The Journal of Experimental Biology 206, 2675-2684

doi:10.1242/jeb.00468

Angiotensin II-induced inotropism requires an endocardial endothelium-nitric oxide mechanism in the *in-vitro* heart of *Anguilla anguilla*

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Accepted 28 April 2003

Summary

Using an isolated working heart preparation we show that angiotensin II (ANG II), at concentrations of 10^{-10}–10^{-7} mol l^{-1}, elicits negative chronotropism and inotropism in the freshwater eel *Anguilla anguilla*. The negative inotropism was insensitive to losartan and CGP42112 (AT_1 and AT_2 ANG II receptor antagonists, respectively), and was abrogated by the AT_1 receptor antagonist CV11974, the G protein blocker pertussis toxin (PTx) and the muscarinic antagonist atropine. In contrast, it was not affected by the adrenoceptor antagonists propanolol, sotalol and phentolamine. Using donors (L-arginine) and inhibitors [N^G-monomethyl-L-arginine (L-NMMA), L-N^5(1-iminoethyl)ornithine (L-NIO)] of nitric oxide synthase (NOS), and haemoglobin as NO scavenger, we demonstrate that NO signalling is involved in ANG II-mediated inotropism. Pretreatment with Triton X-100, a detergent that damages the endocardial endothelium (EE), or with 1H-(1,2,4)oxadiazolo-(4,3-a)quinoxalin-1-one (ODQ), a specific inhibitor of soluble guanylate cyclase, or with the cGMP-activated protein kinase (PKG) inhibitor KT5328, abolished ANG II-mediated inotropism. Thus, ANG II-mediated inotropism occurs *via* an EE-NO-cGMP-PKG mechanism. ANG II did not affect the mechanical performance influenced by preload changes (i.e. the Frank–Starling response), which in the eel heart is modulated by NO. This EE-paracrine-mediated cardio-suppressive action of endoluminal ANG II suggests that the hormone plays an important intracardiac role in the fish heart.

Key words: fish heart, angiotensin, nitric oxide, endocardial endothelium, Frank–Starling response, European eel, *Anguilla anguilla.*

Introduction

The renin–angiotensin system (RAS) is a master regulator of cardiovascular homeostasis and hydromineral balance in vertebrates. It also acts as a universal regulator of cardiac morphogenesis (Crackower et al., 2002). Its principal effector is the octapeptide angiotensin II (ANG II), a pluripotential hormone whose biological actions, including blood pressure regulation, fluid osmolarity and cardiac function, have been extensively studied in mammalian and non-mammalian vertebrates (for references, see Kobayashi and Takei, 1996). The presence of RAS components in myocardial and non-myocardial tissue (Meulemans et al., 1990, and references therein), together with the local production of ANG II, confirms that the intracardiac RAS directly modulates various cardiac functions in an autocrine/paracrine manner (Baker et al., 1992). In mammals, the cardiac actions of ANG II, as viewed in the context of circulating or locally produced hormone, include chronotropic and inotropic effects (Saavedra et al., 1993; Sekine et al., 1999), myocardial growth (Grinstead and Young, 1992) and coronary vasoconstriction (Timmermans et al., 1993). These effects are attributed to the interaction with specific plasma membrane receptors called AT_1 and AT_2, classified according to their selectivity for specific ligands such as losartan, CV11974, TCV116 (AT_1) and CGP42112, PD123177, PD123319 (AT_2) (see references in De Gasparo et al., 1995; Cerra et al., 2001).

Both ANG II receptors have been identified in the heart. Cardiac AT_1 is responsible for most of the ANG II-mediated effects on cardiac performance (i.e. chronotropism and inotropism) and rate of protein synthesis in isolated myocyte preparations (Schorb et al., 1993). AT_1 receptors belong to the superfamily of seven-transmembrane-spanning-domain receptors and are coupled to a classical second messenger system *via* G-protein. The main post-receptor signal transduction pathways include activation of the slow Ca^{2+} channel (Freer et al., 1976), acceleration of phosphoinositide hydrolysis (Baker and Aceto, 1989; Baker et al., 1989) and stimulation of nitric oxide synthase activity (Paton et al., 2001).

In fish, RAS components have been identified in teleosts (Nishimura, 1985; Olson, 1992) and elasmobranchs (Kobayashi and Takei, 1996). In bony fish, the RAS is active in multiple effector systems and there are numerous examples

of parallel actions in ANG II-mediated responses in teleosts and mammals (for references, see Kobayashi and Takei, 1996). Several fish ANG II receptors are now being cloned (Marsigliante et al., 1996; Tran van Chuoi et al., 1999). An eel angiotensin receptor cDNA sequence in the GenBank (Accession no. AJ05132; Tran Van Chuoi et al., 1999) shows 60% homology with the mammalian AT_1 receptor (Russell et al., 2000). In the cardiovascular system, species-specific ANG II effects have been documented in teleosts, most notably in synergy with the adrenergic system (Oudit and Butler, 1995; Bernier and Perry, 1999). ANG II has been found to exert both direct and indirect (i.e. *via* cardiac adrenoceptors) stimulatory effects on the heart of the American eel *Anguilla rostrata* and of the trout *Oncorhynchus mykiss* (Oudit and Butler, 1995; Bernier et al., 1999). Evidence for an intracardiac RAS in teleosts comes from angiotensin-converting enzyme (ACE) activity in the ventricles of a variety of species (Lipke and Olson, 1988). However, apart from these few studies, no data have been obtained in the fish heart about the direct effects of ANG II, the site(s) of action of the hormone, either local or systemic, and the signal transduction mechanisms involved. Eels represent an extraordinary example of flexibility in hydromineral and cardiovascular regulation and therefore the cardiac role of ANG II in these organisms may be of particular interest.

The aim of this study was to analyse the role of ANG II in modulating cardiac performance in isolated and perfused working heart preparations of the European eel *Anguilla anguilla* both under basal (i.e. non-stimulated) conditions and after chemical and mechanical stimuli.

As in a previous study (Imbrogno et al., 2001), we used juvenile eel hearts with a compact outer ventricular layer and a poorly developed coronary circulation. With this system, one may analyse the effects of cardioactive substances such ANG II without interference from the coronary vasculature and also explore the involvement of the endocardial endothelium (EE), i.e. the single-cell-thick lining of the cardiac chambers, in sensing and transducing ANG II stimuli. We previously reported that in the eel heart the EE acts as an endoluminal modulator of mechanical performance by way of a nitric oxide (NO)-cGMP mechanism (Imbrogno et al., 2001). We now demonstrate that ANG II exerts a direct suppressive effect on eel heart performance; this effect involves AT_1-like receptors, $G_{i/o}$ proteins and the cholinergic system, and occurs *via* an EE-NO-cGMP-cGMP-activated protein kinase (PKG) cascade. Preliminary results of this study have been presented in abstract form (Imbrogno et al., 2002).

