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THE ROLE OF ANGIOTENSIN IN ARTERIAL BLOOD PRESSURE REGULATION IN THE TOAD *BUFO MARINUS*

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Summary

Little is known about the role of the renin–angiotensin system in anuran amphibians, although they appear to possess the functional components of such a system. We investigated the role of angiotensin (ANG) in arterial blood pressure regulation in the conscious toad *Bufo marinus* using the angiotensin-converting enzyme blocker captopril. We found that conversion of endogenous ANG I to ANG II made a significant contribution to mean arterial pressure in undisturbed animals. The vascular tone contributed by ANG II was not mediated *via* alpha adrenergic mechanisms because increases in pressure in response to ANG infusion were unaffected by the presence of the alpha antagonist phentolamine. Angiotensin-induced vasoconstriction was shown to be an important mechanism in arterial blood pressure regulation in the face of an acute hypotensive perturbation of pressure brought about by sodium nitroprusside. Blockade of the conversion of ANG I to ANG II significantly delayed the recovery of mean arterial pressure after sodium nitroprusside-induced hypotension. This suggests that the renin–angiotensin system may play an important role in the initial responses to hypotension in anurans, whether brought about by haemorrhage or dehydration.

Key words: toad, *Bufo marinus*, angiotensin, blood pressure, captopril, vasoconstriction, hypotension.

Introduction

The functional components of the renin–angiotensin system have been found in all vertebrates other than the cyclostomes and elasmobranchs. Renin secretion is stimulated by challenges such as haemorrhage or dehydration. The actions of angiotensin II (ANG II) include stimulation of both short- and long-term mechanisms safeguarding adequate rates of tissue perfusion, including cardiovascular effects and actions on the behavioural, renal and other mechanisms regulating total body Na^+ and water content (Nishimura, 1980; Wilson, 1984).

Renin has been found in the kidneys of several species of amphibians (Grill *et al.* 1972), and in *Bufo bufo* it has been localized to the juxtaglomerular cells (Lamers *et al.* 1985). There is also evidence that angiotensin-converting enzyme (ACE) activity is present in the genera *Rana* and *Necturus* (Fruchter *et al.* 1980). The native angiotensin of *Rana catesbeiana* is the decapeptide ($Asp^1Val^5Asn^9$) angiotensin I (ANG I), which is converted to the vasoactive octapeptide ANG II (Asp^1Val^5) by ACE (Hasegawa *et al.* 1983). The structures of ANG I and ANG II in *Bufo marinus* are unknown. In most amphibians, reptiles, birds and mammals studied, the structure of the octapeptide ANG II is either Asp^1Val^5 ANG II or Asp^1Ile^5 ANG II (Kobayashi and Takei, 1996). We used Asp^1Ile^5 ANG II in the experiments described here.

The amino acid in position 9 of the decapeptide ANG I is quite variable across species. Perhaps this should not be surprising, because cleavage of the ninth and tenth amino acids, catalyzed by ACE, is necessary for the formation of the biologically active form of the peptide, ANG II. In this study, we used $Asp^1Ile^5His^9$ ANG I. His^9 ANG I has been identified in mammals, a turtle (*Pseudemys scripta*) and the goosefish *Lophius litulon* (Kobayashi and Takei, 1996). We confirmed in our study that the pressor response to $Asp^1Ile^5His^9$ ANG I in *Bufo marinus* was blocked by captopril inhibition of ACE, as it is in other species (Kobayashi and Takei, 1996).

Little is known of the role of the renin–angiotensin system in cardiovascular control in anuran amphibians. The purpose of this study was therefore threefold: to investigate the contribution of endogenous angiotensin to the control of arterial pressure in undisturbed toads; to determine whether exogenous ANG II raised arterial pressure by increasing sympathetic outflow or by acting directly on vascular smooth muscle, and to assess the role of the endogenous ANG system in the cardiovascular responses to a hypotensive challenge.

Materials and methods

Animals

Twenty-nine toads (*Bufo marinus*) of either sex, weighing

327±16 g (mean ± S.E.M.), were used in the study. The toads were obtained from a commercial supplier in the United States and maintained on a 16 h:8 h light:dark cycle at 20–22 °C with free access to water. They were force-fed canned dog food once a week. Experiments were performed all year round at a room temperature of 22–25 °C.

