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Cardiac angiotensin II receptors: studies on functional coupling in Sprague-Dawley rats and TGR(αMHC-hAT$_1$) transgenic rats

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Abstract

The renin-angiotensin system plays an important role in the pathogenesis of cardiac hypertrophy and chronic heart failure as angiotensin II has been shown to induce cardiac hypertrophy and fibrosis. Besides these structural alterations, functional effects on cardiomyocytes have been reported in different mammalian species. Angiotensin II is known to produce a positive inotropic effect in some species, and differences in atrial and ventricular myocardium have been described. So far, the molecular events which govern angiotensin II-mediated changes in cardiac contractility are not completely understood. In order to study the dependency of the angiotensin II-induced positive inotropic effect on receptor density, we examined the effect of angiotensin II on cardiac function in atria, papillary muscles and isolated ventricular cardiomyocytes from adult Sprague-Dawley rats and TGR(αMHC-hAT$_1$) transgenic rats, which expressed the human angiotensin AT$_1$ receptor (hAT$_1$) specifically in the heart. In atrial myocardium from adult Sprague-Dawley rats, angiotensin II (30 μmol/l) produced an AT$_1$-mediated positive inotropic effect (38.5% of control), whereas in papillary muscles and isolated ventricular myocytes, no inotropic response was observed. As shown by polymerase chain reaction (PCR) and radioligand binding, the human angiotensin AT$_1$ receptor was exclusively expressed in transgenic animals, which markedly overexpressed the angiotensin AT$_1$ receptor. However, in transgenic rats the positive inotropic effect in atrial preparations was similar to the controls, and neither in papillary muscles nor in isolated cardiomyocytes the increase in receptor density led to an inotropic effect induced by angiotensin II. These data suggest that the existence of functionally uncoupled receptors rather than the low density of receptors at the ventricular site is responsible for the inability of ventricular myocardium to respond to angiotensin II. © 1997 Elsevier Science B.V.

Keywords: Angiotensin II; Angiotensin AT$_1$ receptor; Contractile function; Cardiomyocyte, isolated; Transgenic rat

1. Introduction

The renin-angiotensin system plays an important role in the pathogenesis of arterial hypertension, cardiac hypertrophy and chronic heart failure. Since all of its components can be generated or activated at the tissue level, the structural and functional consequences of its activation in organs appear to be due to endocrine as well as paracrine or autocrine mechanisms (Dzau, 1987; Jin et al., 1987). In addition to the well-documented vasoconstrictor effects, angiotensin II also influences cardiovascular homeostasis inducing cardiac hypertrophy and fibrosis (Timmermans et al., 1993), as well as positive chronotropic and inotropic effects in various mammalian species (Dempsey et al., 1971; Freer et al., 1976; Kobayashi et al., 1978; Rogers, 1984; Hirakata et al., 1990; Holubarsch et al., 1993; Zerkowski et al., 1993; Ishihata and Endoh, 1995; Lenz et al., 1995). Based on different affinities for the specific nonpeptide receptor antagonists losartan (2-*n*-butyl-4-chloro-5-hydroxymethyl-1-[2′((1*H*-tetrazol-5-yl)biphenyl-4-yl)-methyl]imidazole) and PD 123177 (1-[(4-amino-3-methylphenyl)methyl]-5-(diphenyl-acetyl)-4,5,6,7-tetrahydro-1*H*-imidazole[4,5-*c*]pyridine-6-carboxylic acid), two subtypes of angiotensin II receptors, AT$_1$ and AT$_2$, have been identified (Bumpus et al., 1991; Smith et al., 1992). Both are expressed in the rat heart, but subtype distribution and the presence on cardiac myocytes coupled to force of contraction remain controversial (Sechi et al., 1992; Meggs et al., 1993; Suzuki et al., 1993; Lopez et al.,

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1994). Most of the known biological effects of angiotensin II including inotropic effects are mediated by the angiotensin AT_1 receptor (Feolde et al., 1993), which is a member of the family of G-protein-coupled receptors. The function of the angiotensin AT_2 receptor, whose coupling to G-proteins is controversial (Bottari et al., 1991; Mukojama et al., 1993; Zhang and Pratt, 1996), remains unclear, although there is evidence that it antagonizes the growth effects of the angiotensin AT_1 receptor and mediates inhibition of cell proliferation (Nakajima et al., 1995; Stoll et al., 1995).

Cardiac hypertrophy can be stimulated by hemodynamic processes like mechanical stress as well as by various endogenous mediators such as endothelin-1 (Shubeita et al., 1990), α-adrenoceptor stimulants (Simpson and McGrath, 1983) and angiotensin II (Sadoshima and Izumo, 1993a). However, the translation of mechanical to biochemical stimuli via paracrine release of angiotensin II and endothelin-1 is a current subject of debate (Sadoshima and Izumo, 1993b; Yamazaki et al., 1995). Angiotensin II has been reported to stimulate cell growth leading to structural remodeling of the cardiac interstitium in hypertensive heart disease (Weber and Brilla, 1991; Morgan and Baker, 1991), and angiotensin converting-enzyme inhibitors are effective in both reduction of myocardial hypertrophy and improvement of cardiac function (The CONSENSUS Trial Study Group, 1987; Mukherjee and Sen, 1990; Sumimoto et al., 1992).

Besides these structural changes, direct functional effects on cardiomyocytes have been reported in different mammalian species. Since it is not clear whether the left ventricular diastolic dysfunction, which precedes systolic dysfunction in hypertensive heart disease (Fouad, 1987), is due to interstitial changes or to intrinsic abnormalities of cardiomyocytes, it is important to differentiate between the effects of angiotensin II on myocardial interstitial cells and on cardiac myocytes. The effects of angiotensin II on myocardial contractility are not completely understood and appear to differ among species. Positive inotropic and chronotropic effects have been reported in dogs (Kobayashi et al., 1978), cats (Dempsey et al., 1971), rabbits (Freer et al., 1976; Ishihata and Endoh, 1995), hamsters (Hirakata et al., 1990), cows (Rogers, 1984) and human atrial myocardium (Moravec et al., 1990; Holubarsch et al., 1993; Zerkowski et al., 1993; Lenz et al., 1995), whereas no such effects could be observed in guinea pig hearts or human ventricular myocardium (Baker and Singer, 1988; Holubarsch et al., 1993; Lenz et al., 1995). In the rat, the contractile response to angiotensin II remains a subject of controversy since opposing results have been obtained by different investigators (Kobayashi et al., 1995; Neyses and Vetter, 1990). The fact that there are functionally coupled AT_1 receptors in rat cardiac myocytes has been shown by recent experiments demonstrating that angiotensin II causes an increase in protein synthesis, alterations in sarcoplasmatic reticulum protein gene expression and induction of proto-oncogene expression (Sadoshima and Izumo, 1993c). These effects are most pronounced and well characterized in neonatal myocytes. Nevertheless, the molecular events which govern the angiotensin II-mediated changes in cardiac contractility are not fully understood. In order to study the dependency of the angiotensin II-induced contractile response on AT_1 receptor density, we examined the effect of angiotensin II on cardiac function in atrial and ventricular myocardium from adult Sprague-Dawley rats as well as transgenic rats which markedly overexpress the angiotensin AT_1 receptor.