Materials and methods

Isolated and perfused working heart preparations

We used specimens of freshwater European eel *Anguilla anguilla* L., weighing 74.33±2 g (mean ± S.E.M., N=90). Fish were provided by a local hatchery and kept at room temperature (18–20°C) without feeding, for 5–7 days. Experiments were performed from November to April. Each eel was anaesthetised in benzocaine (0.2 g l^{-1}) for approximately 15 min. The hearts, isolated and connected to a perfusion apparatus as previously described (Imbrogno et al., 2001), received Ringer's solution from an input reservoir and pumped against an afterload pressure given by the height of an output reservoir. The Ringer's solution contained the following in g l^{-1}: NaCl 6.68, KCl 0.15, KH_2PO_4 0.05, $MgSO_4$ 0.35, $(NH_4)_2SO_4$ 0.05, $CaCl_2$ 0.14, glucose 1, Na_2HPO_4 0.227; pH was adjusted to 7.7–7.9 by adding $NaHCO_3$ (approximately 1 g l^{-1}); the Ringer's solution was equilibrated with a mixture of O_2:CO_2 at 99.5:0.5%. Experiments were carried out at room temperature (18–20°C). For paced experiments, hearts were stimulated with an LE 12006 stimulator (Ugo Basile, Comerio, Italy) (frequency identical to that of control, non-paced hearts; pulse width fixed at 0.1 ms; voltage: 1.2±0.1 V, means ± S.E.M.).

Measurements and calculations

Pressure was measured through T-tubes placed immediately before the input cannula and after the output cannula, and connected to two MP-20D pressure transducers (Micron Instruments, Simi Valley, CA, USA) in conjunction with a Unirecord 7050 (Ugo Basile, Comerio, Italy). Pressure measurements (input and output) (kPa) were corrected for cannula resistance. Heart rate (f_H) was calculated from pressure recording curves. Cardiac output was collected over 1 min and weighed; values were corrected for fluid density and expressed as volume measurements. The afterload (mean aortic pressure) was calculated as two-thirds diastolic pressure plus one-third maximum pressure. Stroke volume (cardiac output/heart rate, V_S, in ml kg^{-1}) was used as a measure of ventricular performance; changes in V_S were considered to be inotropic effects. Cardiac output and V_S were normalized per kg wet body mass. Ventricular stroke work [W_S; mJ g^{-1}; (afterload – preload)×V_S/ventricle mass] served as an index of systolic functionality.

Experimental protocols

Basal conditions

Isolated perfused hearts were allowed to maintain a spontaneous rhythm for up to 15–20 min. In all experiments the control conditions were a mean output pressure of approximately 3.00 kPa, with a cardiac output rate set to 10 ml min^{-1} kg^{-1} body mass by appropriately adjusting the filling pressure. These values are within the physiological range (for references, see Imbrogno et al., 2001). Cardiac variables were simultaneously measured during experiments. To analyse the inotropic effects as distinct from the chronotropic actions of substances, the preparations were electrically paced. Hearts that did not stabilize within 20 min from the onset of perfusion were discarded.

Drug application

After the 15–20 min control period, both spontaneously beating and paced hearts were perfused for 20 min with Ringer's solution enriched with ANG II at increasing

concentrations to construct cumulative concentration–response curves. We used the homologue teleost octapeptide ANG II (Oudit and Butler, 1995).

Paced heart preparations were used to test the effects of 10^{-8} mol l^{-1} of ANG II in the presence of the ANG II receptor antagonists [CGP42112, Losartan, Candesartan (CV11974)], the specific NOS substrate L-arginine, the NO scavenger haemoglobin, the NOS inhibitors [L-N^5(1-iminoethyl)ornithine (L-NIO) and N^G-monomethyl-L-arginine (L-NMMA)], the soluble guanylate cyclase (GC) specific inhibitor [1H-(1,2,4)oxadiazole-(4,3-a)quinoxalin-1-one (ODQ)], and after inhibition of protein kinase G (PKG) by KT5823. The effects of ANG II (10^{-8} mol l^{-1}) were also analysed after pre-treatment with isoproterenol (ISO), phenylephrine, propanolol, phentolamine, sotalol and atropine. In the above-mentioned protocols the hearts were perfused for 20 min with Ringer's solution enriched with the specific drug at the given concentrations before the addition of ANG II.

In another set of experiments the effects of ANG II (10^{-8} mol l^{-1}) were tested after inhibition of G-proteins by pertussis toxin (PTx); in this case the hearts were pre-incubated for 60 min with PTx (10^{-11} mol l^{-1}).

The effect of ANG II (10^{-8} mol l^{-1}) was also studied after inducing functional damage of the ventricular EE with the detergent Triton X-100. 0.1 ml of 0.05% Triton X-100 was injected through a needle inserted into the posterior ventral region of the ventricular wall to avoid damage to the atrium (for further details, see Imbrogno et al., 2001). At this concentration the detergent does not affect the subjacent myocardium, as assessed by viability tracer and confocal microscopy (Sys et al., 1997).

Frank–Starling response

To study the interaction between ANG II and the Frank–Starling response, we generated a Starling curve (baseline condition) by varying the atrial reservoir height to alter the preload on the *in-vitro* heart. After baseline assessment, the atrial reservoir height was returned to basal conditions and a second Starling curve (untreated time-control) was generated. These time-control curves were compared with Starling curves constructed in the presence of ANG II.

Statistics

Percentage changes were evaluated as means ± S.E.M. of percentage changes obtained from individual experiments. Because each heart acted as its own control, the statistical significance of differences was assessed using the paired Student's *t*-test ($P<0.05$). We used the Student's *t*-test on absolute values for within-group comparisons of the Starling curves; between-group comparisons were made using two-way analysis of variance (ANOVA). Significant differences from the time-control group were detected with Duncan's multiple-range test.

Drugs and chemicals

All the solutions were prepared in double-distilled water [except for ODQ, which was prepared in ethanol]; dilutions were made in Ringer's solution immediately before use. ANG II, CGP42112, Losartan, L-arginine, haemoglobin, L-NIO, L-NMMA, PTx, Triton X-100, ISO, phenylephrine, propanolol, phentolamine, sotalol and atropine sulphate salt were purchased from Sigma Chemical Company (St Louis, MO, USA). KT5823 (used in a darkened perfusion apparatus to prevent degradation) was purchased from Calbiochem (Milan, Italy). Candesartan (CV11974) was a generous gift from Takeda Pharmaceutical Company, Ltd. (Osaka, Japan).