Surgical preparation

Toads were anaesthetized by immersion in 0.25 % ethyl *m*-aminobenzoate methanesulphonic acid (MS-222; Sigma no. A 5040) buffered to pH 7 with NaOH. A polyethylene cannula (PE-50, Intramedic) was inserted occlusively into a sciatic artery and advanced into the abdominal aorta to a position just below the renal artery. The distance the cannula was to be advanced had been determined previously in preliminary experiments. Tip placement was confirmed *post mortem*. A thinner cannula (PE-10, Intramedic) was inserted into the sciatic vein. The cannulae were filled with heparinized saline (arterial, 100 i.u. ml^{-1} 0.65 % NaCl; venous, 50 i.u. ml^{-1} 0.65 % NaCl). The free ends were coiled and attached to the dorsal surface of the toad with silk sutures until use. Incisions were closed with silk sutures or wound clips and treated with 10 % proviodine solution to prevent post-operative infection.

Instrumentation and recorded and calculated variables

Pulsatile arterial pressure was recorded with Beckman 4-327-0 pressure transducers and Beckman 9853A couplers. Pressure was recorded on a Beckman 511A pen recorder writing on rectilinear coordinates. Mean arterial pressure was either recorded simultaneously on a separate channel or calculated as diastolic + one-third pulse pressure. Instantaneous heart rate was measured as the reciprocal of the time between sequential systolic pressure pulses.

Drugs

Appropriate drug doses were determined in preliminary experiments. Vasoactive compounds were delivered from separate Hamilton 710 syringes (0.1 ml) followed by a 0.2 ml saline flush. $Asp^1Ile^5His^9$ ANG I and Asp^1Ile^5 ANG II (Peninsula Laboratories Inc., CA, USA) were dissolved in 0.65 % saline and kept frozen at −80 °C. Phenylephrine, phentolamine and sodium nitroprusside were dissolved in 0.65 % saline and refrigerated. Captopril solutions were made fresh as required.

Experimental protocol

Animals were allowed to recover for 3 days after surgery in a translucent white plastic box (20 cm×26 cm×45 cm), which also served as the experimental chamber. The floor of the box was kept moist with tap water. The animals were exposed to a low uniform noise level during both recovery and experimentation. On the morning of an experimental day, the pressure transducers were calibrated against a mercury-filled U-tube manometer. Cannulae were led out of the box containing the toad and reduced in length to minimize dead space. The arterial cannula was attached to the pressure transducer (arterial) and the venous cannula was attached to a 1 ml syringe filled with saline (0.65 % NaCl). The animals were allowed at least 30 min to settle, and control injections (0.1 ml, 0.2 ml flush) were made *via* the venous cannula to check for vasoactivity in response to saline.

Series 1

In the first series of experiments, ANG I or ANG II was injected *via* the venous cannula (50 pmol in 0.1 ml, followed by a 0.2 ml saline flush). At least 45 min was allowed between sequential injections of ANG I and ANG II, and the order of treatment was alternated. To measure the effect of blocking the conversion of exogenous or endogenous ANG I to ANG II, captopril (20 μg kg^{-1}) was injected intravenously 30 min before ANG I or ANG II.

Series 2

To investigate the role of the alpha receptors in mediating increases in vascular tone, alpha receptors were blocked by phentolamine (5 mg kg^{-1}), which was delivered at least 30 min before subsequent ANG I, ANG II or L-phenylephrine (0.25 μmol kg^{-1}) injections.

Series 3

In the third series of experiments, the role of endogenous ANG II in the cardiovascular responses to hypotension was investigated. Sodium nitroprusside (5 μg kg^{-1}) was injected to produce hypotension in controls and in trials in which the conversion of ANG I to ANG II had been blocked by pretreatment with captopril (20 μg kg^{-1}).

Statistical analyses

Data are reported as means ± S.E.M. *N* represents the number of animals used in a trial. Statistical equivalence was assessed by analysis of variance (ANOVA). The fiducial level of statistical significance was considered to be $P<0.05$.

Results

Effect of angiotensin-converting enzyme inhibition on resting arterial pressure and heart rate

Captopril, 20 μg kg^{-1} intravenously, caused a small but statistically significant reduction in resting arterial pressure in toads. Mean arterial pressure 30 min after captopril injection was 22.4±0.6 mmHg (2.99±0.08 kPa) (33 experiments) compared with 25.8±0.8 mmHg (3.44±0.11 kPa) under control conditions (39 experiments, $P<0.001$). The heart rates of toads treated with captopril (15.2±0.5 min^{-1}, 39 experiments), however, were not significantly different from those of controls (15.7±07 min^{-1}, 33 experiments).