2. Materials and methods

2.1. Animals

Adult Sprague-Dawley and Wistar Kyoto rats were obtained from Bayer (Wuppertal, Germany). Transgenic rats [TGR(αMHC-hAT_1) 594-17] expressed the human angiotensin AT_1 receptor (hAT_1) under the regulatory control of a 1030 bp α-myosin heavy chain (αMHC) promotor specifically in the heart (Hoffmann et al., 1996). They were housed and bred in the animal laboratories of the Max Delbrück Centrum in Berlin (Germany). Age-matched Sprague-Dawley control rats were obtained from Tierzucht Schönwalde (Germany). This was the same strain into which the transgene was originally introduced. Only male rats were used. All animals were held on a standard laboratory animal diet (Altromin®) and tap water ad libitum. They were exposed to 12 h dark and light cycles at 20–22°C. Adult Sprague-Dawley rats had a mean body weight of 192 ± 9 g and the mean heart weight was 2.5 ± 0.2 g. Transgenic rats and Sprague-Dawley control rats were investigated at an age of 4–5 weeks, because at this age overexpression of the AT_1 receptor in the transgenic animals was maximal decreasing thereafter. However, these animals showed no signs of hypertension or cardiac hypertrophy and there was no significant difference in body and heart weights compared to Sprague-Dawley rats (body weight 77 ± 24 g vs. 78 ± 6 g, heart weight 328 ± 75 mg vs. 339 ± 23 mg, heart/body weight ratio 4.4 ± 0.4 vs. 4.4 ± 0.2 mg/g).

2.2. Isolated cardiac muscle strip preparation and measurement of force of contraction

Atrial trabeculae and papillary muscles were obtained from the left hearts of the above animals. Immediately after excision, the papillary muscles and atrial trabeculae were placed in ice-cold pre-aerated Tyrode solution. The experiments were performed on isolated electrically driven muscle preparations. Muscle strips of uniform size with muscle fibers running approximately parallel to the length of the strips were dissected under microscopic control using scissors in aerated modified Tyrode solution (composition see below) at room temperature. Connective tissue

was trimmed away carefully. The muscles were suspended in an organ bath (75 ml) maintained at 37°C and containing a modified Tyrode solution of the following composition (in mmol/l): NaCl 119.8, KCl 5.4, $CaCl_2$ 1.8, $MgCl_2$ 1.05, Na_2HPO_4 0.42, $NaHCO_3$ 22.6, Na_2EDTA 0.05, ascorbic acid 0.28, glucose 5.0. The bathing solution was continuously aerated with 95% O_2 and 5% CO_2. The muscles were stimulated by two platinum electrodes (frequency 1 Hz, impulse duration 5 ms; intensity 10–20% greater than threshold) using field stimulation from an electronic stimulator (Grass S88, Quincy, MA, USA). Each muscle was stretched to the length at which force of contraction was maximal. Isometric force of contraction was measured with an inductive force transducer (W. Fleck, Mainz, Germany) attached to a Gould recorder (Brush 2400, Gould, Cleveland, OH, USA). All preparations were allowed to equilibrate for at least 90 min, with the bathing solution being changed once after 45 min. Concentration-dependent mechanical effects were obtained. Control strips kept in Tyrode's solution with identical composition as original experiments revealed maximally 10% reduction of baseline isometric tension over the period necessary to complete pharmacological testing. Agents were applied cumulatively to the organ bath. Each muscle was used only once to record a concentration-response curve. Experimental details have been described elsewhere (Böhm et al., 1992).

2.3. Isolation of cardiac myocytes

Ventricular cardiomyocytes from the above-described animals were isolated according to the method described by Piper et al. (1982) with slight modifications. In brief, animals were killed by cervical dislocation and the hearts were rapidly excised, mounted on a Langendorff apparatus and perfused retrogradely through the aorta for about 5 min at 37°C with oxygenated Powell medium containing in mmol/l: 110 NaCl, 2.5 KCl, 1.2 KH_2PO_4, 1.2 $MgSO_4$, 25.0 $NaHCO_3$, 11.1 glucose. After the blood was removed, collagenase (Type CLS II, 375 U/mg, Biochrom, Berlin, Germany) was added to the above medium and the heart was perfused at 10 ml/min for about 20 min. Subsequently the softened heart was removed from the perfusion system, and ventricles were chopped and incubated in the oxygenated enzyme solution at 37°C for another 9 min. The dispersed cells were then filtered through a nylon mesh of 210 μm, and the resultant cell suspension was centrifuged (Minifuge AF, Heraeus Sepatech Instruments, Osterode, Germany) at $22.4 \times g$ for 2.5 min. The resultant cell pellet was resuspended in 40 ml Powell medium (composition see above) containing 0.6 mmol/l calcium and again centrifuged at $22.4 \times g$ for 2.5 min to wash out the remaining collagenase. In order to increase the proportion of rod-shaped cells albumin gradient centrifugation was performed at $12.6 \times g$ for 1.5 min. During the series of centrifugation steps Ca^{2+} concentration was gradually increased in steps of 0.6 mmol/l. Finally, the isolated cells were suspended in M199 medium containing 1.8 mmol/l Ca^{2+} (Gibco, Berlin, Germany) with 4% fetal bovine serum and maintained at 37°C.

To avoid measurement of damaged cells in a hypercontractile state, morphological exclusion criteria were used to choose cells for measurement. Only those cells were used in the study, which showed a rod-shaped appearance, less than 2 spontaneous contractions per minute, no cell vacuoles and no areas of hypercontracted myofilaments. Cells that were not rod-shaped, had obvious sarcolemmal blebs or showed variations in cell length or irreversible morphological change to round cells during the experiment were excluded from the study (Harding et al., 1988). In our preparation, the amount of rod-shaped cells was approximately 60–70%. Mean cell length was 134.6 ± 5.6 μm ($n = 43$) for Wistar Kyoto rats and 94.3 ± 4.8 μm ($n = 20$) for transgenic and Sprague-Dawley control rats, and the calculated sarcomere length of all cells was between 1.7–2.2 μm.