Results

The isolated and perfused working heart preparation

The *in-vitro* isolated and perfused whole heart preparation works at physiological loads and generates values of output pressure, cardiac output, *V*S, *W*S and power that mimic the physiological values of the animal, as previously described (Imbrogno et al., 2001).

Effects of ANG II on basal cardiac performance

A concentration–response curve of the effect of ANG II (10^{-10} mol l^{-1} to 10^{-7} mol l^{-1}) on spontaneously beating eel heart preparations revealed that f_H was significantly reduced at concentrations as low as 10^{-9} mol l^{-1} (Fig. 1). In electrically paced preparations, ANG II (at concentrations from 10^{-11} to 10^{-7} mol l^{-1}) induced a negative inotropism, as shown by a significant decrease in *V*S and *W*S only at higher concentrations (10^{-8} and 10^{-7} mol l^{-1}) (Fig. 2). These effects appeared between 5 and 10 min of exposure of the preparation to ANG II.

Transducing receptors and G-protein interactions

To identify the receptors involved in the ANG II-dependent inotropic response we used a classic mammalian AT_1 antagonist, losartan, and CV11974 and CGP42112, two AT_1

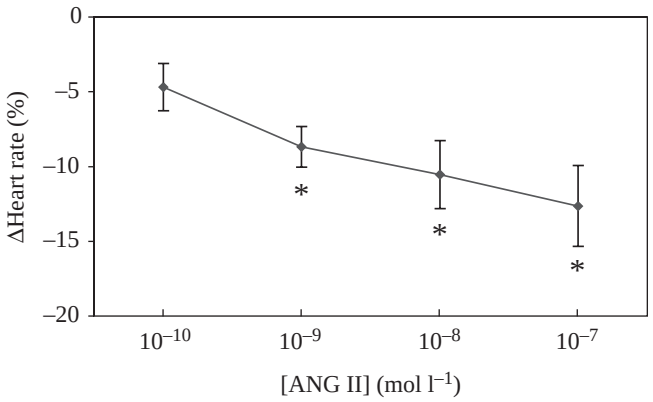

Fig. 1. Cumulative dose–response curve showing the effect of angiotensin II (ANG II; from 10^{-10} to 10^{-7} mol l^{-1}) on heart rate in isolated and perfused unpaced eel hearts. Percentage changes were evaluated as means ± S.E.M. of five experiments. *Significantly different from the control value ($P<0.05$).

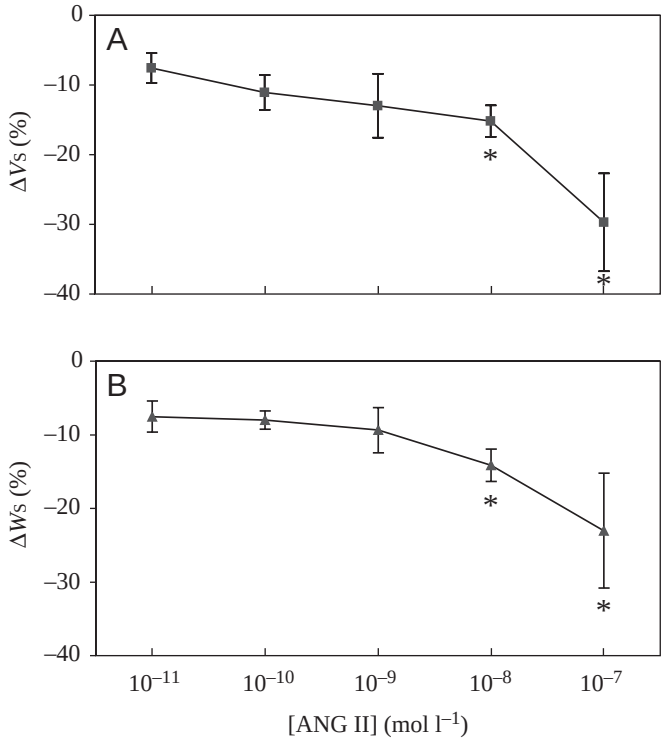

Fig. 2. Cumulative dose–response curve showing the effect of for angiotensin II (ANG II; from 10^{-11} to 10^{-7} mol l^{-1}) on (A) stroke volume (*V*S) and (B) stroke work (*W*S) in isolated and perfused paced eel hearts. Percentage changes were evaluated as means ± S.E.M. of five experiments. *Significantly different from the control value (P<0.05).

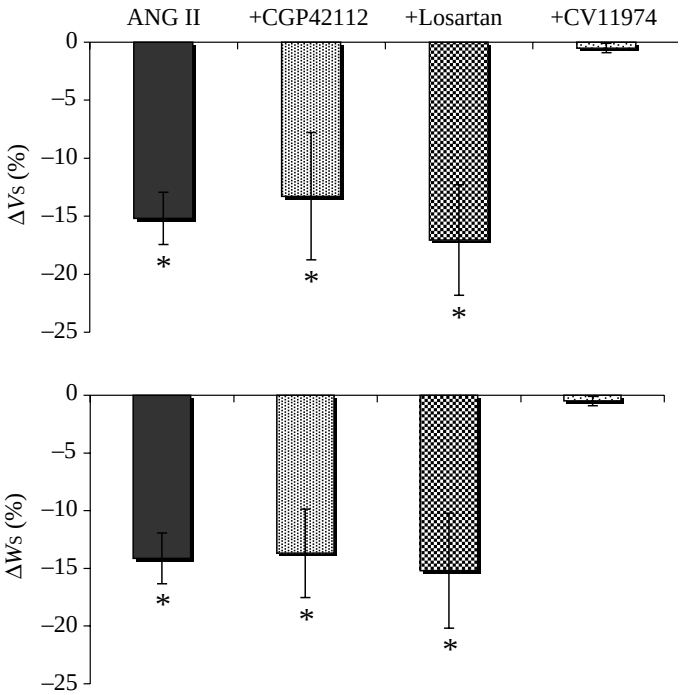

Fig. 3. Effects of angiotensin II (ANG II; 10^{-8} mol l^{-1}) before and after treatment with CGP42112 (10^{-6} mol l^{-1}), losartan (10^{-6} mol l^{-1}) and CV11974 (10^{-7} mol l^{-1}) on stroke volume (*V*S) and stroke work (*W*S) in isolated and perfused paced eel hearts. For details, see Materials and methods. Percentage changes are shown as means ± S.E.M. of 4–5 experiments for each drug. *Significantly different from the control value (P<0.05).

