caudal artery (post-branchial) and ventral aorta (pre-branchial) pressures were measured simultaneously by using a second pressure transducer.

Ventilation was assessed in two different ways. One method involved using an impedance converter (Transmed Scientific, Model 2992) to measure the changes in impedance between the two wire leads sutured to each gill pouch. The impedance data were converted to measurements of ventilation amplitude by a calibration procedure that compared linear deflections (in cm) of the wire leads to the corresponding impedance changes. Thus, ventilation amplitude in these experiments refers to the absolute change in distance between the two gill pouch covers during a breathing cycle. Breathing frequency (V_f) was determined automatically using the acquisition software. A second method used to monitor ventilation in some fish involved measuring pressure changes in the gill pouches associated with the breathing cycle. One of the gill pouch cannulae was filled with seawater and connected to a pressure transducer (Elcomatic Model EM751 with a Harvard transducer amplifier) linked to the data acquisition system. The pressure transducer was calibrated daily against a static column of water. V_f was determined automatically using the data acquisition software.

Oxygen uptake (MO_2) was determined using flow-through respirometry. Inflowing water PO_2 was measured by siphoning water from the tube providing water to the respirometer into a temperature-controlled cuvette containing a PO_2 electrode (Radiometer E5046) linked to an O_2 meter (Cameron Instruments Inc.). Output from the O_2 meter was collected by the data acquisition system. Outflowing water (i.e. the water exiting the respirometer) PO_2 was determined by siphoning water from the exit tube of the respirometer into a second temperature-controlled cuvette housing an identical O_2 electrode. MO_2 (in mmol kg^{-1} h^{-1}) was calculated according to the following equation:

$$MO_2 = \frac{\left[(\text{inflowing } PO_2 - \text{outflowing } PO_2) \times \alpha O_2 (\text{mmol } l^{-1} \text{ torr}^{-1}) \times \text{water flow rate } (l\ h^{-1})\right]}{\text{fish mass (kg)}}$$

where αO_2 is the solubility coefficient of O_2 in seawater (35 ppt) at the appropriate temperature (from Boutilier et al. 1984).

Nitrogen excretion

Urea excretion (J_{urea}) was monitored by measuring the appearance of ^{14}C-urea in the outflowing water of the respirometer. Wood et al. 1997 have shown that the appearance of ^{14}C-urea in the external seawater provides a valid measure of net urea excretion when the specific activity of the blood is taken into account. Approximately 2.5 h prior to starting an experiment, fish were injected via the arterial cannula with 1.48×10^7 B_9 kg^{-1} of ^{14}C-urea (NEN; specific activity = 3.2×10^8 B_9 $mmol^{-1}$) in 0.2 ml saline (Wood et al. 1997). Just prior to beginning an experiment, a blood sample was removed for subsequent analysis of plasma ^{14}C activity and urea/ammonia concentrations. To commence an experiment, a total of five consecutive 1-min outflowing water samples (3 ml) were taken to reflect the pre-injection period. After injection of the appropriate drug into the arterial cannula, outflowing water samples were collected over an ensuing 35 min period. All water samples were subsequently analysed for ^{14}C activity (cpm ml^{-1}) and urea/ammonia concentrations.

J_{urea} (in μmol kg^{-1} h^{-1}) was calculated according to the following equation:

$$J_{urea} = \frac{\text{outflowing water urea cpm } ml^{-1} \times \text{water flow rate (ml } h^{-1})}{\text{plasma urea specific activity (cpm } \mu mol^{-1}) \times \text{fish mass (kg)}}$$

Ammonia excretion (J_{amm}; in μmol kg^{-1} h^{-1}) was determined by measuring the levels of total ammonia (NH_3 and NH_4^+) in the inspired and outflowing water and applying the following equation:

$$J_{amm} = \frac{\left[\text{outflowing water [amm]} - \text{inflowing water[amm]}(\mu mol\ l^{-1}) \times \text{water flow rate } (1\ h^{-1})\right]}{\text{fish mass (kg)}}$$

where [amm] is the concentration of total ammonia.

Tritiated water excretion

In several experiments, fish also were injected with $2.2 - 3.0 \times 10^6$ B_9 of [^{3}H]-H_2O (New England Nuclear, 3.1×10^7 B_9 g^{-1}). The rate of diffusive water efflux (J_{water}; μmol kg^{-1} h^{-1}) was calculated assuming that [water] in body tissues was 1000 g/l, or 55.5 mol/l using the following equation:

$$J_{water} = \frac{\text{outflowing water } ^3H \text{ cpm } ml^{-1} \times \text{water flow rate (ml } h^{-1})}{\text{Plasma water specific activity (cpm } \mu mol^{-1}) \times \text{fish mass (kg)}}$$

Analytical procedures

The concentrations of urea or ammonia in seawater and plasma were determined using standard colorometric assays (Ivancic, Degobbis 1984; Price and Harrison 1987) employing a micro-plate spectrophotometer (Molecular Devices Thermomax).

For experiments involving ^{14}C-urea single labelling, plasma (10 μl plus 1 ml seawater plus 10 ml Ecolume fluor) or water (1 ml seawater plus 10 ml Ecolume fluor) radioactivity was determined using a liquid scintillation counter (TM Analytic Beta Trac 6895). For experiments incorporating ^{14}C-urea and ^{3}H-water dual labelling, samples were prepared for counting as above but a Beckman LS1801 liquid scintillation counter was used for simultaneous determination of ^{14}C and ^{3}H activities. Quench was corrected using onboard programs.

Data presentation and statistical analyses

Mean data were compiled for 1 and 5 min intervals corresponding to the sampling periods during the pre- and post-injection periods. Data were analysed by repeated measures one-way analysis of variance followed by Dunnet's post-hoc multiple comparison test, which compared all data points to the final point of the pre-injection period. Where assumptions of normality or equal variance were violated, the data were analysed by equivalent non-parametric tests. In all cases, the statistical level of significance was set at 5% ($P < 0.05$).