Effect of angiotensin-converting enzyme inhibition on cardiovascular responses to ANG I and ANG II

Intravenous injections of 50 pmol of ANG I or ANG II increased arterial pressure and decreased heart rate in conscious toads (all values of $P<0.001$, Fig. 1). There were no

statistically significant differences in the magnitude or time course of responses to the two peptides when given alone. Captopril pretreatment, however, abolished the pressor and bradycardic responses to ANG I (P<0.001), but had no effect on responses to ANG II (Fig. 1).

Effect of alpha-adrenergic inhibition on the pressor responses to L-phenylephrine, ANG I and ANG II

A dose of L-phenylephrine (0.25 μmol kg^{-1} intravenously) was chosen that stimulated a prompt increase in mean arterial pressure similar to that caused by ANG I or ANG II (Fig. 2). Pretreatment with phentolamine (5 mg kg^{-1} intravenously) abolished the pressor responses to L-phenylephrine (P<0.001) but had no significant effect on the responses to ANG I or ANG II (Fig. 2).

Effect of angiotensin-converting enzyme inhibition on cardiovascular responses to hypotension

To investigate the role of endogenous ANG II in cardiovascular control during hypotension, the cardiovascular responses to sodium nitroprusside (5 μg kg^{-1} intravenously) were compared before and after blockade of the renin–angiotensin system with captopril (Fig. 3). Because captopril reduced baseline arterial pressure, as noted above, the statistical analysis was performed on the change in mean arterial pressure (Fig. 3B). Although captopril pretreatment had no significant effect on the initial fall in blood pressure caused by nitroprusside, it significantly prolonged the time taken for recovery. Arterial pressures in the two treatment groups were significantly different at all time periods from 2 min after nitroprusside treatment until the end of the 15 min observation period (P<0.05). In control experiments, mean arterial pressure had returned to pre-nitroprusside values by 4.6±1.1 min (range 2–12 min). After captopril treatment, arterial pressure had returned to pre-nitroprusside values in only half the toads by 15 min.

Hypotension was accompanied by tachycardia in both control and captopril trials. Again, the period of recovery was prolonged in captopril-treated toads. Heart rates returned to within 2 beats min^{-1} of pre-nitroprusside values by 4.2±0.6 min in control experiments but not until 7.6±0.9 min when the renin–angiotensin system was blocked by captopril (P<0.01).

Discussion

The results show (1) that ongoing conversion of endogenous ANG I to ANG II in conscious, undisturbed *Bufo marinus* contributes significantly to the resting level of mean arterial