and AT_2 antagonists, respectively, which are selective toward non-mammalian cardiac (Cerra et al., 2001) and non-cardiac (Tierney et al., 1997; Hazon et al., 1997) ANG II receptors. The negative inotropism induced by ANG II (10^{-8} mol l^{-1}) was not affected by either losartan (10^{-6} mol l^{-1}) or CGP42112 (10^{-6} mol l^{-1}), whereas it was abolished by CV11974 (10^{-7} mol l^{-1}) (Fig. 3). These results implicate an AT_1-like receptor in the effects of ANG II on the heart. The AT_1 receptor belongs to the guanine nucleotide-binding protein (G protein)-coupled receptor superfamily characterised by seven-transmembrane segments (Sasaki et al., 1991). To examine whether G proteins mediate the inhibitory inotropic action of ANG II (10^{-8} mol l^{-1}), the cardiac preparations were pre-incubated with pertussis toxin (PTx; 10^{-11} mol l^{-1}), which uncouples signal transduction between several families of receptors and G_i or G_o proteins (Ai et al., 1998 and references therein). PTx alone did not influence mechanical performance (data not shown), whereas pre-treatment with the toxin abolished the effects of ANG II (Fig. 4), suggesting that they are mediated by the G protein system.

Involvement of the adrenergic and cholinergic systems

It has been suggested that the cardiovascular effects of ANG II in teleosts may be mediated by catecholamines (Oudit and Butler, 1995; Bernier and Perry, 1999) or by modulation of cholinergic tone (Reid, 1992). We assessed the relative contributions of cholinergic and adrenergic activation to the cardiac effects induced by ANG II (10^{-8} mol l^{-1}) in *A. anguilla*. Pre-treatment with atropine (an unspecific muscarinic antagonist; 10^{-6} mol l^{-1}) abolished the negative effects of ANG II on *V*S and *W*S (Fig. 4). In contrast, treatment with both adrenoceptor antagonists phentolamine (10^{-8} mol l^{-1}), propanolol (10^{-8} mol l^{-1}) and sotalol (10^{-7} mol l^{-1}) and agonists phenylephrine (10^{-9} mol l^{-1}) and ISO (10^{-9} mol l^{-1}), did not modify the ANG II-mediated inotropic response (Fig. 5).

Involvement of an EE-NO-cGMP-PKG signal transduction pathway

Nitric oxide, *via* activation of GC, is an important modulator of cardiac performance in the working eel heart *in vitro* (Imbrogno et al., 2001). There is evidence of cross talk between ANG II and nitric oxide synthase (NOS) in the downstream transduction cascade activated by AT_1 (Paton et al., 2001). To analyse whether the ANG II response involves a NO-cGMP pathway, the paced preparations were pre-treated with the natural NOS substrate L-arginine (10^{-6} mol l^{-1}), the NO scavenger haemoglobin (10^{-6} mol l^{-1}), the NOS inhibitors L-NIO and L-NMMA (10^{-5} mol l^{-1}) and the guanylyl cyclase blocker ODQ (10^{-5} mol l^{-1}). The inotropic effect of ANG II

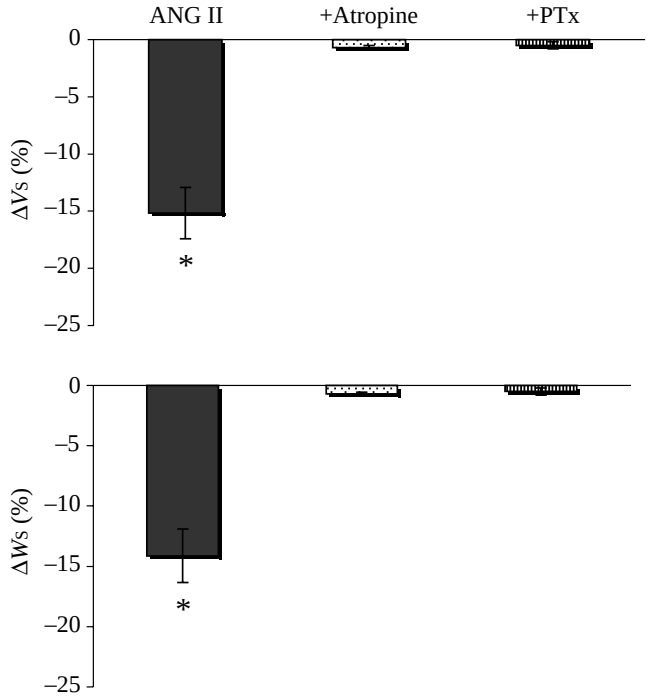

Fig. 4. Effects of angiotensin II (ANG II; 10^{-8} mol l^{-1}) before and after treatment with atropine (10^{-6} mol l^{-1}) and pertussis toxin (PTx; 10^{-11} mol l^{-1}) on stroke volume (*V*S) and stroke work (*W*S) in isolated and perfused paced eel hearts. For details, see Materials and methods. Percentage changes are shown as means ± S.E.M. of four experiments for each drug. *Significantly different from the control value (P<0.05).

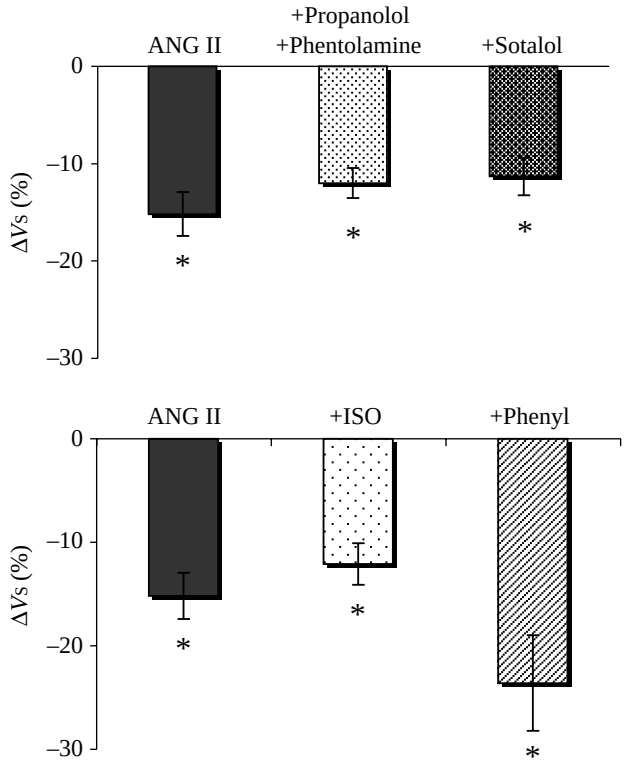

Fig. 5. Effects of angiotensin II (ANG II; 10^{-8} mol l^{-1}) before and after treatment with propanolol (10^{-8} mol l^{-1}) and phentolamine (10^{-8} mol l^{-1}), sotalol (10^{-7} mol l^{-1}), isoproterenol (ISO; 10^{-9} mol l^{-1}) and phenylephrine (Phenyl; 10^{-9} mol l^{-1}) on stroke volume (*V*S) and stroke work (*W*S) in isolated and perfused paced eel hearts. For details, see Materials and methods. Percentage changes are shown as means ± S.E.M. of 4–5 experiments for each drug. *Significantly different from the control value (P<0.05).