Results

Effects of isoproterenol

Intra-arterial injections of isoproterenol caused large and persistent cardiovascular changes that were consistent with stimulation of β-adrenoceptors (Figs. 1, 2). In all fish that were examined, isoproterenol elicited a pronounced reduction in arterial blood pressure (Fig. 1A) despite a concomitant sustained increase in H_f

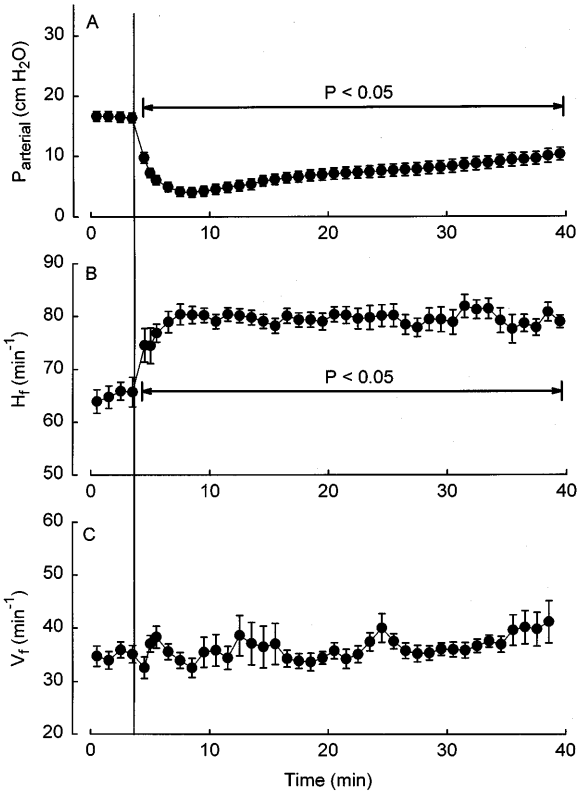

Fig. 1A–C The effects of intra-arterial injection of isoproterenol (nominal circulating concentration after injection = 10^{-7} mol l^{-1}) on (**A**) arterial blood pressure ($P_{arterial}$), (**B**) cardiac frequency (H_f) and (**C**) breathing frequency (V_f) in gulf toadfish (*Opsanus beta*). The *vertical line* indicates the time of injection. Data are shown as mean values ± 1 SEM (n = 11); the *horizontal double arrows* indicate data points that are statistically different (P < 0.05) from the value immediately prior to injection

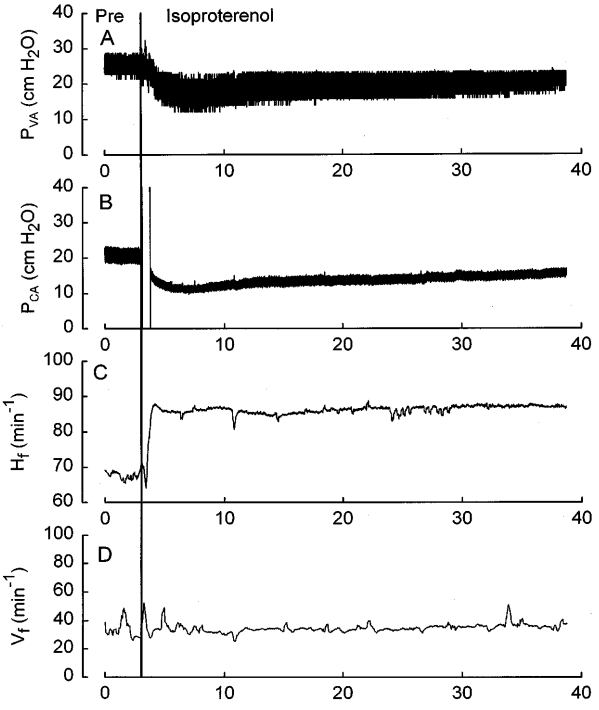

Fig. 2A–D A representative original data recording illustrating the effects of intra-arterial injection of isoproterenol (nominal circulating concentration after injection = 10^{-7} mol l^{-1}) on (**A**) ventral aorta blood pressure (P_{VA}), (**B**) caudal artery blood pressure (P_{CA}), (**C**) cardiac frequency (H_f) and (**D**) breathing frequency in gulf toadfish (*O. beta*). The *vertical line* indicates the time of injection

of approximately 15 beats min^{-1} (Fig. 1B). Blood pressure gradually increased towards pre-injection values but nevertheless remained significantly depressed upon termination of the experiment. V_f was unaltered by isoproterenol treatment (Fig. 1C). Mean values for ventilation amplitude were not compiled owing to the difficulty in obtaining stable recordings (principally as a result of sudden baseline shifts during impedance measurements) and the incomparability between the two different measuring techniques (impedance versus pressure measurements). Figure 2 depicts an original representative data recording from a toadfish fitted with caudal artery (post-branchial) and ventral aorta (pre-branchial) cannulae. Note, in particular, the parallel decreases in both pre- and post-branchial blood pressures (Fig. 2A, B) and the increase in H_f (Fig. 2C) associated with the injection of isoproterenol.

Isoproterenol treatment caused an approximate 3-fold increase in whole body tritiated water efflux (Fig. 3A). Urea excretion also was increased by about 3-fold within the initial 15 min after the injection (Fig. 3B); the increase in the variability in the data after 15 min post-injection reflected a single fish that exhibited a sudden and large increase in urea excretion of about 3000 μmol kg^{-1} h^{-1}. J_{amm} (Fig. 3C) and MO_2 (Fig. 3D) were unaffected by isoproterenol injection.

Effects of adrenaline

The injection of adrenaline caused a transient doubling of arterial blood pressure (Fig. 4A) that was accompanied by a transient increase (10 beats min^{-1}) of H_f frequency (Fig. 4B); V_f was unaffected (Fig. 4C). J_{urea} and J_{amm} were not affected by adrenaline injection (Figs. 5A, B) while MO_2 was significantly lowered by approximately 30% (Fig. 5C).