2.4. Contractility measurements in isolated cells

Cardiac myocytes were suspended in a thermostatically controlled Perspex chamber of 400 μl volume and superfused at 0.6 ml/min with a modified Tyrode's solution containing in mmol/l: 119.6 NaCl, 5.36 KCl, 1.0 $CaCl_2$, 1.05 $MgCl_2$, 22.6 $NaHCO_3$, 0.42 $NaHPO_4$, 5.046 glucose, 0.283 ascorbic acid and 0.05 EDTA. The medium was oxygenated with carbogen (95% O_2, 5% CO_2) and temperature was maintained at 32 ± 0.5°C. The cells were electrically field stimulated with platinum electrodes at 0.5 Hz with 5-ms pulses by an electronic stimulator (Stim2, Scientific Instruments, Heidelberg, Germany). Contraction amplitudes were measured using a phase-contrast microscope (Diaphot 300, Nikon, Tokyo, Japan) connected to a one-dimensional camera (ZK4, Scientific Instruments, Heidelberg, Germany), which detects the edges of the cell by light-dark contrasts in the microscope image. These signals were digitalized on an oscilloscope (HM 205-3, Hameg Instruments, Kowloon, Hong Kong) and cell images were analyzed by computer using the MUCELL program (Scientific Instruments, Heidelberg, Germany), which measured the diastolic length of the myocyte and cell shortening synchronized to electrical stimulation. The twitch amplitude was calculated as the maximal shortening length subtracted from the resting length. In order to evaluate contractile function precisely, twitch amplitudes were normalized by dividing each by the resting length (Sakai et al., 1992).

2.5. RNA isolation and polymerase chain reaction

Atrial and ventricular myocardium were isolated, homogenized, and RNA was isolated with RNA-clean according to the manufacturer's protocol in order to obtain

total cellular RNA. This was quantified spectrophotometrically by measuring absorbance values at 260 and 280 nm. 2-μg aliquots were electrophoresed through 1.2% agarose-0.67% formaldehyde gels and stained with ethidium bromide to verify the quantity and quality of the RNA. 2 μg of the isolated total RNA were reverse transcribed using random primers. The single-stranded cDNA was amplified by polymerase chain reaction (PCR) using Taq DNA polymerase (Boehringer). 25 cycles were performed under the following conditions: 30 s, 94°C; 60 s, 65°C; 45 s, 72°C. The sequences for rat AT_1 receptor sense and antisense primers were: 5′-ACCCCTCTACAGCATCTT-TGTGGTGGGGA-3′ and 5′-GGGAGCGTCGAATTC-CGAGACTCATAATGA-3′, respectively. Human AT_1 receptor cDNA was amplified using the primers 5′-CCT-TCGACGCACAATGCTTG-3′ and 5′-AGCCCTATCG-GAAGGGTTGA-3′. The same samples were used for β-actin cDNA amplification to confirm that equal amounts of RNA were reversely transcribed. The primers employed were 5′-GTTCCGATGCCCCGAGGATCT-3′ and 5′-GCATTTGCGGTGCACGATGGA-3′. PCR amplification gave 479 bp, 173 bp, and 361 bp of fragments originated from the rat angiotensin AT_1 receptor mRNA, the human angiotensin AT_1 receptor mRNA and β-actin mRNA, respectively.

2.6. Membrane preparation and radioligand binding assay

Membrane preparation and receptor binding assay were performed as previously described (Suzuki et al., 1993). In brief, hearts were removed and frozen in liquid nitrogen. A crude membrane preparation was prepared by homogenizing tissue in 0.25 mol/l sucrose and 25 mmol/l Tris (pH 7.5) containing 0.5 mmol/l EDTA, 0.5 mmol/l phenylmethylsulfonyl fluoride, 10 mg/l bacitracin, 4 μg/ml leupeptin, 4 μg/ml pepstatin and 40 U/ml trasylol. The pellet was resuspended in 0.6 mol/l KCl and 30 mmol/l histidine (pH 7.0) containing 0.5 mmol/l EDTA, 0.5 mmol/l phenylmethylsulfonyl fluoride, 10 mg/l bacitracin, 4 μg/ml leupeptin, 4 μg/ml pepstatin and 40 U/ml trasylol and centrifuged at $45\,000 \times g$ for 30 min. The pellets were washed three times and resuspended in 25 mmol/l Tris (pH 7.5) containing 10 mmol/l $MgCl_2$, 0.5 mmol/l phenylmethylsulfonyl fluoride, 10 mg/l bacitracin, 4 μg/ml leupeptin, 4 μg/ml pepstatin and 40 U/ml trasylol. Protein concentrations were determined according to Bradford (1976). Membranes (50 μg of protein) were incubated for 90 min at room temperature in suspension with 90–1800 pmol/l ^{125}I-[Sar^1,Ile^8]angiotensin II in 150 μl of incubation buffer as above containing in addition 2 g/l bovine serum albumin and the peptase inhibitors antipain, phosphoramidon, leupeptin, pepstatin, bestatin and amastatin, each at 1 μg/ml. The binding reaction was determined by addition of 5 ml of ice-cold 25 mmol/l Tris (pH 7.5) and the mixtures were immediately filtered over a Whatman GF/C filter presoaked with 0.4% polyethylenimine by vacuum filtration. Filters were washed three times with ice-cold buffer and bound radioactivity was counted in a Beckman gamma counter (Beckman Instruments, Fullerton, CA, USA). Nonspecific binding