(10^{-8} mol l^{-1}) was significantly enhanced in the presence of L-arginine, but it was abolished by haemoglobin, L-NIO, L-NMMA and ODQ (Fig. 6).

cGMP modulates cardiac contractility through several intramyocardial mechanisms, one of which is *via* activation of a cGMP-PKG pathway (Hove-Madsen et al., 1996). We studied the ANG II inotropic response before and after treatment with a specific inhibitor of PKG (KT5328, 10^{-7} mol l^{-1}). This treatment reduced the inotropic effect of ANG II (Fig. 7), which suggests that the NO-cGMP-PKG pathway plays a role in the effects of ANG II on the heart.

The avascular heart of *A. anguilla* possesses a highly trabeculated ventricle with an extensive EE surface which, being an important source of NO, modulates cardiac performance (Imbrogno et al., 2001). The EE impairment caused by Triton X-100 (0.05%), a detergent which, at this concentration, damages the EE functionally but not structurally (see Sys et al., 1997), abolished the ANG II (10^{-8} mol l^{-1})-mediated inotropic effects (Fig. 6), thereby implicating EE in the transduction of endoluminal ANG II signalling.

ANG II and the Frank–Starling response

Intracardiac NO increases the sensitivity of the *in-vitro* eel heart to filling pressure changes, i.e. to the Frank–Starling response (preload-induced increases in cardiac output at constant afterload and heart rate; see Imbrogno et al., 2001). The influence of ANG II on the Frank–Starling response of the isolated and perfused eel heart was studied by increasing the preload (see Materials and methods) in the presence and absence of ANG II (10^{-8} mol l^{-1}). Two-way ANOVA showed no significant differences between the Frank–Starling curves obtained with and without ANG II (Fig. 8). To separate the time factor (i.e. the heart's 'memory') of loading stimulation, we generated baseline and time-control curves. ANOVA showed that the curves were identical within the limits of experimental error.

Discussion

Isolated working heart preparations

Since haemodynamic stresses on the heart enhance the release of factors, including NO, that affect cardiac performance, it is important to use a 'physiological' working heart preparation to evaluate autocrine and/or paracrine regulation of cardiac performance (Imbrogno et al., 2001). This kind of working heart preparation may also elicit activation of a silencing endothelial NOS (eNOS). There is evidence from

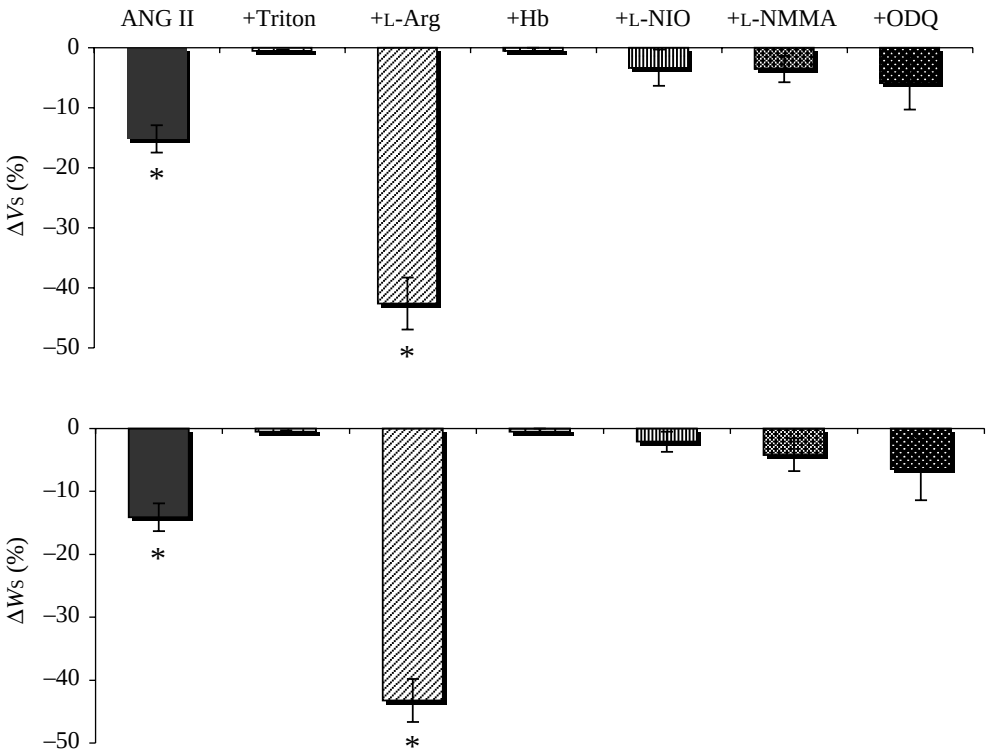

Fig. 6. Effects of angiotensin II (ANG II; 10^{-8} mol l^{-1}) before and after injection with Triton X-100 (0.05%), or perfusion with L-arginine (L-Arg; 10^{-6} mol l^{-1}), haemoglobin (Hb; 10^{-6} mol l^{-1}), L-N^5(1-iminoethyl)ornithine (L-NIO; 10^{-5} mol l^{-1}), N^G-monomethyl-L-arginine (L-NMMA; 10^{-5} mol l^{-1}) and 1H-(1,2,4)oxadiazolo-(4,3-a)quinoxalin-1-one (ODQ; 10^{-5} mol l^{-1}) on stroke volume (*V*S) and stroke work (*W*S) in isolated and perfused paced eel hearts. For details of treatment and perfusions, see Materials and methods. Percentage changes are shown as means ± S.E.M. of 4–5 experiments for each drug. *Significantly different from the control value (P<0.05).