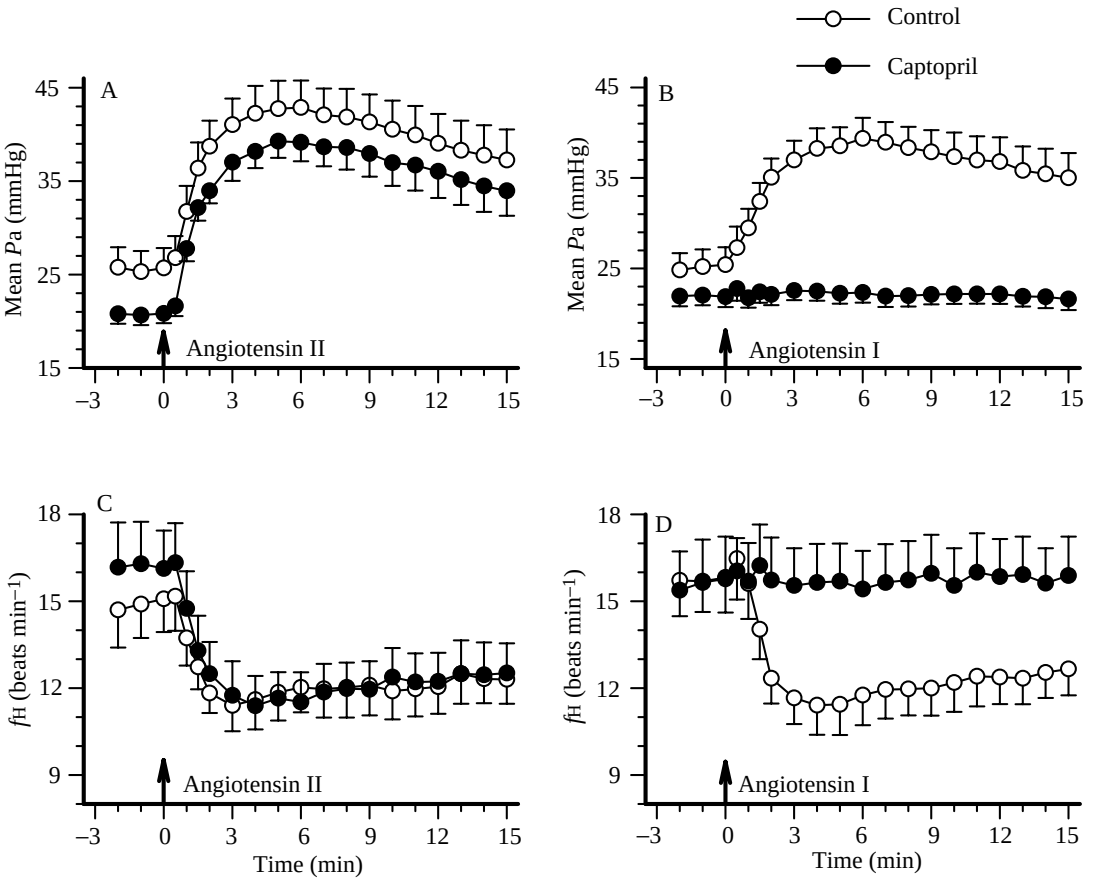

Fig. 1. Effects of intravenous injections (at the arrows) of 50 pmol of angiotensin II (A,C) or angiotensin I (B,D) on mean arterial blood pressure (mean Pa) (A,B) and instantaneous heart rate (f_H) (C,D) in conscious toads *Bufo marinus* under control conditions (open circles, N=15) and after pretreatment with captopril (20 μg kg^{-1} intravenously; filled circles, N=12). Values are means ± S.E.M. 1 mmHg=0.1333 kPa.

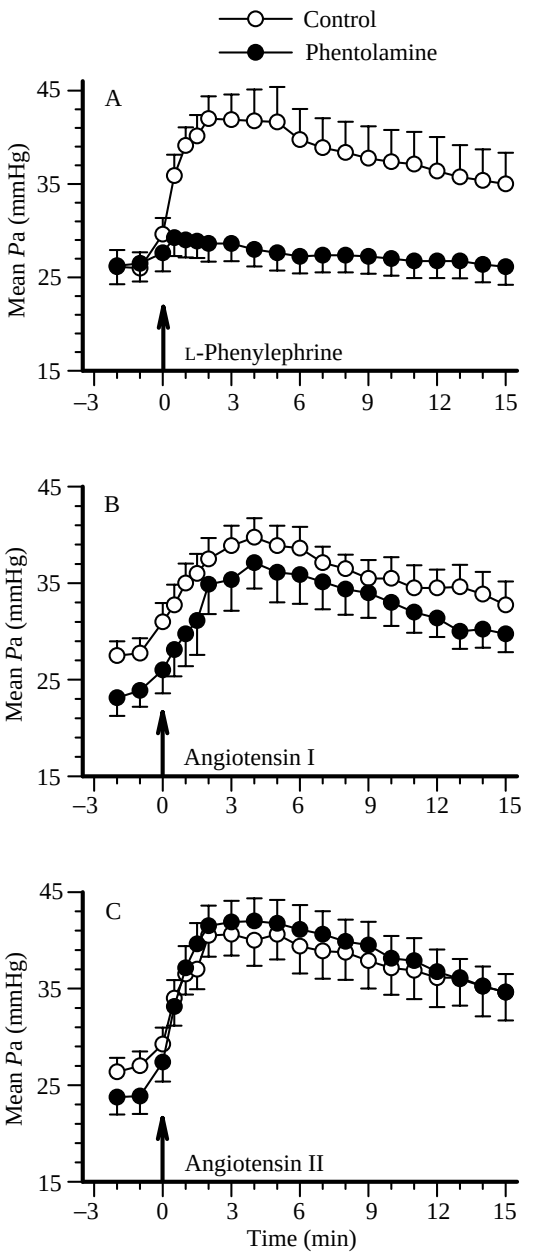

Fig. 2. Mean arterial blood pressure responses (mean *P*a) to intravenous injections (at the arrows) of L-phenylephrine (0.25 μmol kg^{-1}, A), angiotensin I (50 pmol, B) and angiotensin II (50 pmol, C) in conscious toads *Bufo marinus* under control conditions (open circles, *N*=8) and after alpha-adrenergic blockade with phentolamine (5 mg kg^{-1} intravenously; filled circles, *N*=8). Values are means ± S.E.M. 1 mmHg=0.1333 kPa.