Effects of AVT

Injections of AVT caused dose-dependent increases in arterial blood pressure (Fig. 6). At the highest dose (3×10^{-10} mols kg^{-1} yielding estimated levels in the blood of 1×10^{-9} mol l^{-1}), AVT caused a 2-fold increase in pressure during the maximal phase of the response; blood pressure remained significantly elevated upon termination of the experiment. At the lowest dose (3×10^{-12} mols kg^{-1} yielding estimated levels in the blood of 1×10^{-11} mol l^{-1}), AVT caused a 1.5-fold increase in pressure that was short-lived.

Figure 7 illustrates a representative data recording from a fish fitted with pre- and post-branchial blood

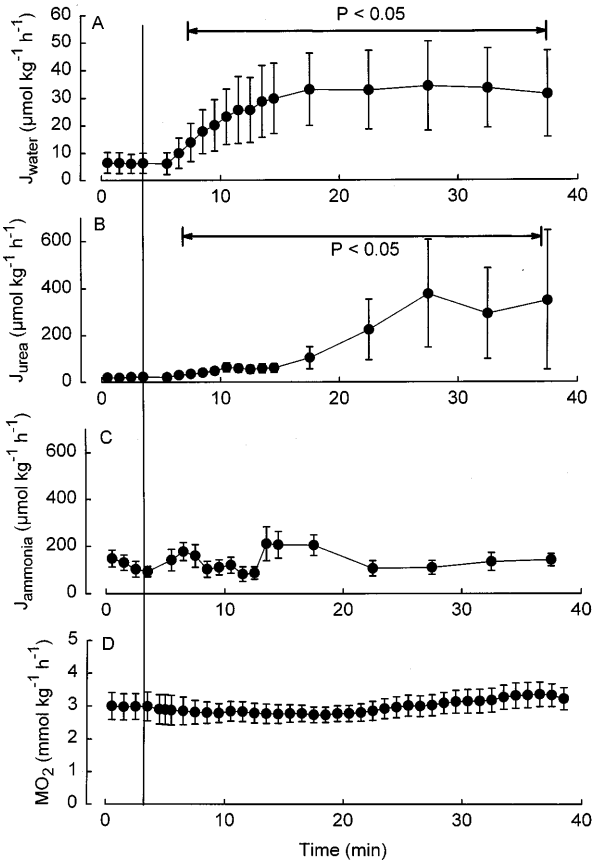

Fig. 3A–D The effects of intra-arterial injection of isoproterenol (nominal circulating concentration after injection = 10^{-7} moll^{-1}) on whole body (**A**) diffusive water efflux (J_{water}; $n = 6$), (**B**) urea efflux (J_{urea}; $n = 12$), (**C**) ammonia efflux ($J_{ammonia}$; $n = 12$) and (**D**) oxygen uptake (MO_2; $n = 5$) in gulf toadfish (*O. beta*). The *vertical line* indicates the time of injection. Data are shown as means ± 1 SEM; the *horizontal double arrows* indicate data points that are statistically different ($P < 0.05$) from the value immediately prior to injection

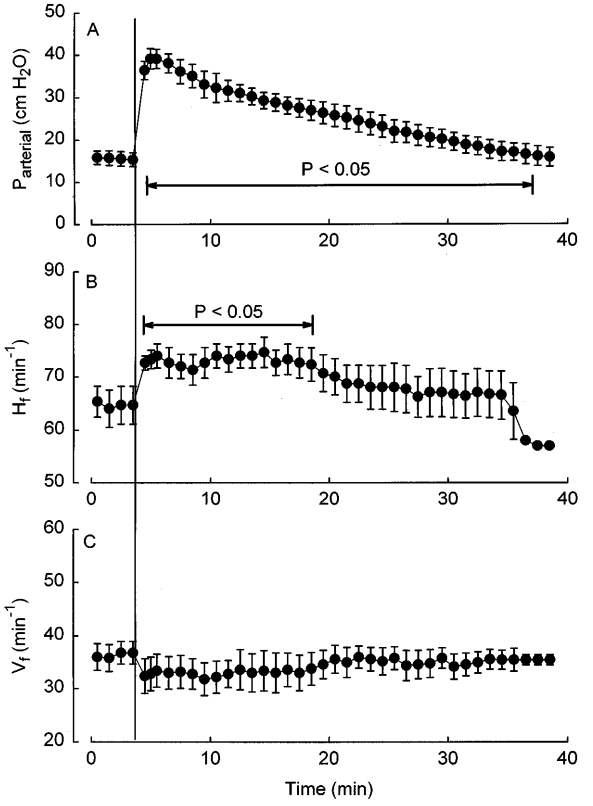

Fig. 4A–C The effects of intra-arterial injection of adrenaline (nominal circulating concentration after injection = 10^{-7} moll^{-1}) on **(A)** arterial blood pressure ($P_{arterial}$), **(B)** cardiac frequency (H_f) and (**C**) breathing frequency (V_f) in gulf toadfish (*O. beta*). The *vertical line* indicates the time of injection. Data are shown as mean values ± 1 SEM ($n = 5$); the *horizontal double arrows* indicate data points that are statistically different ($P < 0.05$) from the value immediately prior to injection

pressure cannulae and injected with 3×10^{-11} mols kg^{-1} AVT. Although the pattern of the blood pressure increase in both vessels was similar, the absolute change in pressure in the ventral aorta (pre-branchial vessel) was greater (13 cm H_2O compared to 10 cm H_2O). H_f and V_f and MO_2 were not affected by any of the doses of AVT (Table 1).

The dose-dependent effects of AVT on urea/ammonia/tritiated water fluxes are shown in Fig. 8. J_{amm} was unaltered by any of the injected doses of AVT whereas J_{urea} was markedly stimulated in a transitory manner at the two highest doses. At the lowest dose of AVT (Fig. 8C), the apparent increase in J_{urea} was not statistically significant owing to the high degree of variability in the data set. The effect of AVT on tritiated water efflux was examined only at the intermediate dose (3×10^{-11} mols kg^{-1}). Although there appeared to be a tendency for increased tritiated water efflux, these changes were not statistically significant. Nevertheless the correspondence between increases in urea and water effluxes after AVT injection was striking in several individual fish. For example, Fig. 9 demonstrates the response of a single fish in which AVT elicited simultaneous increases in urea and tritiated water effluxes without affecting ammonia efflux.