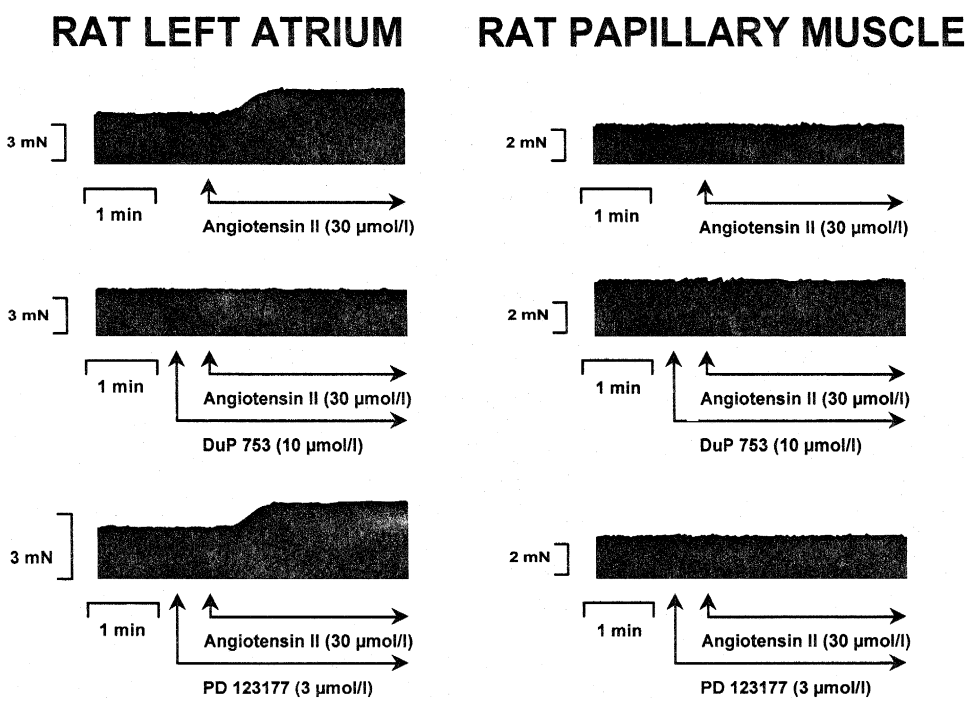

Fig. 1. Original recordings of isometric force of contraction in isolated rat left atria (left panel) and isolated rat papillary muscles (right panel) following application of angiotensin II (30 μmol/l) alone or in presence of the selective angiotensin AT_1 receptor antagonist Dup 753 (10 μmol/l) and the selective angiotensin AT_2 receptor antagonist PD 123177 (3 μmol/l).

was determined in the presence of 10 μmol/l unlabeled competitor.

2.7. Materials

Angiotensin II was obtained from Sigma (Deisenhofen, Germany). DuP 753 (losartan) was purchased from Merck Sharp and Dohme (Munich, Germany) and PD 123177 was from Parke Davis (Berlin, Germany). Collagenase (Type CLS II, 375 U/mg, Cat. No. CII-24, Lot No. P 037) was purchased from Biochrom (Berlin, Germany), and albumin (bovine albumin, 96–99%, A-8022, Lot 34 H 0275) was from Sigma (Deisenhofen, Germany). M199 medium and fetal bovine serum were obtained from Gibco (Berlin, Germany). The ligand ^{125}I-[Sar^1,Ile^8]angiotensin II was obtained from New England Nuclear (Braunschweig, Germany). All other compounds used were of analytical or best grade commercially available. Only deionized and distilled water was used throughout.

2.8. Statistical analysis

All data are expressed as means ± S.E.M. Statistical significance was estimated using Student's *t*-test for paired and unpaired observations. A P value of less than 0.05 was considered significant.

3. Results

3.1. Effects of angiotensin II on force of contraction in atrial and ventricular myocardium of the rat

The effects of angiotensin II on force of contraction in isolated electrically stimulated atrial and ventricular preparations of the rat are demonstrated in Fig. 1. In atrial trabeculae ($n = 5$), angiotensin II at 30 μmol/l had a positive inotropic effect which was maximal within 1 min (upper left panel). In contrast, in left ventricular preparations ($n = 5$) no such effect on force of contraction could be observed at 30 μmol/l angiotensin II (upper right panel). To functionally characterize the positive inotropic effect of angiotensin II observed in atrial myocardium, the effect was investigated in the presence of the selective angiotensin AT_1 and AT_2 receptor antagonists DuP 753 (losartan) and PD 123177. The positive inotropic effect of angiotensin II in atrial trabeculae was antagonized by 10 μmol/l of the angiotensin AT_1 receptor antagonist DuP 753 (losartan) but remained unchanged in the presence of 3 μmol/l of the angiotensin AT_2 receptor antagonist PD 123177 ($n = 5$). These observations provide evidence for an angiotensin AT_1 receptor-mediated positive inotropic effect of angiotensin II in atrial myocardium of the rat. In contrast, in ventricular myocardium no inotropic effect of angiotensin II could be shown. In order to investigate whether the contractile response to angiotensin II is due to the density of angiotensin AT_1 receptors, we performed the same experiments in a transgenic model with an overexpression of the angiotensin AT_1 receptor.

3.2. Expression of the AT_1 receptor mRNA in atrial and ventricular myocardium of Sprague-Dawley rats and TGR(αMHC-hAT$_1$) transgenic rats

First, we investigated the differential cardiac angiotensin AT_1 receptor gene expression in transgenic and control rats. The AT_1 receptor mRNA levels were measured by means of a semi-quantitative PCR technology ($n = 3$). Fig. 2 shows that the human angiotensin AT_1 receptor mRNA is exclusively expressed in left atrium and left ventricle of the transgenic animals. The wild-type rat angiotensin AT_1 receptor mRNA expression is not significantly altered in both control and transgenic animals. The detection of β-actin mRNA served as control for the experimental setup.

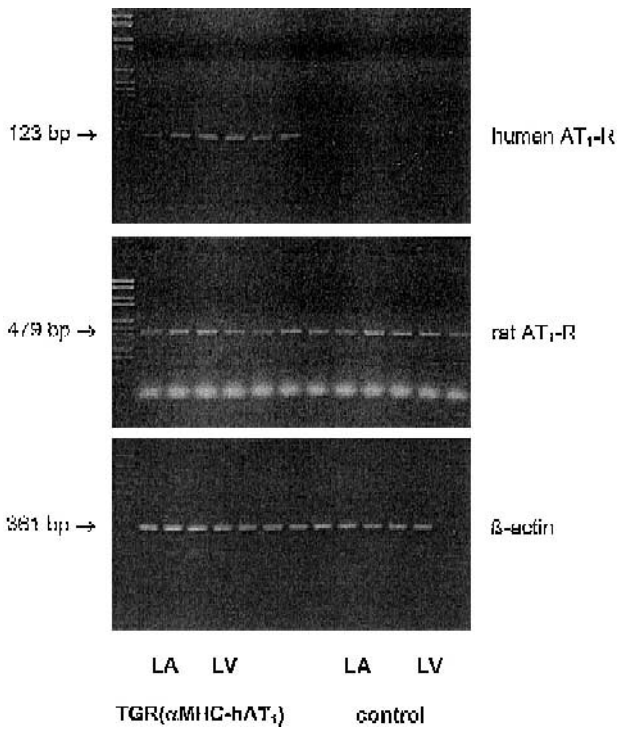

Fig. 2. Representative ethidium bromide-stained agarose gels of PCR reactions. The upper panel shows the expression of the human angiotensin AT_1 receptor mRNA, the middle panel the rat angiotensin AT_1 receptor mRNA, and the β-actin mRNA is demonstrated in the lower panel. PCR reactions have been performed following reverse transcription reactions. Three left ventricular and left atrial preparations have been investigated for each control and transgenic animal. Lane 1, marker; lanes 2–4, LA transgenic rats, lanes 5–7, LV transgenic rats, lanes 8–10, LA controls, lanes 11–13, LV controls. LA, left atrium; LV, left ventricle.