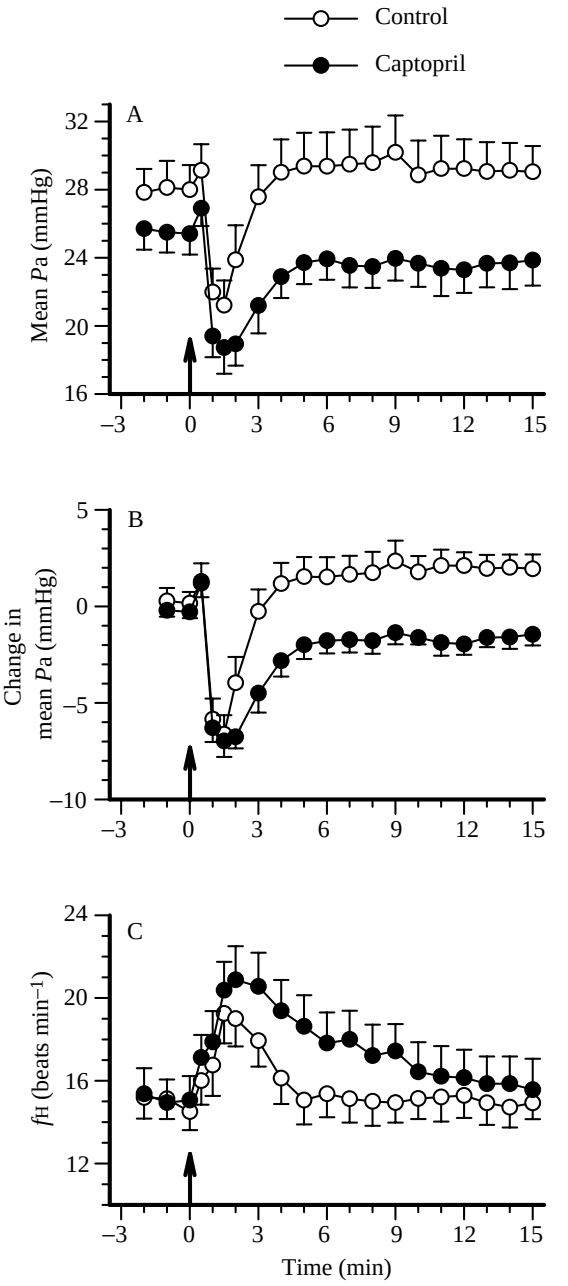

Fig. 3. Effect of sodium nitroprusside injections (5.0 μg kg^{-1} intravenously) on mean arterial blood pressure (mean *P*a) (A), the change in mean *P*a (B) and instantaneous heart rate (f_H) (C) in conscious toads *Bufo marinus* under control conditions (open circles, *N*=8) and after pretreatment with captopril (20 μg kg^{-1} intravenously; filled circles, *N*=8). Values are means ± S.E.M. 1 mmHg=0.1333 kPa.

blood pressure; (2) that the pressor response to ANG II in *Bufo marinus* is not mediated significantly by alpha-adrenergic mechanisms, in contrast to previous findings in the frog *Rana catesbeiana*; and (3) that conversion of ANG I to ANG II is an important effector limb for arterial blood pressure homeostasis in the face of a hypotensive perturbation in *Bufo marinus*.

Captopril has previously been shown to be an effective angiotensin-converting enzyme inhibitor in anuran amphibians (Harper and Stephens, 1985) and abolished the pressor response to exogenous ANG I in our animals. The reduction we observed in mean arterial pressure in resting toads treated with captopril is comparable to that seen in the more aquatic frog *Rana catesbeiana* (Harper and Stephens, 1985). Angiotensin also contributes to the control of resting arterial pressure in mammals (Murthy *et al.* 1977; Oliverio *et al.* 1997)

and eels *Anguilla anguilla* (Nishimura *et al.* 1978). Angiotensin-related vascular tone appears to be less significant in maintaining resting arterial pressure in a shark *Squalus acanthius* (Opdyke and Holcombe, 1976), a freshwater turtle (Stephens, 1981) and the turkey (Fregley *et al.* 1981). Too few species have been studied in any group to enable a definitive judgment to be made on whether the data accumulated thus far reflect any phylogenetic trends in the relative importance of angiotensin in maintaining vascular tone at rest.