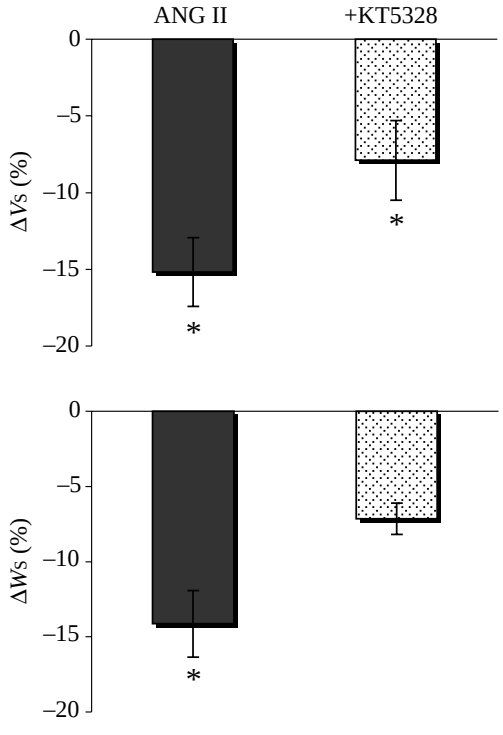

Fig. 7. Effects of angiotensin II (ANG II; 10^{-8} mol l^{-1}) before and after treatment with KT5328 (10^{-7} mol l^{-1}) on stroke volume (*V*S) and stroke work (*W*S) in isolated and perfused paced eel hearts. Percentage changes are shown as means ± S.E.M. of four experiments. *Significantly different from the control value (P<0.05).

mammalian studies that eNOS, due to its intrinsic molecular properties (i.e. anchorage to caveolin-1, myristoylation and palmytoilation, auto-inhibition of calmodulin binding), is inactive at the basal cellular level (Wu, 2002, and references therein). The failure to detect nitrergic tone in several *in vitro* cardiovascular preparations in fish could be partly due to activation of this silencing eNOS.

Effects of ANG II on basal cardiac performance

Exogenous ANG II exerted direct chronotropic and inotropic effects on the isolated working heart of freshwater *A. anguilla*. In spontaneously beating heart preparations, ANG II induced negative chronotropism, which became significant at a concentration of 10^{-9} mol l^{-1}. Nanomolar concentrations of ANG II had a negative chronotropic effect in pacemaker cells of rabbit through modulation of the L-type Ca^{2+} current (Habuchi et al., 1995). In electrically paced preparations, ANG II at 10^{-8} moll^{-1} and 10^{-7} mol l^{-1} caused a significant decrease in *V*S and *W*S, indicating a direct negative modulation of mechanical performance. The cardiac effects of ANG II are species-specific in mammals. For example, ANG II elicits positive inotropism in dogs, cats, rabbits, chickens and humans, while it has no effect in rats or guinea-pigs (Ai et al., 1998, and references therein). The mechanisms whereby ANG II exerts its effect, however, are only partially understood (Meulemans et al., 1990). Results obtained with mammalian ventricular myocardia suggest that this wide range of species variations in ANG II-mediated inotropism is due either to different intracardiac endocrine stores, e.g. the release of catecholamines responsible for the indirect positive inotropism

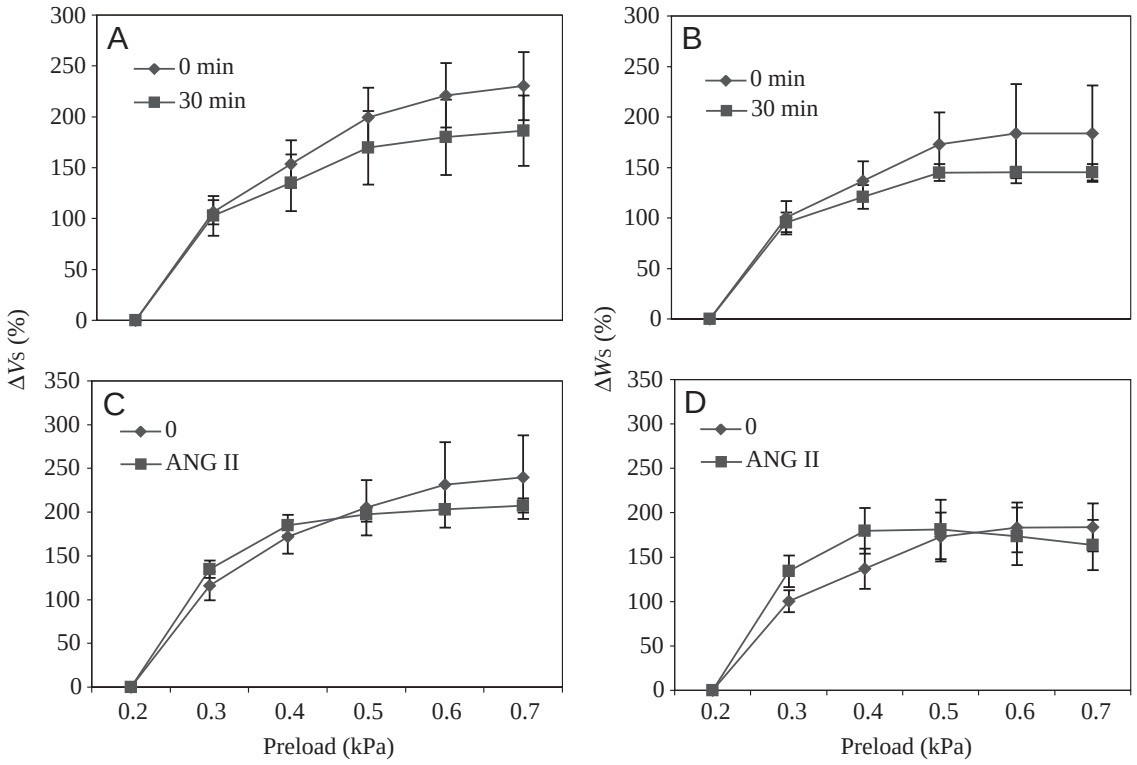

Fig. 8. Effects of preload elevation on (A,C) stroke volume (*V*S) and (B,D) stroke work (*W*S) (A,B) in control conditions and (C,D) after treatment with angiotensin II (ANG II; 10^{-8} mol l^{-1}) in isolated and perfused paced eel hearts. Percentage changes are shown as means ± S.E.M. of five experiments for each group. A paired Student's *t*-test was used for comparison within groups; a two-way ANOVA analysis was used for comparison between groups. (A,B) The baseline control (0 min) and the untreated time-control (30 min) values (see Materials and methods). (C,D) Values without ANG II (0 min) and with ANG II (30 min).

of the peptide in dog papillary muscle (Drimal and Boska, 1973), or to different AT receptor patterns, tissue localization and coupling to divergent facilitatory or inhibitory intracellular signal-transduction pathways (Nishimura, 2001).