Correlation analysis performed on all available data revealed that J_{urea} was not correlated with J_{amm} (Fig. 10A) or MO_2 (Fig. 10B). A significant correlation was observed between J_{urea} and tritiated water efflux (Fig. 10C). However, the nature of the correlation varied markedly between the isoproterenol- and AVT-treated fish with the latter group displaying much higher rates of J_{urea} for any given rate of water efflux. J_{amm} and water efflux were not correlated (Fig. 10D).

Discussion

In the present study, adrenoceptor agonists were used as tools to increase the general conditions for solute transfer across the gill. These experiments were performed to test the hypothesis that pulsatile urea excretion in the gulf toadfish (Wood et al. 1995, 1997) might be caused, at least in part, by sudden and non-specific

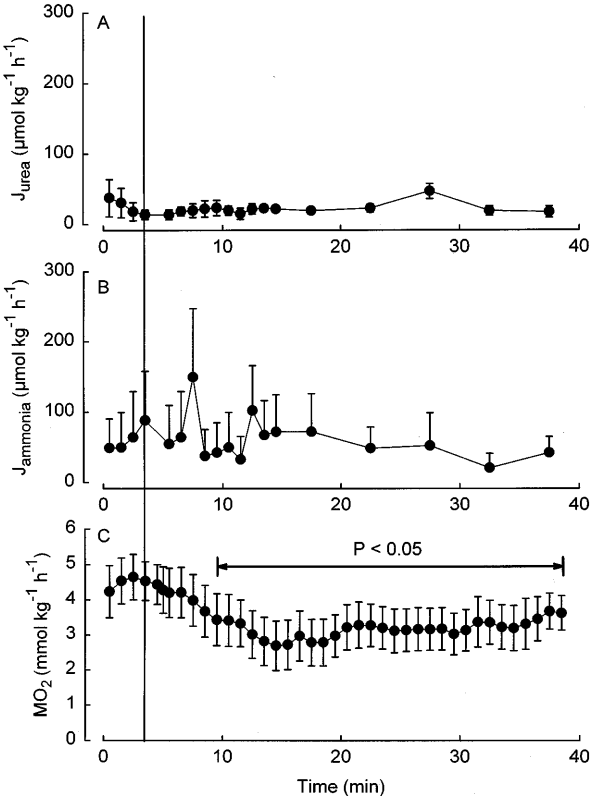

Fig. 5A–C The effects of intra-arterial injection of adrenaline (nominal circulating concentration after injection = 10^{-7} mol l^{-1}) on whole body (**A**) urea efflux (J_{urea}), (**B**) ammonia efflux ($J_{ammonia}$) and (**C**) oxygen uptake (MO_2) in gulf toadfish (*O. beta*). The *vertical line* indicates the time of injection. Data are shown as means ± 1 SEM (n = 5); the *horizontal double arrows* indicate data points that are statistically different ($P < 0.05$) from the value immediately prior to injection

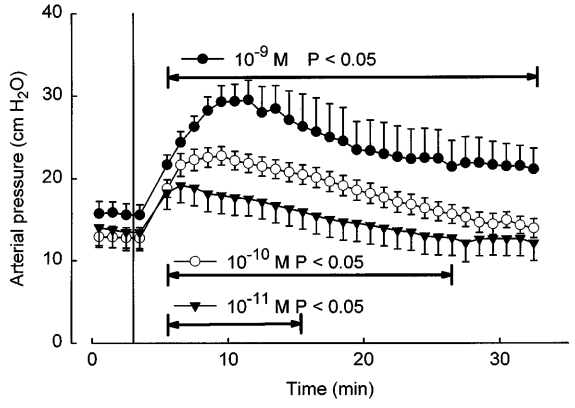

Fig. 6 The effects of intra-arterial injection of arginine vasotocin (nominal circulating concentration after injection = 10^{-11} mol l^{-1}–10^{-9} mol l^{-1}) on arterial blood pressure in gulf toadfish (*O. beta*). *Filled triangles* = 10^{-11} mol l^{-1}, n = 9; *unfilled circles* = 10^{-10} mol l^{-1}, n = 14; *filled circles* = 10^{-9} mol l^{-1}, n = 6. The *vertical line* indicates the time of injection. Data are shown as means ± 1 SEM; the *horizontal double arrows* indicate data points that are statistically different ($P < 0.05$) from the value immediately prior to injection

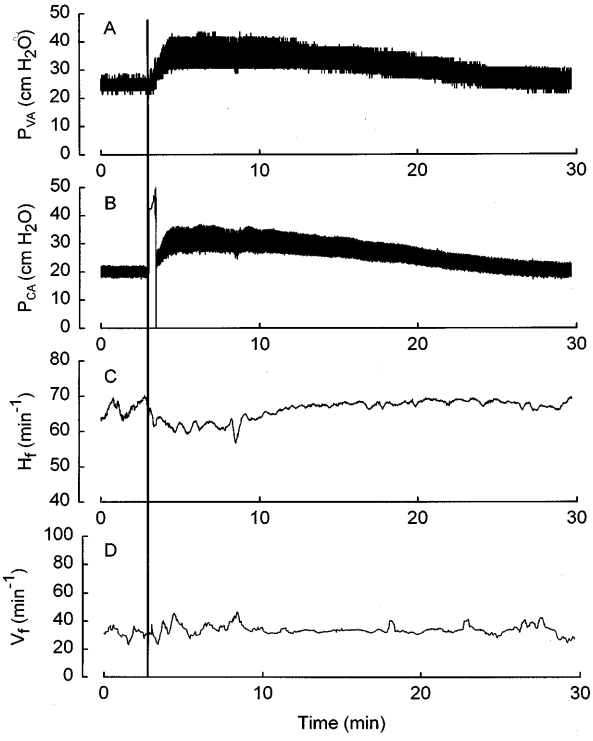

Fig. 7A–D A representative original data recording illustrating the effects of intra-arterial injection of arginine vasotocin (nominal circulating concentration after injection = 10^{-10} mol l^{-1}) on (**A**) ventral aorta blood pressure (P_{VA}), (**B**) caudal artery blood pressure (P_{CA}), (**C**) cardiac frequency (H_f) and (**D**) breathing frequency (V_f) in gulf toadfish (*O. beta*). The *vertical line* indicates the time of injection

