Paper No. BJP 127996

British Journal of Pharmacology (1997) 121, 118–124

Trophic effect of angiotensin II in neonatal rat cardiomyocytes: role of endothelin-1 and non-myocyte cells

Klaus Pönicke, Ingrid Heinroth-Hoffmann, Karin Becker & [1]Otto-Erich Brodde

Institute of Pharmacology, Martin-Luther-University Halle-Wittenberg, Germany

1 Angiotensin II (AII) and the endothelins (ET) are known to be potent trophic stimuli in various cells including cardiomyocytes. In order to characterize further these effects we studied, in neonatal rat ventricular cardiomyocytes, the effects of several endothelin-receptor antagonists and the AT_1-receptor antagonist losartan on AII- and endothelin-induced inositol phosphate (IP)-formation (assessed as accumulation of total [^{3}H]-IPs in myo-[^{3}H]-inositol prelabelled cells) and increase in rate of protein synthesis (assessed as [^{3}H]-phenylalanine incorporation).

2 Endothelin (10 pM – 1 μM) concentration-dependently increased IP-formation (max. increase at 100 nM ET-1: 130 ± 14% above basal, $n=25$) and [^{3}H]-phenylalanine incorporation (max. increase at 1 μM: 52 ± 4% above basal, $n=16$) with an order of potency: ET-1 > > ET-3. Both effects were antagonized by the ET_A/ET_B-receptor antagonist bosentan and the ET_A-receptor antagonist BQ-123, but not affected by the ET_B-receptor antagonist IRL 1038 and the AT_1-receptor antagonist losartan.

3 Pretreatment of the cells with 500 ng ml^{-1} pertussis toxin (PTX) overnight that completely inactivated PTX-sensitive G-proteins did not attenuate but rather enhance ET-1-induced IP-formation. On the other hand, in PTX-pretreated cardiomyocytes ET-1-induced [^{3}H]-phenylalanine incorporation was decreased by 39 ± 5% ($n=5$).

4 AII (1 nM – 1 μM) concentration-dependently increased IP-formation (max. increase at 1 μM: 42 ± 7% above basal, $n=16$) and [^{3}H]-phenylalanine incorporation (max. increase at 1 μM: 29 ± 2%, $n=9$). These effects were antagonized by losartan, but they were also antagonized by bosentan and BQ-123.

5 In well-defined cultures of cardiomyocytes (not contaminated with non-myocyte cells) AII failed to increase [^{3}H]-phenylalanine incorporation; addition of non-myocyte cells to the cardiomyocytes restored AII-induced increase in [^{3}H]-phenylalanine incorporation.

6 We conclude that, in rat neonatal ventricular cardiomyocytes, (a) the ET-1-induced increase in rate of protein synthesis (through ET_A-receptor stimulation) involves at least two signalling pathways: one via a PTX-insensitive G-protein coupled to IP-formation, and the other one via a PTX-sensitive G-protein, and (b) the trophic effects of AII are brought about via local ET-1 secretion upon AT_1-receptor stimulation in neonatal rat ventricular non-myocyte cells.

Keywords: Endothelin; angiotensin II; neonatal rat cardiomyocytes; protein synthesis; inositol phosphates; G-proteins

Introductions

Angiotensin II (AII) and the endothelins (ET) are potent vasoconstrictors. However, during the years growing evidence has accumulated that both peptides also exert a number of effects in the myocardium including positive inotropism (for recent review see Brodde *et al*., 1995) and regulation of cardiomyocyte growth during development and hypertrophy (for reviews see Baker *et al*., 1992; Sugden & Bogoyevitch, 1996). These effects are mediated by binding to specific membrane receptors. At least two different receptor subtypes have been cloned for each peptide designated ET_A and ET_B (Bax & Saxena, 1994) and AT_1 and AT_2 (Timmermans *et al*., 1993), respectively, and it appears that the cardiac effects of endothelins and AII are mediated predominantly by ET_A- and AT_1-receptors (Timmermans *et al*., 1993; Brodde *et al*., 1995; Sugden & Bogoyevitch, 1996). However, the mechanism underlying the trophic effects of AII in rat cardiomyocytes is still a matter of debate. Thus, in rat isolated ventricular cardiomyocytes AII-induced protein synthesis via direct activation of AT_1-receptors has been demonstrated (Sadoshima & Izumo, 1993). However, it has also been shown that the trophic effects of AII in rat cardiomyocytes are mediated by ET-1 (Ito *et al*., 1993). Recent studies suggest that AT_1-receptors are localized predominantly on non-myocyte cells (mainly cardiac fibroblasts, Villarreal *et al*., 1993) and that AT_1-receptors on these cells play a critical role in AII-induced hypertrophic response in the neonatal rat cardiomyocytes (Kim *et al*., 1995).