There are very few studies on the cardiotropic effects of ANG II in fish. In conscious freshwater *Anguilla rostrata*, physiological doses of the homologous peptide increased cardiac output mainly by increasing *V*S, a finding that was attributed to positive inotropism and/or to the Frank–Starling mechanism (Oudit and Butler, 1995). Using *in situ* heart preparations, Bernier and Perry (1999) observed that rapid changes in systemic vascular resistance and slower longer-lasting changes in cardiac output contributed to the ANG II-mediated vasopressor responses in *Oncorhynchus mykiss* and *A. rostrata*. However, in the latter species, unlike in the trout, changes in cardiac output were responsible for the indirect adrenergically mediated vasopressor action of ANG II. The discrepancy between these and our results might be due to species-related differences, as documented in mammals, and/or to the organizational level under study (i.e. intact cardiovascular system or *in situ* heart *versus* isolated and denervated working heart) and functional interactions among its key components. For example, in *A. anguilla* the ANG II-mediated cardio-suppressive effect observed at heart level *in vitro* could be overridden *in vivo* by an overall cardiovascular excitatory stimulation due to the synergism of the adrenergic and RAS pathways, both of which are activated under stress and emergency conditions (for the vascular district, see Hazon et al., 1995). The intriguing possibility that ANG II exerts divergent cardiovascular effects at local and systemic levels, i.e. local cardio-inhibitory protection *versus* systemic cascades of convergent excitatory stimuli targeting the heart, would not be surprising in view of the recently proposed concept of a counter-regulatory hormone in 'zero steady-state error' homeostasis (Koeslag et al., 1999). Additional studies are needed to test this hypothesis.

Transducing receptors and G protein interactions

We used the AT_1-selective receptor antagonists losartan and CV11974 and the AT_2-selective antagonist CGP42112 to identify the receptor subtypes involved in the ANG II inotropic response. In agreement with functional studies conducted with teleosts (Olson et al., 1994; Bernier and Perry, 1997; Cobb et al., 1999), we found that losartan did not prevent the ANG II-mediated effects. This finding can be explained by the functional restrictions of the antagonist site of the receptor (GenBank Accession No. AJ05132; Tran van Chuoi et al. 1999) that in the eel does not contain residues associated with losartan binding (Russell et al., 2000). For this reason we used as AT_1 antagonist CV11974, which blocks the vascular ANG

II-mediated effects *in vivo* and *in vitro* (Maillard et al., 2002) and also binds cardiac ANG II receptors in an elasmobranch (Cerra et al., 2001). CV11974, but not GCP42112, abolished the negative inotropism elicited by ANG II, suggesting that it may be mediated by an AT_1-like receptor. This result agrees with those of functional studies conducted with non-cardiac tissues of other teleosts (see Nishimura, 2001; Russell et al., 2000); however other AT_2 antagonists should be tested before ruling out any role for AT_2 receptors in the cardiac effects of ANG II.

The negative inotropic action of ANG II was prevented by pre-treatment with PTx, pointing to the involvement of a PTx-sensitive G protein. In mammalian myocytes, AT_1 receptors, acting through a G_i protein, mediate the inhibitory effect of ANG II on L-type Ca^{2+} currents and on the adenylate cyclase system (Ai et al., 1998). Whether the ANG II-induced negative inotropism in the eel heart involves these mechanisms or other intracellular cascades (i.e. the phosphoinositide pathway) remains to be established.

Involvement of the adrenergic and cholinergic systems

There is evidence that some of the cardiovascular effects of ANG II in teleost are mediated by catecholamines (Bernier and Perry, 1999; Oudit and Butler, 1995) or by changes in cholinergic tone (Reid, 1992). However, it is not clear whether the interactions occur at the adrenergic nervous endings, including the intramyocardial terminals, at the chromaffin tissue level, or both (Nishimura et al., 1978). Whereas in *A. anguilla* inhibition of α-adrenergic receptors only attenuates the ANG II response (Oudit and Butler, 1995), in *Squalus acanthias* the α-adrenergic antagonist phentolamine blocks the ANG II effect (Opdyke and Holcombe, 1976). Moreover, in *A. rostrata* β-adrenergic receptor blockade did not affect the cardiovascular response to ANG II (Oudit and Butler, 1995). In the denervated eel heart preparation, our finding that ANG II-mediated inotropism was insensitive to α- and β-adrenergic agonists (phenylephrine and isoproterenol) and to adrenergic antagonists (phentolamine, propanolol and sotalol) argues against intra-cardiac adrenergic involvement. To what extent this adrenergically independent inotropic response to blood-borne ANG II may apply to the innervated heart remains to be elucidated.

In the heart, cholinergic stimuli, mediated by the M_2 and M_4 muscarinic receptor subtypes (mAChR), preferentially located on the myocardiocytes and coupled to adenylate cyclase inhibition, elicit negative chronotropic and inotropic effects (mammals: Hove-Madsen et al., 1996; teleosts: Imbrogno et al., 2001). Little is known about how the cholinergic system influences ANG II-mediated inotropy. Antagonism between ANG II and cholinergic effects has been reported in mammals (Ai et al., 1998). In *A. rostrata*, the ANG II effect was greater after muscarinic receptor blockade (Oudit and Butler, 1995). Our finding that the non-specific muscarinic antagonist atropine blocked ANG II-mediated negative inotropy suggests that in *A. anguilla* the cardiotropic action of ANG II could be partly due to activation of muscarinic receptors.

Involvement of an EE-NO-cGMP-PKG signal transduction pathway

In the *in vitro* working eel heart, the EE under basal conditions, and when activated by chemical (i.e. acetylcholine) or physical stimuli negatively modulates mechanical performance as a result of the tonic release of NO, which in turn increases cGMP levels in cardiomyocytes (Imbrogno et al., 2001).

Interactions between ANG II and endothelial-type NOS have been demonstrated *in vivo* and *in vitro* in the mammalian vascular endothelium (for references, see Li et al., 2002), where the AT_1 subtype receptor has been reported. In contrast, except for a study describing AT_2 receptors in the human EE (Wharton et al., 1998), there are no reports of ANG II receptors in the EE. The obligatory role played by the eel EE in transducing the intracavitary ANG II signal suggests that AT_1-like receptors could be located at the EE level.