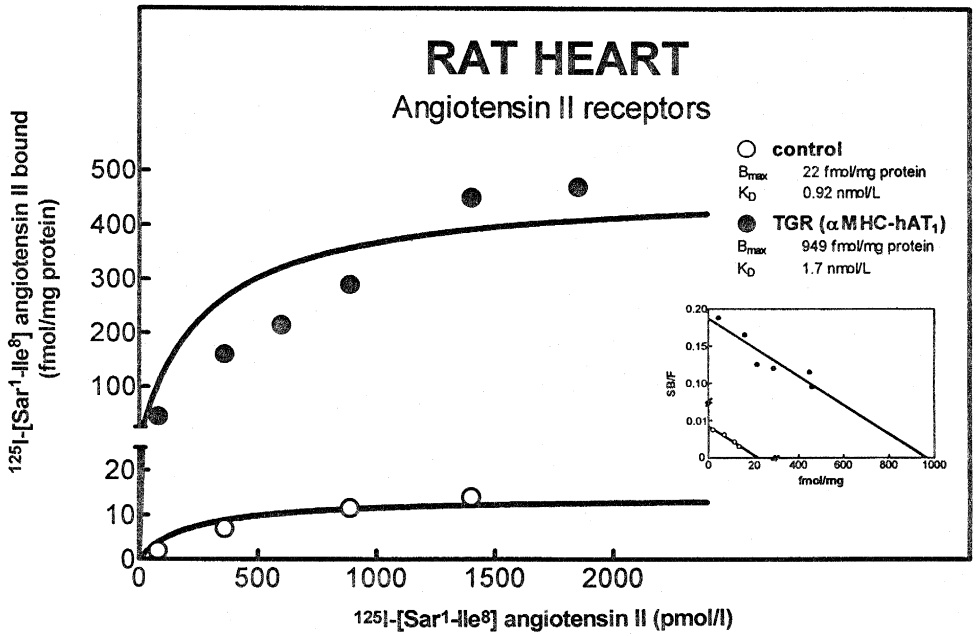

Fig. 3. Representative saturation binding of ^{125}I-[Sar^1,Ile^8]angiotensin II to membranes prepared from whole-heart homogenates from 4-week-old Sprague-Dawley rat (control) (B_{max} = 22 fmol/mg protein, K_d = 0.92 nmol/l) and 4-week-old TGR(αMHC-hAT$_1$) transgenic rat (TGR) (B_{max} = 949 fmol/mg protein, K_d = 1.7 nmol/l). Binding is expressed as fmol/l. Inset: Scatchard plot of ^{125}I-[Sar^1,Ile^8]angiotensin II bound per mg protein was plotted as a function of the ratio (SB/F) of bound ^{125}I-[Sar^1,Ile^8]angiotensin II and free ^{125}I-[Sar^1,Ile^8]angiotensin II (nmol/l). The intercept with the ordinate is the maximal number of binding sites (B_{max}), the slope is the apparent affinity (K_d).

3.3. Binding characteristics of ^{125}I-[Sar^1-Ile^8]angiotensin II to membranes from Sprague-Dawley rats and TGR(αMHC-hAT$_1$) transgenic rats

The radioligand binding assays illustrate the overexpression of angiotensin AT$_1$ receptors in transgenic rats. Binding characteristics of ^{125}I-[Sar^1,Ile^8]angiotensin II to membranes prepared from whole hearts in 4-week-old Sprague-Dawley rats and 4-week-old TGR(αMHC-hAT$_1$) transgenic rats are shown in Fig. 3. In transgenic rats overexpressing the human angiotensin AT$_1$ receptor, binding of ^{125}I-[Sar^1,Ile^8]angiotensin II was significantly increased compared to age-matched controls. B_{max} was 27 $\pm$ 7 fmol/mg protein (K_d 1.05 $\pm$ 0.2 nmol/l) in control rats (n = 3) and 854 $\pm$ 173 fmol/mg protein (K_d 2.2 $\pm$ 1.5 nmol/l) in transgenic animals (n = 3), indicating significantly increased angiotensin AT$_1$ receptor density in hearts from transgenic rats at the age of 4 weeks, when functional studies on isolated ventricular myocytes were performed.

3.4. Comparison of the inotropic effects of angiotensin II in atrial myocardium from Sprague-Dawley rats and TGR(αMHC-hAT$_1$) transgenic rats

Fig. 4 shows the cumulative concentration-response curves for angiotensin II in left atrial trabeculae from normal and transgenic Sprague-Dawley rats. In normal rats (n = 5), basal force of contraction of 1.9 $\pm$ 0.4 mN was enhanced to a maximal value of 2.5 $\pm$ 0.3 mN. The positive inotropic effect induced by angiotensin II was maximal at 1 μmol/l increasing force of contraction by 0.8 $\pm$ 0.08 mN (35.8 $\pm$ 5.5%). In transgenic animals (n = 5), the concentration-dependent response to angiotensin II was similar. Application of angiotensin II led to an increase from 2.9 $\pm$ 0.9 mN to 3.6 $\pm$ 1.1 mN, resulting in a maximal increase in force of contraction of 25% at 0.1 μmol/l.

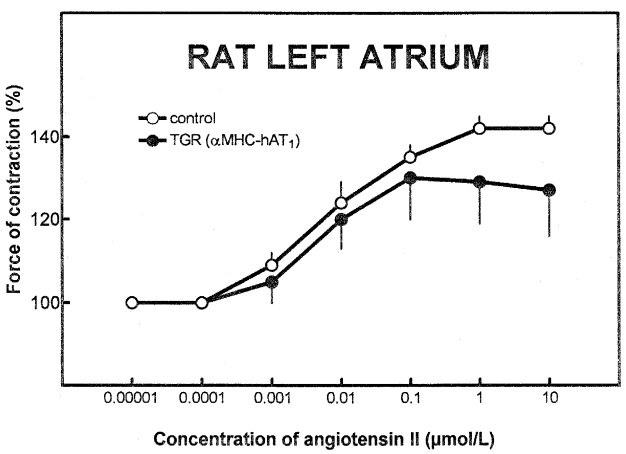

Fig. 4. Concentration-response curves for the effects of angiotensin II (0.00001–10 μmol/l) on force of contraction in left atrium from Sprague-Dawley and AT$_1$ transgenic rats. Ordinate: Force of contraction in % of basal value. Abscissa: Concentration of drug in μmol/l.