Angiotensin stimulates the release of catecholamines in both mammals and non-mammalian vertebrates (Peach *et al.* 1966; Carroll and Opdyke, 1982; Wilson and West, 1986). The pressor response associated with angiotensin infusion could therefore be at least partially mediated by stimulation of vascular smooth muscle alpha receptors rather than by a direct effect of angiotensin stimulation. In frogs *Rana catesbeiana*, approximately 40 % of the arterial blood pressure response was blocked by pretreatment with the alpha antagonists phentolamine or phenoxybenzamine (Carroll and Opdyke, 1982; Harper and Stephens, 1985). Sham *et al.* (1984) concluded, on the basis of significant attenuation of the ANG I pressor response by phentolamine and the failure of captopril to produce any hypotensive effect under control conditions, that the renin–angiotensin system has no direct involvement in blood pressure homeostasis in the bullfrog *Rana catesbeiana*.

In contrast, our results show that in *Bufo marinus* the peripheral vasoconstrictor effect of angiotensin is mediated by the direct action of angiotensin, rather than through adrenergic mechanisms. Phentolamine pretreatment resulted in only a non-significant reduction in mean arterial pressure. This accords well with previous findings that there is no adrenergic vasoconstrictor tone present in resting *Bufo marinus* (Wahlqvist and Campbell, 1988). Infusion of either ANG I or ANG II in the presence of phentolamine resulted in increases in arterial pressure that were of the same magnitude as those in the absence of the antagonist, also indicating that the vasopressor responses to exogenous angiotensin are not mediated by catecholamines in *Bufo marinus*. The contribution of catecholamines to the angiotensin pressor response has been shown to be highly variable in both teleosts and higher vertebrate groups (Carroll and Opdyke, 1982). The overall trend appears to be a change in the angiotensin pressor response from that found in primitive fishes, which is catecholamine-mediated, to that in mammals, which is due to the direct effect of angiotensin on vascular smooth muscle. The response in *Bufo marinus* is closer to that of mammals, in which only approximately 10 % of the response to exogenous angiotensin is inhibited by alpha blockade, than to that of agnathans and elasmobranchs, in which the entire response is abolished (Carroll and Opdyke, 1982).

In mammals, angiotensin-induced vasoconstriction is of prime importance in the maintenance blood pressure under conditions of acutely imposed hypotension (Averill *et al.* 1983). In amphibians, hypotension is usually associated with the hypovolaemia caused by haemorrhage or dehydration. Hypotension reduces renal perfusion, resulting in the secretion of renin from the juxtaglomerular apparatus and in the formation of angiotensin from plasma angiotensinogen. Renin is present in amphibian kidneys (Grill *et al.* 1972) and in *Bufo bufo* is localized to the juxtaglomerular cells (Lamers *et al.* 1985). Angiotensin stimulates water absorption in *Bufo*, ultimately restoring blood volume, and therefore pressure, in a similar way to increased drinking in other tetrapods (Hoff and Hillyard, 1991; Parsons *et al.* 1993). However, an increase in vascular tone mediated by angiotensin could play an important role in counteracting the initial reduction in arterial pressure. To test this, we blocked the conversion of endogenous ANG I, produced in response to nitroprusside-induced hypotension, to vasoactive ANG II. Arterial pressure did not recover in captopril trials. In control trials, mean arterial pressure returned to control values by about 3 min after the nadir of pressure. Therefore, in *Bufo marinus*, acute-onset hypotension causes an increased angiotensin-mediated contribution to vascular tone that serves to regulate mean arterial pressure back to normal values. This suggests that the renin–angiotensin system plays an important role in the initial responses to dehydration or haemorrhage in these animals.

Angiotensin does not have positive cardiac chronotropic effects in anuran atrial preparations (Wilson *et al.* 1987). It differs in this respect from arginine vasotocin, which increases atrial contraction rate in *Bufo*, possibly by a mechanism not involving beta receptor stimulation (Chiu *et al.* 1988). Angiotensin-induced hypertension caused bradycardia in our intact, conscious toads, presumably by stimulating pulmocutaneous baroreceptors (West and Van Vliet, 1994). Sodium-nitroprusside-induced hypotension caused tachycardia of a similar magnitude to that observed previously (Van Vliet and West, 1989).

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