ANG II-mediated negative inotropism was significantly enhanced in the presence of the NOS substrate L-arginine, but abolished by pre-treatment with NO scavenger (Hb), specific NOS (L-NIO and L-NMMA) or soluble GC (ODQ) inhibitors, or by exposure to Triton X-100, which are all consistent with stimulation of EE-NO-cGMP signalling induced by endoluminal ANG II. The integrity of the EE is a prerequisite for triggering the ANG II signal transduction pathway, and is further evidence that EE-NO plays an intracavitary autocrine–paracrine role in the control of fish heart function (Imbrogno et al., 2001).

An important intramyocardial target of NO signalling is PKG. In addition to its direct effect on calcium influx in isolated mammalian ventricular cardiomyocytes (Méry et al., 1991), PKG, through phosphorylation of troponin I, reduces the affinity of troponin C for calcium, thereby negatively regulating cardiac contractility (Hove-Madsen et al., 1996). Pre-treatment with the inhibitor KT5328 attenuated the effect of ANG II, thereby implicating PKG in ANG II-mediated negative inotropism.

AT_1 receptors, G-proteins, G-protein-linked receptors (e.g. mAChR) and several signalling molecules (e.g. eNOS, protein kinase C, Ca^{2+} channels, plasmalemmal Ca^{2+}-ATPase) are structurally and functionally localized in the endothelial cell caveolae (Ishizaka et al., 1998). Being the location of many proteins involved in signal-transduction cascades, the caveolae could be the domain where ANG II signalling is generated. In the light of our earlier results showing intracardiac cross talk between EE-NO-cGMP and cholinergic stimuli (Imbrogno et al., 2001), we postulate that the colocalization of AT_1 receptors, muscarinic cholinergic receptors and eNOS in the restricted space of the EE caveolae (see Fig. 9) may represent a temporally and spatially delimited pathway for signal transduction.

ANG II and the Frank–Starling response

Like most fish hearts, the eel heart is very sensitive to the Frank–Starling response, i.e. the heterometric regulation that contributes to the increased cardiac output associated with increased filling pressure, as occurs during periods of exercise or increased venous return (Farrell and Jones, 1992). ANG II is a potent effector of venoconstriction in teleosts (Oudit and

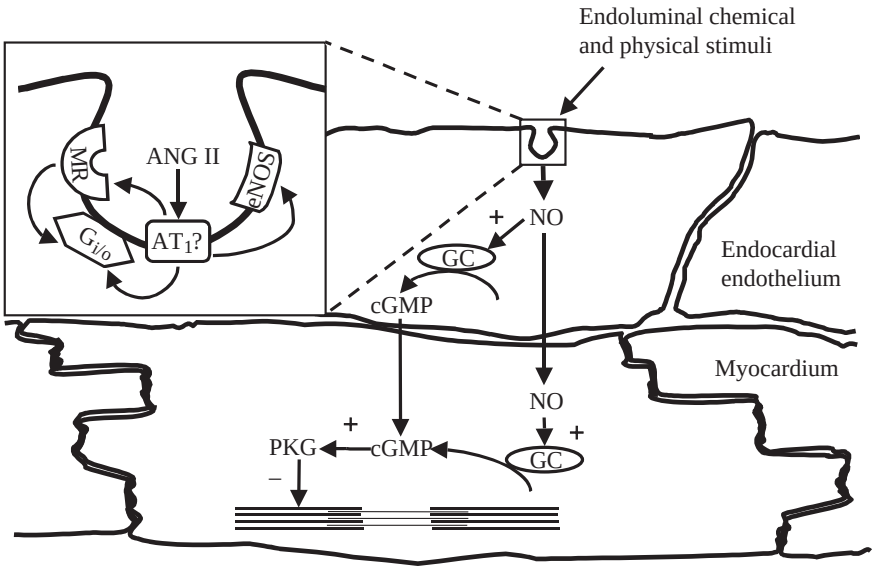

Fig. 9. Cross talk between the endocardial endothelium (EE) and the myocardium in *A. anguilla*. Endoluminal ANG II activates AT_1-like receptors in the EE that stimulate NO release, eliciting in the subjacent myocardium the negative inotropism through a cGMP-PKG mechanism. NO, nitric oxide; $G_{i/o}$, $G_{i/o}$ proteins; MR, muscarinic receptor; PKG, cGMP-activated protein kinase; GC, guanylate cyclase; eNOS, endothelial nitric oxide synthase.

Butler, 1995). In the *in vitro* isolated working heart of *A. anguilla* the basal release of endogenous NO greatly affects the Frank–Starling response by making the heart more sensitive to preload-induced increases in cardiac output at a constant afterload and heart rate (Imbrogno et al., 2001). It is therefore notable that, although exerting NO-dependent negative inotropism, ANG II *per se* did not affect the Frank–Starling mechanism in the eel heart. This indicates that the NO-cGMP mechanism underlying ANG II negative inotropism may differ from the mechanism underlying the nitrergic modulation of the Frank–Starling response. The finding that in the eel heart the local cardio-suppressive modulatory action of ANG II is exerted without detriment of the intrinsic heterometric modulation may be of great physiological interest. It is also compatible with the hypothesis that the local ANG II cardio-inhibitory modulation is part of a homeostatic loop that protects the heart from excessive haemodynamic loads such as those deriving from activation of the systemic RAS itself and the adrenergic system.

In conclusion, this study provides the first evidence that endoluminal ANG II exerts a direct cardio-suppressive effect on the mechanical performance of the fish heart *via* interaction with the endocardial endothelium. This interaction activates G protein-coupled AT_1-like receptors, which in turn trigger a NO-cGMP-PKG signal transduction pathway. The cardio-depressive effect of ANG II does not influence the Frank–Starling response. These data, together with the involvement of the muscarinic receptors in mediating ANG II inotropic stimulation, suggest that the EE, through its sensory function, is able to adapt cardiac performance to the peripheral demands of the fish. The EE caveolae are prime candidates as the domain at which the tonic-phase ANG II-NO signalling is generated.

Abbreviations

ACE	angiotensin-converting enzyme
ANG II	angiotensin II
EE	endocardial endothelium
GC	guanylate cyclase
ISO	isoproterenol
L-NIO	L-N^5(1-iminoethyl)ornithine
L-NMMA	N^G-monomethyl-L-arginine
NO	nitric oxide
NOS	nitric oxide synthase
ODQ	1H-(1,2,4)oxadiazolo-(4,3-a)quinoxalin-1-one
PKG	cGMP-activated protein kinase
PTx	pertussis toxin
RAS	renin–angiotensin system

Grants for S.I. was from ‘Progetto Giovani Ricercatori, UNICAL, 2001’. B.T. and M.C.C. were supported by PNRA (Programma Nazionale di Ricerche in Antartide, 2001). We are grateful to Jean Ann Gilder for editing the text.

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