changes in branchial conductance. Such changes could reflect modification of gill surface area, diffusion distance, permeability, ventilation or perfusion (reviewed by Gilmour 1997). The inability of the β-adrenoceptor agonist, isoproterenol, or the endogenous non-selective adrenoceptor agonist, adrenaline, to reproduce the natural pattern of pulsatile urea excretion suggests that a generalised increase in branchial conductance is not a significant underlying mechanism of this phenomenon. On the other hand, the results of experiments employing the neurohypophyseal peptide, AVT, indicate that urea excretion across the toadfish gill may be markedly enhanced in the absence of any associated increases in general branchial permeability. The relative involvement of generalised conductance changes (i.e. those caused by catecholamines) and specific modulation of urea permeability (i.e. that caused by AVT) are discussed in detail below.

Effects of adrenergic stimulation

Isoproterenol

The teleost gill is under dual (α and β) adrenergic control although β-adrenoceptors appear to be more important in determining the net response of the gill to adrenergic

Table 1 The effects of arginine vasotocin (*AVT*) (values shown are nominal circulating concentrations) on cardiac frequency (H_f), ventilation frequency (V_f) and oxygen uptake (MO_2) in gulf toadfish (*Opsanus beta*) before (pre) and 2 or 5 min after a bolus intravascular injection. Data are shown as means ± SEM; *n* indicated in parentheses

		10^{-11} mol l^{-1} AVT (10)	10^{-10} mol l^{-1} AVT (14)	10^{-9} mol l^{-1} AVT (4)
H_f (min^{-1})	Pre	62.6 ± 9.3	64.6 ± 11.3	67.0 ± 5.7
	2 min	59.2 ± 11.8	62.4 ± 10.9	67.8 ± 3.9
	5 min	60.5 ± 10.7	62.6 ± 9.5	67.0 ± 5.4
V_f (min^{-1})	Pre	35.9 ± 12.1	36.0 ± 6.7	34.5 ± 1.8
	2 min	33.6 ± 10.0	36.7 ± 11.5	33.2 ± 1.2
	5 min	34.9 ± 13.4	38.7 ± 9.8	37.7 ± 1.4
MO_2 (mmol kg^{-1} h^{-1})	Pre	3.6 ± 1.4	3.6 ± 1.8	3.0 ± 0.4
	2 min	3.5 ± 1.4	3.5 ± 1.8	3.1 ± 0.4
	5 min	3.8 ± 2.4	3.5 ± 1.4	3.2 ± 0.5

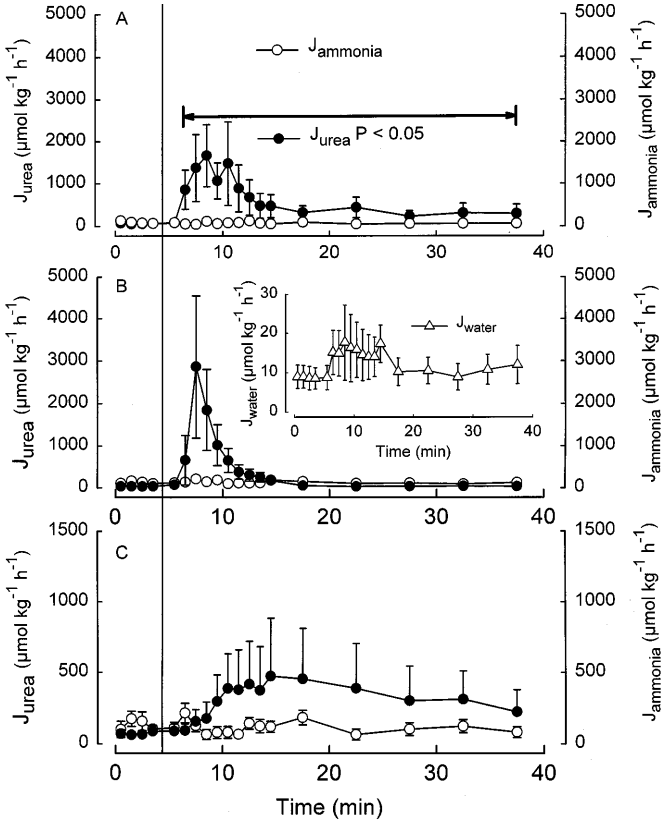

Fig. 8A–C The effects of intra-arterial injection of arginine vasotocin (*AVT*; nominal circulating concentration after injection = 10^{-11} mol l^{-1}–10^{-9} mol l^{-1}) on whole body urea efflux (J_{urea}; *filled circles*) and ammonia efflux ($J_{ammonia}$; *unfilled circles*) in gulf toadfish (*O. beta*). (**A**) 10^{-9} mol l^{-1} *AVT* (n = 5), (**B**) 10^{-10} mol l^{-1} *AVT* (n = 11), (**C**) 10^{-11} mol l^{-1} *AVT* (n = 9). The *figure inset* in panel **B** illustrates the effect of 10^{-10} mol l^{-1} AVT on tritiated water efflux (J_{water}; *unfilled triangles*). The *vertical line* indicates the time of injection. Data are shown as means ± 1 SEM; the *horizontal double arrows* indicate data points that are statistically different ($P < 0.05$) from the value immediately prior to injection