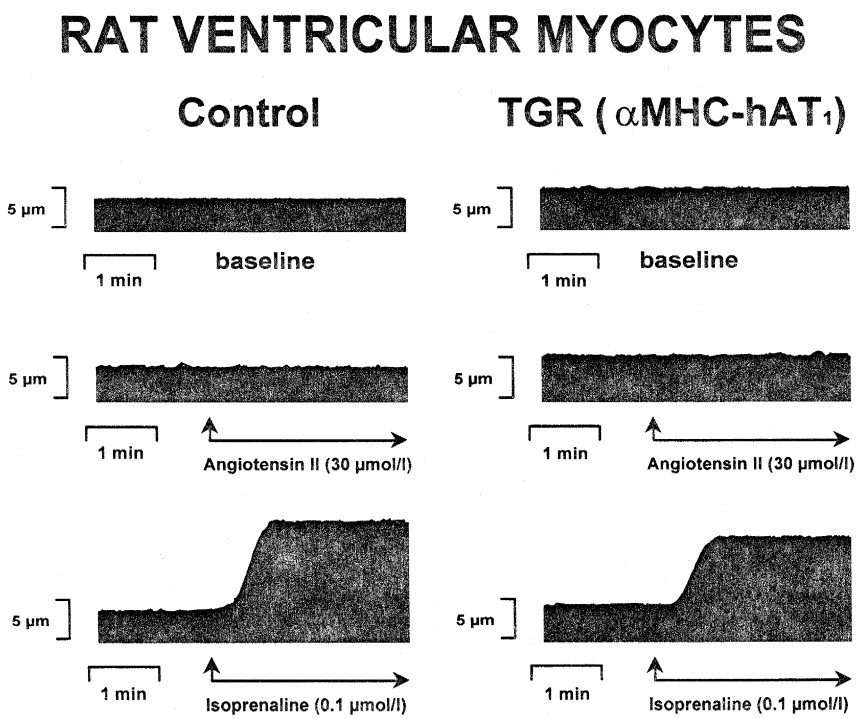

Fig. 5. Original recordings of cell shortening of isolated ventricular cardiomyocytes from Sprague-Dawley rats (left panel) and TGR(αMHC-hAT$_1$) transgenic rats (right panel). Illustrated are basal contraction (upper panel) and the responses to application of angiotensin II at 30 µmol/l (middle panel) and isoprenaline at 0.1 µmol/l (lower panel).

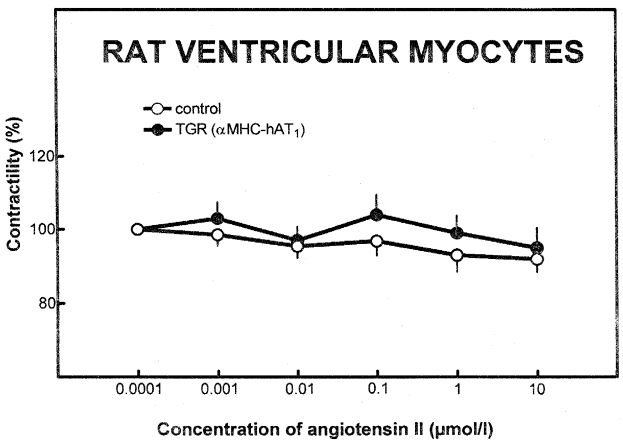

Fig. 6. Concentration-response curves for the effects of angiotensin II (0.0001–10 µmol/l) on contractility of isolated ventricular cardiomyocytes from adult Sprague-Dawley rats and from transgenic rats overexpressing the angiotensin AT$_1$ receptor. Ordinate: Contractility in % of basal value. Abscissa: Concentration of drug in µmol/l.

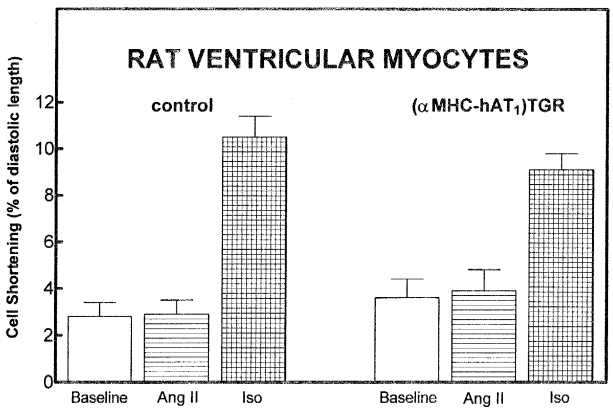

Fig. 7. Inotropic responses to angiotensin II (30 µmol/l) and isoprenaline (0.1 µmol/l) in isolated ventricular cardiomyocytes from Sprague-Dawley and TGR(αMHC-hAT$_1$) transgenic rats. Cell shortening is expressed in % of diastolic length.

Table 1
Cell shortening of isolated ventricular myocytes from 4-week-old Sprague-Dawley rats and TGR(αMHC-hAT$_1$) transgenic rats

	Baseline cell shortening (% of diastolic length)	Angiotensin II (30 µmol/l)	Isoprenaline (0.1 µmol/l)
Sprague-Dawley control	2.8 ± 0.6 (100%)	2.9 ± 0.6 (103%)	10.5 ± 0.9 (375%)
TGR(αMHC-hAT$_1$)	3.6 ± 0.8 (100%)	3.9 ± 0.9 (108%)	9.1 ± 0.7 (253%)

Maximal responses to angiotensin II (30 µmol/l) and isoprenaline (0.1 µmol/l).