The aim of the present study was to characterize further the trophic effects of endothelins and AII. For this purpose we studied, in neonatal rat isolated ventricular cardiomyocytes, the effects of endothelins and AII on inositol phosphate (IP)-formation and incorporation of [^{3}H]-phenylalanine (as a measure of rate of protein synthesis) in the absence and presence of the ET_A-receptor antagonist, BQ-123 (Moreland, 1994), the ET_B-receptor antagonist, IRL 1038 (Urade *et al*., 1992), the ET_A/ET_B-receptor antagonist, bosentan (Clozel *et al*., 1994), and the AT_1-receptor antagonist, losartan (Timmermans *et al*., 1993). Moreover, pertussis toxin (PTX)-induced ADP-ribosylation was used to clarify which G-protein is involved in endothelin-induced IP-formation and protein synthesis. And finally, in order to obtain some information on the role of non-myocyte cells for the trophic effects of endothelins and AII, we performed experiments in well defined cultures of cardiomyocytes with and without addition of non-myocyte cells.

Methods

Cell culture

Primary culture of neonatal rat cardiomyocytes was prepared as originally described by Toraason *et al*. (1990). Hearts from

[1]Author for correspondence at: Institute of Pharmacology, Martin-Luther-University of Halle-Wittenberg, Magdeburger Str. 4, D-06097 Halle/Saale, Germany.

1–2 days old Wistar rats were excised, atria were dissected away and the ventricles were minced in an ice-cold culture dish and stored overnight in Hanks' balanced salt solution without calcium and magnesium containing 0.1% trypsin at 4°C. Thereafter they were removed from the medium and incubated for 25 min at 37°C in culture medium M199 supplemented with 10% newborn calf serum and 100 u ml^{-1} penicillin-streptomycin. After dispersion the single cell suspension was preplated for 60 min at 37°C in a 5% CO_2 incubator to reduce the percentage of non-muscle cells. The percentage of viable cardiomyocytes estimated by trypan-blue exclusion was >95%.

The isolated ventricular myocyte suspension was seeded into 6-well-plates (1×10^6 cells/well for [^{3}H]-phenylalanine incorporation) or 75 cm^2 cell culture flasks (1.6×10^5 cells/cm^2) and incubated for a further 24 h in culture medium M199 supplemented with 10% newborn calf serum and 100 u ml^{-1} penicillin-streptomycin. In certain experiments we prepared cultures of almost pure cardiomyocytes by the continuous use of 10 μM cytosine-β-D-arabinofuranoside.

Preparation of non-myocyte rich culture

Cultures of non-myocyte cells were prepared as described by Sadoshima *et al.* (1992). Adhered cells during the preplating procedure were cultured in the cardiomyocyte culture medium (see above). After the second passage cells were seeded into 6 well-plates (1×10^5 cells/well) that contained cardiomyocytes cultured for 48 h (i.e. 24 h before [^{3}H]-phenylalanine was added, see below).

Inositol phosphate formation

After the 24 hour incubation (see above) cells were washed with culture medium M199 supplemented with 10% newborn calf serum and 100 u ml^{-1} penicillin-streptomycin and incubated for 24 h with myo-[^{3}H]-inositol (2.9 μCi ml^{-1}) at 37°C. In some experiments 500 ng ml^{-1} PTX was added to the culture medium. Thereafter adherent cells were peeled off by trypsin-EDTA treatment and non-incorporated myo-[^{3}H]-inositol was washed out by centrifugation and resuspension in Hanks buffered saline solution (composition in mM: NaCl 140, KCl 5, $NaHCO_3$ 25, $MgSO_4$ 1.2, KH_2PO_4 1.2, $CaCl_2$ 2.5 and HEPES 10) supplemented with 10 mM LiCl and 1% bovine serum albumin. Aliquots (970 μl) of the cardiomyocyte suspension (2.3×10^5 cells ml^{-1}) were then incubated with the indicated agonists in the presence or absence of antagonists for 60 min at 37°C in a final volume of 1 ml. (±)-Propranolol (10 μM) was present in all experiments. The incubation was stopped by addition of 1 ml ice-cold methanol and 2 ml chloroform. The mixture was vigorously vortexed twice, and thereafter the phases were separated by centrifugation at $820 \times g$ for 10 min at 4°C. Aliquots (1.6 ml) of the upper phase were placed on Dowex AG 1-X8 columns (200 mg/column). Free inositol was eluted twice each with 5 ml H_2O and 5 ml of 60 mM ammonium formate. Total inositol phosphates were eluted by addition of 2×1 ml 1 M ammonium formate dissolved in 100 mM formic acid. Each data point was determined in quadruplicate in each experiment.