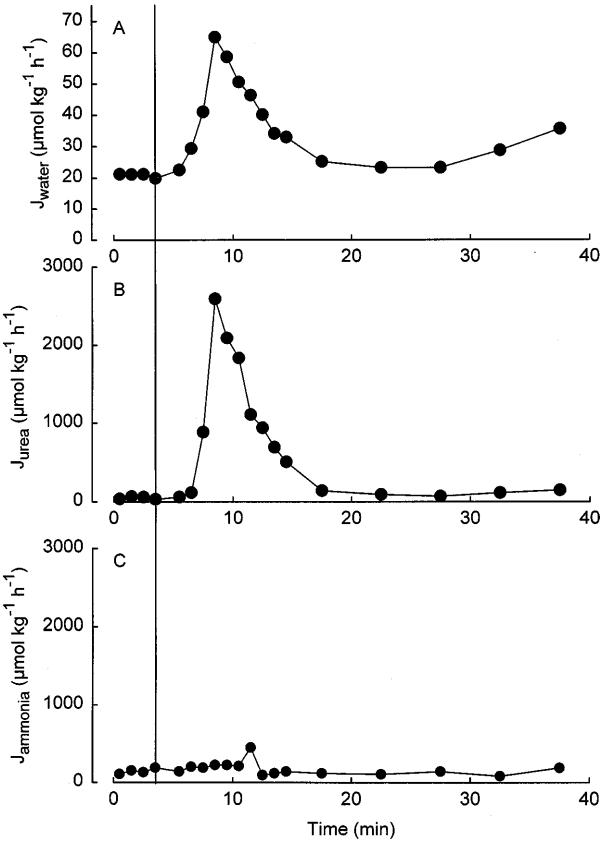

Fig. 9A–C The effects of intra-arterial injection of arginine vasotocin (*AVT*; nominal circulating concentration after injection = 10^{-10} mol l^{-1}) on whole body (**A**) diffusive water efflux (J_{water}), (**B**) urea efflux (J_{urea}) and (**C**) ammonia efflux ($J_{ammonia}$) in a representative individual gulf toadfish (*O. beta*). The *vertical line* indicates the time of injection

stimulation. In all teleost species that have been examined either in vivo (e.g. Wood and Shelton 1980) or using perfused preparations (Wood 1974, 1975; Oduleye et al. 1982; Stagg and Shuttleworth 1984; Bennett and Rankin 1987), stimulation of branchial β-adrenoceptors causes a reduction in vascular resistance owing to vasodilatation of the arterio-arterial circulation (see reviews by Nilsson 1983; Laurent 1984). This leads to an increase in the functional surface area of the gill (Bergman et al. 1974; Booth 1979). An increase in the functional surface area of the gill, coupled with an apparent specific effect of β-adrenergic stimulation on the permeability of the gill to small lipophilic molecules (Haywood et al. 1977; Bennett and Rankin 1987), is therefore expected to maximise the conditions for diffusive solute

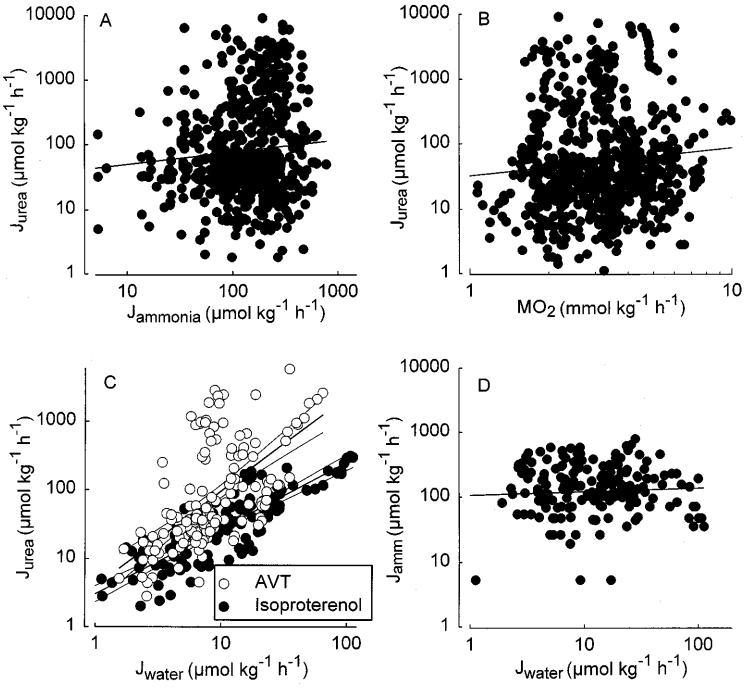

Fig. 10A–D The interrelationships among urea efflux (J_{urea}), ammonia efflux ($J_{ammonia}$), diffusive water efflux (J_{water}) and oxygen uptake (MO_2) in gulf toadfish (*O. beta*) using all available data from control and drug-treated animals. In panel **C**, the *unfilled circles* represent data from fish treated with AVT whereas the *filled circles* represent data from fish treated with isoproterenol. In panel **C**, the regressions are accompanied by 95% confidence intervals. **A** Log $J_{urea} = 0.053 \times \log J_{ammonia} + 2.02$ ($r^2 =$ 2.18e−30); **B** log $J_{urea} = 0.417 \times \log J_{ammonia} + 1.54$ ($r^2 =$ 9.33e−3); **C** AVT: log $J_{urea} = 1.376 \times \log J_{water} + 0.60$ ($r^2 = 0.387$, $P < 0.05$), ISO: log $J_{urea} = 0.949 \times \log J_{water} +$ 0.49 $r^2 = 0.710$, $P < 0.05$); **D** log $J_{ammonia} = 0.053 \times \log J_{water} + 2.02$ ($r^2 =$ 2.18e−3)