3.5. Response to angiotensin II on contractility of isolated papillary muscles and isolated rat ventricular myocytes from Sprague-Dawley rats and TGR(αMHC-hAT$_1$) transgenic rats

In isolated, electrically driven papillary muscle strips from Sprague-Dawley rats, there was no inotropic effect of angiotensin II (0.1–10 μmol/l, not shown). To investigate whether the expression level of angiotensin II receptors in ventricular myocardium is crucial for an inotropic response, we examined the effects on isolated cardiomyocytes from transgenic rats overexpressing the angiotensin AT$_1$ receptor compared to age-matched Sprague-Dawley control rats (Fig. 5). Angiotensin II did not alter cardiac contractility independently of AT$_1$ receptor density. Neither in cardiomyocytes from transgenic ($n = 5$) nor from control animals ($n = 5$) application of angiotensin II in increasing concentrations produced a positive inotropic response. Fig. 6 demonstrates the cumulative dose-response curves for angiotensin II in transgenic and control ventricular myocytes. No inotropic response was observed at concentrations from 0.0001 to 10 μmol/l in both transgenic and control cells. Cell shortening ranged from 106 to 84% of control values in all cells. To assure that the isolated cardiomyocytes were responsive to positive inotropic stimulation, all cells were stimulated with isoprenaline (0.1 μmol/l) after angiotensin was washed out. As demonstrated in Fig. 7, responses to angiotensin II and isoprenaline were similar in cells from both groups. Baseline values and maximal responses to angiotensin II (30 μmol/l) and isoprenaline (0.1 μmol/l) are given in Table 1.

4. Discussion

Our data illustrate that angiotensin II exerts a positive inotropic effect via activation of angiotensin AT$_1$ receptors in atrial but neither in ventricular tissue nor in isolated ventricular cardiomyocytes of the rat heart. The lacking effect of angiotensin II is independent of the AT$_1$ receptor density, as shown in experiments on rats overexpressing the AT$_1$ receptor.

Angiotensin II has been shown to induce cardiac fibrosis and collagen remodeling in cardiac interstitium (Weber and Brilla, 1991; Morgan and Baker, 1991), but the involvement of direct effects on cardiac myocytes remains unclear. The presence of angiotensin II receptors in myocardial tissue has been reported in various mammalian species (Baker et al., 1984; Baker and Singer, 1988; Urata et al., 1989) including the rat (Ishihata and Endoh, 1995; Sechi et al., 1992). They are expressed on cardiac fibroblasts as well as on cardiomyocytes (Fareh et al., 1996). Angiotensin II is also reported to affect cardiac function. A positive inotropic effect following angiotensin AT$_1$ receptor stimulation with angiotensin II has been observed in hearts from dogs (Kobayashi et al., 1978), cats (Dempsey et al., 1971), rabbits (Freer et al., 1976; Ishihata and Endoh, 1995) and human atrial but not ventricular myocardium (Moravec et al., 1990; Holubarsch et al., 1993; Zerkowski et al., 1993; Lenz et al., 1995). However, the inotropic response to angiotensin II appears to be species-dependent, and differences have been reported between atrial and ventricular preparations.

In the present study, angiotensin II produced a positive inotropic response of about 40% only in atrial preparations, whereas no effect was observed in ventricular myocardium of the rat. This effect was prevented by the specific angiotensin AT$_1$ receptor antagonist losartan but not by the angiotensin AT$_2$ receptor antagonist PD 123177, indicating that the positive inotropic response was mediated via angiotensin AT$_1$ receptor stimulation. This is in accordance with Feolde et al. (1993), who observed a positive inotropic effect of angiotensin II in guinea pig atria which could also be abolished by losartan but not by PD 123319. Similar responses have also been shown in human myocardium (Holubarsch et al., 1993; Lenz et al., 1995). The physiological relevance of the observed inotropic effect in atrial myocardium appears questionable since the concentrations required to elicit an inotropic response were by far above those producing vasoconstrictory properties in vessels. However, it is difficult to extrapolate from systemic to local concentrations, because a paracrine release may lead to high local concentrations of angiotensin II at atrial AT$_1$ receptors (Urata et al., 1994). Thus, a relevant inotropic effect in atrial myocardium is nevertheless possible.

The overexpression of the angiotensin AT$_1$ receptor in atrial myocardium did not lead to an augmentation of the positive inotropic effect. This may be explained by the fact that these overexpressed receptors are either spare receptors or are poorly coupled. The existence of spare receptors in normal as well as transgenic rats is not known and difficult to measure. Determination of spare receptors would require a system in which receptors are reduced depending on disease stage or pharmacological treatment, while a certain physiological response has to be measured in the tissue. This approach is not possible to perform with cardiac angiotensin AT$_1$ receptors, who do not mediate a positive inotropic effect in the ventricle, and whose effect in the atria is limited. A second approach would be the use of nonreversible ligands with antagonistic properties for angiotensin II receptors. Unfortunately, these compounds are not available. In addition, reduction of angiotensin II effects depending on inactivation of angiotensin AT$_1$ receptors is difficult to perform, because the effect in the atria is only small. Therefore, to date we are unable to distinguish whether the described observations are due to spare or uncoupled receptors. A second explanation for the lack of augmentation of the inotropic response in atria is that translation only occurs in ventricles. However, polymerase chain reaction shows similar amounts of RNA in

atrial and ventricular myocardium. Although possible it seems unlikely that translation should be different in atria and ventricles.

To further elucidate why ventricular myocardium does not respond to angiotensin II, we examined the effects of angiotensin II on contractile function of isolated ventricular cardiomyocytes of the rat. Isolated myocytes respond more sensitively to positive inotropic interventions than multicellular preparations, as exemplarily shown by the lower EC_{50} values of isoprenaline in isolated human cardiomyocytes (Harding et al., 1988) compared to multicellular preparations (Böhm et al., 1989). However, our experiments on adult Sprague-Dawley rats showed no angiotensin effect on contractile function in isolated ventricular cardiomyocytes of the rat. To exclude the possibility of strain-specific responses to angiotensin II we also investigated Wistar Kyoto rats and obtained similar results in these two strains. Other investigators obtained contradictory results: Ishihata and Endoh (1995) did not observe any inotropic response to angiotensin II in adult Wistar Kyoto rats, and Kobayashi et al. (1995) found no effect of angiotensin II in normal Wistar Kyoto rats but a negative inotropic response in spontaneously hypertensive rats (SHR). In contrast, Neyses and Vetter (1990) observed a positive inotropic response to angiotensin II in adult Sprague-Dawley rats. They suggested that the maximal contractility increased at rates above 80 beats/min and remained constant up to 200 beats/min. Thus, the discrepancy in the inotropic response may be explained by different stimulation frequencies resulting in beating rates of 30/min in the present study versus 60 and 80/min in the other studies. However, in further experiments an increase in stimulation frequency did not alter the contractile response to angiotensin II (not shown). Also, no positive force-frequency relationship has been observed in the rat heart (Schouten and Ter Keurs, 1986). Temperature is another important issue in experimental conditions, since temperature-depending differences in the intracellular Ca^{2+} homeostasis have been discussed. Therefore, we also investigated the effect of angiotensin II in isolated cardiomyocytes at room temperature (25–27°C) and found no change in the inotropic response. One possible explanation for the negative inotropic effect in SHR is a possible suppression of adenylate cyclase activity via coupling of angiotensin AT_1 receptors to inhibitory G-proteins, as described for endothelin receptors (Hilal-Dandan et al., 1994). However, there was no negative inotropic effect of angiotensin II (anti-adrenergic effect) in the presence of isoprenaline (not shown).

In pathophysiological conditions changes in receptor density or functional coupling may lead to a different response to angiotensin II in the hypertrophied or failing heart. Only recently a down-regulation of angiotensin AT_1 receptors in cardiac fibroblasts and an up-regulation in cardiomyocytes has been described in cardiac hypertrophy induced by volume overload in the rat (Fareh et al., 1996), and negative inotropic responses to angiotensin II have been reported in SHR and in pacing-induced heart failure in the dog (Kobayashi et al., 1995; Cheng et al., 1996). In our experiments the total amount of angiotensin AT_1 receptors appears to be similar in atria and ventricles as assessed by semi-quantitative polymerase chain reaction. However, minor differences cannot be determined using this method. Binding experiments were performed in whole hearts and thus, differences between atria and ventricles cannot be detected by these experiments. The exact localisation of angiotensin AT_1 receptors in cardiac tissue is not known, and their distribution in terms of expression in different cell types may be different in atrial and ventricular myocardium. Although the total amount of receptors is similar in atria and ventricles, their distribution on cardiomyocytes and fibroblasts may be different. Thus, the qualitative difference in atria and ventricles may be dependent on receptor density and their relative distribution on different cell types. In order to examine whether the lack of response to angiotensin II in rat ventricular myocytes in our experiments was due to a low density of angiotensin AT_1 receptors we conducted the same experiments in transgenic animals overexpressing the human angiotensin AT_1 receptor under the regulatory control of an αMHC promotor, which is specific for cardiomyocytes. However, the increase in receptor density did not lead to changes in the inotropic response, and no angiotensin II-induced effect on cell shortening could be observed. Thus, it seems very unlikely that the low receptor density is the reason for the inability of ventricular myocardium to respond to angiotensin II.

Beside the cyclic AMP system, cardiac contractility is regulated via receptor agonists that stimulate the phosphatidylinositol hydrolysis, including α-adrenergic stimulants, endothelin-1 and possibly angiotensin II. Angiotensin AT_1 receptors are coupled to receptor signal transducing G-proteins (G_q) and phospholipase C, and receptor activation leads to an acceleration of the hydrolysis of phosphatidylinositol (Griendling et al., 1986). The resultant production of diacylglycerol and inositol 1,4,5-trisphosphate (IP_3) causes an activation of protein kinase C and an intracellular Ca^{2+} release (Brock et al., 1985; Griendling et al., 1993). Various mechanisms including various ion channels and the responsiveness of contractile proteins to Ca^{2+} are involved in the transduction process (Endoh, 1991, 1995) which varies among mammalian species and potentially causes changes in myocyte cell growth and cardiac function. In isolated rat cardiac cells, angiotensin II has been shown to lead to an increase in inositol phosphate (IP_3) formation and intracellular calcium transients (Baker and Aceto, 1989; Feolde et al., 1993).

While the reasons for the differences in the angiotensin II-induced inotropic effect between mammalian species as well as between atrial and ventricular myocardium have not been determined, various aspects of the signal trans-

duction process including receptor density, distribution of AT_1 and AT_2 subtypes and second messengers have to be taken into account.

Focusing on species-dependent variations, Ishihata and Endoh (1995) showed that neither the angiotensin II receptor density nor the relative proportion of subtypes is the reason for the distinct response to angiotensin II in different species. Moreover, it has been suggested that the positive inotropic effect is dissociated from the angiotensin II-induced acceleration of phosphatidylinositol hydrolysis (Baker and Singer, 1988; Baker and Aceto, 1989; Ishihata and Endoh, 1993), and that species-dependent variations in the inotropic response seem to be mediated by signaling events downstream of phosphatidylinositol hydrolysis. Similar differences in the dissociation of accelerated phosphatidylinositol hydrolysis and positive inotropic effect have also been reported between atria and ventricles. Whereas an angiotensin-induced acceleration of phosphatidylinositol hydrolysis was apparent in both atrial and ventricular muscle, a positive inotropic effect via angiotensin AT_1 receptors was observed only in atrial (Feolde et al., 1993) but not in ventricular myocardium (Baker et al., 1992). In accordance with our results in cells from transgenic animals, these findings suggest that neither the density nor subtype distribution of receptors, but a signal transduction process distal to the acceleration of the phosphatidylinositol hydrolysis may be responsible for the different inotropic responses in atrium and ventricle. Possible sites for dissociation from the inotropic response are the further coupling mechanisms including Na^+-H^+ exchange, Ca^{2+}, K^+ and Cl^- channels and Ca^{2+} sensitivity of contractile proteins (Endoh, 1991, 1995). Further studies are needed to investigate the exact differences in intracellular signaling mechanisms that lead to different responses to angiotensin II in atrial and ventricular myocytes. Possibly, the atrial angiotensin AT_1 receptor mediates force generations, while the role of the ventricular angiotensin AT_1 receptor is confined to the regulation of cardiac growth and ventricular remodeling processes. In addition, the latter effects of angiotensin II could also be largely mediated by paracrine mechanisms leading to a release of other agents, e.g. endothelin, producing direct effects on cardiomyocytes to induce hypertrophy (Kaddoura et al., 1996). In the present study, transgenic rats overexpressing the angiotensin AT_1 receptor did not show cardiac hypertrophy under basal conditions. However, an increased hypertrophic response under pathophysiological conditions with increased afterload, e.g. after aortic banding, is nevertheless possible, so that the receptors in transgenic rats may still be coupled with respect to growth, but this mechanism may only become evident under pathophysiological conditions.

In conclusion, angiotensin II induces a positive inotropic response in atrial myocardium of the rat, which is mediated by angiotensin AT_1 receptors, whereas in ventricular myocardium no effect on contractility has been observed. The existence of functionally uncoupled receptors rather than the low density of receptors at the ventricular site may be responsible for the inability of ventricular myocardium to respond to angiotensin II.

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