transfer across the gill. Although branchial vascular resistance was not measured in the present study, it is likely, based on previous studies on perfused toadfish gills (Oduleye et al. 1982; Oduleye and Evans 1982), that the functional surface area of the gill was indeed increased after isoproterenol treatment. This increase in gill surface area, together with non-specific increases in permeability, presumably contributed to the elevation in urea and water effluxes observed after β-adrenergic stimulation. In addition, changes in ventilation and/or perfusion of the gill may have also contributed to the rise in J_{urea} and water excretion. Although the role of circulating catecholamines in the control of breathing in fish is controversial (see reviews by Randall and Taylor 1991; Perry et al. 1992 for opposing views), there is evidence that stimulation of β-adrenoceptors can elicit hyperventilation in fish under appropriate conditions (Peyreaud-Waitzenegger 1979). In the present study, isoproterenol did not influence V_f. Ventilation amplitude recordings were not rigorously analysed (see Materials and methods) although we did observe obvious increases in ventilation amplitude in several fish treated with isoproterenol. This would have also contributed to the augmentation of water and urea fluxes across the gill. Finally, isoproterenol injection may have enhanced solute transfer across the gill by raising cardiac output (Wood and Shelton 1980). In the present study, isoproterenol treatment was associated with a large rise in H_f that presumably reflected the combined effects of direct stimulation of cardiac β-adrenoceptors (Gamperl et al. 1994) and reduced vagal tone resulting from the barostatic reflex. Wood et al. (1998), using a 10-fold higher dose of isoproterenol and a recording system with much slower time resolution, found somewhat different results; specifically, no effect on J_{urea} but a long-lasting increase in J_{amm}. The reasons for these differences are probably methodological.

In any event, regardless of this discrepancy and of the precise contribution of diffusive versus convective changes, it is clear that the increases in urea efflux across the gill after isoproterenol treatment did not resemble those occurring during natural pulsatile excretion (Wood et al. 1995, 1997, 1998). Specifically, the increases in J_{urea} were much smaller than those occurring during natural pulses. For example, in the present study, the rate of J_{urea} was increased to about 75 μmol kg^{-1} h^{-1} within 15 min of isoproterenol injection, whereas during natural pulses under identical conditions in the respirometer, the average peak rates of urea excretion were 1296 ± 262 μmol kg^{-1} h^{-1} (Gilmour et al. 1998). Furthermore, isoproterenol elicited roughly equivalent changes in J_{urea} and tritiated water excretion (Figs. 2, 10) whereas J_{urea} was stimulated preferentially during natural pulses (Gilmour et al. 1998). The matching of J_{urea} and tritiated water excretion after isoproterenol injection suggests a *generalised* increase in trans-cellular fluxes associated with a functional increase in gill surface area and/or permeability, because the bulk of water movement across the gill is purported to occur by transcellular diffusion (Jackson and Fromm 1981).

J_{amm} and MO_2 were unaltered by isoproterenol, a result which, at first glance, appears to be inconsistent with a generalised increase in the conductance of the gill for solute transfer. However, the absence of any effect on J_{amm} may simply reflect an unusual ammonia-retaining capability of the toadfish gill (Walsh 1997) that has evolved to conserve substrate for the ornithine-urea cycle. If O_2 transfer across the toadfish gill is diffusion-

limited, an increase in branchial diffusive conductance would be expected to increase arterial PO_2, yet this need not be accompanied by a significant increase in MO_2 since haemoglobin is likely to be fully saturated already at normal levels of arterial PO_2.

In the teleosts that have been examined in detail (e.g. rainbow trout, Atlantic cod and eel), the systemic vascular resistance and hence arterial blood pressure are controlled predominantly by vasoconstrictory α-adrenoceptors; the vasodilatory β-adrenoceptors appear to be much less important (Wood 1976; Wood and Shelton 1980). In the present study, isoproterenol injection caused a massive reduction in arterial blood pressure suggesting an unusually important role of β-adrenoceptors in the control of systemic resistance. The exact site and physiological significance of the β-adrenergic control of systemic resistance in toadfish are unclear.

Adrenaline

The effects of adrenaline on J_{urea} and cardiovascular physiology varied markedly from those of isoproterenol. Previous studies employing perfused preparations showed that adrenaline increases branchial urea or water flux owing, at least in part, to the combined effects of increases in the permeability and functional surface area of the gill (Isaia 1984). According to current models of gill circulatory control, an increase in functional surface area following adrenaline treatment is expected for at least two reasons; (1) a passive recruitment of distal lamellae associated with the elevation of blood pressure (Farrell et al. 1979) and (2) an active β-adrenergic vasodilation of the arterio-arterial circulation (Nilsson 1983, 1984; Laurent 1984). Similarly, an increase in permeability is expected as a consequence of β-adrenoceptor stimulation (Haywood et al. 1977; Bennett and Rankin 1987). In this study, therefore, it seems probable that adrenaline injection was accompanied by an increase in gill diffusive conductance (encompassing both surface area and permeability changes) yet J_{urea} was not elevated. This supports the view that sudden and large increases in J_{urea}, as occur during natural pulses, cannot be explained by modification of the diffusive properties of the gill. Furthermore, it suggests that the simple diffusion of urea across the toadfish gill is not an important route of excretion. It is uncertain as to why isoproterenol stimulated J_{urea} while adrenaline was without effect. However, since β-receptor stimulation appears to be more important than α-receptor stimulation in augmenting gill permeability and surface area (e.g. Bergman et al. 1974; Oduleye and Evans 1982), it is possible that inadequate β-receptor stimulation was the underlying cause of the difference. One interesting possibility is that the difference may reflect a specific inhibitory effect of α-adrenoceptor stimulation on urea transport as previously demonstrated for the rat inner medullary-collecting duct (Rouch and Kudo 1996). Finally, the reduction in MO_2 after adrenaline injection implies that other unknown factors may have contributed to a generalised reduction in solute transfer across the gill thereby counteracting the effects of β-adrenoceptor stimulation.

Effects of AVT

Although numerous previous studies have documented the cardiovascular and renal effects of AVT in fishes (Lahlou et al. 1969; Sawyer 1970; Chan 1977; Babiker and Rankin 1978; Lemevel et al. 1993; Amer and Brown 1995; Oudit and Butler 1995; Conklin et al. 1996; Warne and Balment 1997), this is the first to assess the impact of physiological doses of AVT on J_{urea}. The doses of AVT used in the present experiments (3.0×10^{-12}–3.0×10^{-10} mols kg^{-1}) were similar to those used by other investigators (e.g. Lemevel et al. 1993; Oudit and Butler 1995) and were designed to yield circulating levels in the plasma of 1×10^{-11}–1×10^{-9} mol l^{-1}. The concentration range of AVT in the plasma of fishes is about 1×10^{-11} mol l^{-1}–1×10^{-8} mol l^{-1} (Perrott et al. 1991; Pierson et al. 1995; Warne and Balment 1995). Thus we believe that the circulating levels of AVT achieved in the present study, although not measured, were physiologically relevant and able to interact specifically with high affinity AVT receptors (Kd = 1–3 nmol l^{-1}; Guibbolini et al. 1988).

The dose-dependent pressor effects associated with AVT injection were similar to those observed for other fishes including a related toadfish species, *Opsanus tau* (Lahlou et al. 1969). The mechanisms underlying the increased post-branchial blood pressure have not been established unequivocally but are believed to involve an increase in systemic vascular resistance (Chan 1977) coupled with an elevated cardiac output (Oudit and Butler 1995). In the present study, the monitoring of blood pressure was important to ensure that the low doses of AVT employed were indeed capable of exerting physiological effects.

Along with its effect on the systemic vasculature, AVT also is a potent branchial vasoconstrictor (Bennett and Rankin 1986) owing to constriction of sites distal to the arterio-venous anastomoses (Bennett and Rankin 1986). This likely explains the increase in arterio-venous blood shunting that occurs in eels after AVT treatment (Oudit and Butler 1995). Although branchial vasoconstriction was not specifically assessed in these experiments, the larger increase in pre-branchial ventral aortic pressure compared to post-branchial caudal artery pressure that was observed in a doubly cannulated fish (Fig. 7) suggests that an increase in gill resistance did occur.

The effects of AVT on J_{urea} were striking and closely resembled the pattern of excretion observed during natural pulses (Gilmour et al. 1998). Notably, the increases in J_{urea} were transient and much larger than those observed after isoproterenol treatment. Furthermore, unlike the roughly equivalent increases in urea and diffusive water effluxes observed after isoproterenol,

AVT caused an obvious preferential stimulation of urea efflux. Indeed, the AVT-induced increases in tritiated water efflux were not statistically significant owing to the high degree of variability of the data. Nevertheless, the results of the correlation analysis (Fig. 10) revealed a significant relationship between J_{urea} and tritiated water excretion in which small changes in tritiated water efflux were associated with large changes in urea efflux.

It is highly unlikely that the increases in J_{urea} elicited by AVT were caused by generalised increases in the diffusive conductance of the gill. First, the increases in J_{urea} were more than an order of magnitude larger than those evoked by isoproterenol injection, a treatment that is known to maximise the diffusive properties of the gill. Second, any increases in J_{urea} caused by non-specific diffusive changes should have been matched by equivalent changes in tritiated water efflux. Third, a previous in vivo study using ammoniotelic goldfish, *Carassius auratus* (Lahlou and Giordan 1970), demonstrated that AVT reduced branchial fluxes of water, suggesting that AVT diminishes, rather than increases, the conditions for solute transfer across the gill.

It is also improbable that the kidney contributed significantly to the effects of AVT on J_{urea} in this aglomerular fish. Although high doses of AVT are diuretic in some fish (Sawyer 1970), lower doses (as were used in the present study) are antidiuretic (Babiker and Rankin 1978; Amer and Brown 1995). In the related aglomerular toadfish, *O. tau*, AVT injection, at doses sufficient to influence blood pressure, did not affect urine flow (Lahlou et al. 1969). Thus, increases in the already low urine flow rate could not have been responsible for the increase in whole body J_{urea} after AVT treatment. The lack of a significant renal component to whole body J_{urea} also is supported by the findings of Wood et al. (1995) demonstrating that natural urea pulses in toadfish were derived from the head region.

In the mammalian collecting duct, a vasopressin-sensitive urea transporter (UT) has been cloned and characterised in two species (Smith et al. 1995). This transporter plays a key role in the accumulation of urea within the renal medulla and hence is an important component of the urine concentrating mechanism of the mammalian nephron. Like the vasopressin-sensitive water channels of the collecting duct, the RUT is shuttled to the apical membrane in vesicles in response to an increase in the concentration of AVP (Nielsen et al. 1996). Similar neurohypophyseal hormone-dependent UTs and water transporters also may exist in the amphibian bladder (Bentley 1987). Recently, Walsh and colleagues (see Walsh 1997) have used RT-PCR to amplify a 484-bp cDNA fragment from toadfish gill showing a high degree of homology with the mammalian UT. Thus, on the basis of this finding, coupled with the insensitivity of J_{urea} to gill diffusive changes and the profound impact of AVT noted in this study, we speculate that an AVT-sensitive UT mechanism exists on the gill of *O. beta*. In support of this idea, Laurent et al. (1997) demonstrated an obvious fusion of vesicles with the apical membrane of gill cells after AVT treatment, which resembled the pattern of vesicle fusion that occurred during natural pulses. Therefore, it is possible that pulsatile J_{urea} in toadfish reflects periodic AVT-induced migration and insertion of UT-containing vesicles to the apical membrane resulting in sudden and large increases in branchial urea permeability. This idea is supported further by the findings of Wood et al. (1998) from fish displaying natural pulsatile J_{urea}. Wood et al. (1998) showed that the transport system activated in the gills during natural pulses is capable of bi-directional transport, can also transport the urea analogue thiourea, and is strongly inhibited by high concentrations of thiourea. All of these characteristics are supportive of the hypothesis that natural pulse events are due to the insertion or activation by AVT of a UT-like facilitated diffusion urea transporter in the gill epithelium. Furthermore, it has been recently demonstrated (Gilmour et al. 1998) that J_{urea} is abolished by pre-treating toadfish with colchicine, a cytoskeletal disrupter that is known to inhibit AVT-induced water fluxes in amphibian bladder (Bentley 1987). Fusion of vesicles with the apical membrane and subsequent exocytosis of water may explain the significant correlation between urea and tritiated water effluxes that was observed with AVT treatment.

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