Incorporation of [^{3}H]-phenylalanine

Protein synthesis by cardiomyocytes was assessed by incorporation of [^{3}H]-phenylalanine into cells as described by Schlüter & Piper (1992). After the 24 h incubation (see above) cells were washed and incubated for another 48 h in culture medium M199 supplemented with 10% newborn calf serum, 100 u ml^{-1} penicillin-streptomycin and 10 μM cytosine-β-D-arabinofuranoside. Thereafter cells were washed with serum-free Hanks balanced salt solution and cultured in serum-free medium M199 supplemented with 10 μM cytosine-β-D-arabinofuranoside, 10 μg ml^{-1} apo-transferrin, 10 μg ml^{-1} insulin, 1.5 μM vitamin B_{12}, antibiotics (100 u ml^{-1} penicillin and 100 u ml^{-1} streptomycin) after additions of [^{3}H]-phenylalanine (0.5 μCi ml^{-1}) for 72 h at 37°C with various concentrations of endothelins or AII in the presence or absence of antagonists. At the end of the experiments cells were washed twice with ice-cold 0.9% NaCl-solution and incubated for 24 h at 4°C with 10% trichloroacetic acid. Thereafter cells were washed again with 10% trichloroacetic acid and twice with 0.9% NaCl. The remaining precipitate on the culture dishes was solubilized in 1 N NaOH by room temperature for 24 h, and radioactivity was determined in aliquots by the use of a liquid scintillation counter (Beckman LS 6000).

Drugs

L-[2,3,4,5,6-^{3}H]-phenylalanine (spec. activity: 5.03 TBq $mmol^{-1}$) and myo-[^{3}H]-inositol (spec. activity: 4.25 TBq $mmol^{-1}$) were purchased from Amersham Buchler (Braunschweig, Germany) and [^{32}P]-NAD (spec. activity: 1.11 TBq $mmol^{-1}$) from DuPont de Nemours (Bad Homburg, Germany).

ET-1, ET-3, BQ-123 (cyclo(D-Asp-Pro-D-Val-Leu-D-Trp)) and IRL 1038 ([Cys^{11}-Cys^{15}]-endothelin-1(11–21)) were purchased from Saxon Biochemicals (Hannover, Germany), L-phenylalanine, vitamin B_{12}, insulin, apo-transferrin, cytosin-β-D-arabinofuranoside, AII, trypsin (crude) from Sigma-Aldrich (Deisenhofen, Germany), pertussis toxin (PTX) from Calbiochem (Bad Soden, Germany), and Hanks balanced salt solutions, culture medium M199, and penicillin-streptomycin from Life Technologies (Eggenstein, Germany). Bosentan was kindly supplied by Dr Clozel, Pharma Division, Hoffman-LaRoche Ltd. (Basel, Switzerland) and losartan (DUP753) by Dr Timmermanns, DuPont Merck Pharmaceutical Company Research (Wilmington, Delaware, U.S.A.). All others chemicals were of the highest purity grade commercially available.

Statistical evaluations

Data given are means ± s.e.mean of n experiments. Experimental data were analysed by computer-supported iterative non-linear regression analysis by the GraphPad Prism 2.01 programme (GraphPAD software, San Diego, U.S.A.). Statistical significance of differences was analysed by non-paired, two-tailed Student's t test; a P value <0.05 was considered significant. All statistical calculations were performed with the GraphPad Prism 2.01 programme.

Results

Endothelin

In neonatal rat isolated cardiomyocytes endothelins (10 pM–1 μM) concentration-dependently increased IP-formation, maximal increase at 100 nM ET-1 was 130 ± 14% above basal. ET-1 ($pEC_{50} = 9.8 \pm 0.1$) was about 100 times more potent than ET-3 (Figure 1a). To characterize the endothelin-receptor subtypes involved in this action we assessed the effects of several ET-receptor-antagonists on 1 nM ET-1-induced IP-formation. The non-selective ET_A/ET_B-receptor antagonist bosentan inhibited ET-1-induced IP-formation with a pK_i value (7.3 ± 0.2) which was well in the range of affinity of bosentan for ET_A- and ET_B-receptors (Clozel *et al.*, 1994). Similarly, the ET_A-receptor-antagonist BQ-123 (10 nM–10 μM) concentration-dependently inhibited 1 nM ET-1-induced IP-formation; from the concentration-inhibition curve a pK_i-value of 7.9 ± 0.2 was calculated. On the other hand, the ET_B-receptor selective antagonist IRL 1038 (Figure 1b) and the AT_1-receptor antagonist losartan (data not shown) did not affect ET-1-induced IP-formation, not even at high concentrations of 1 μM and 10 μM, respectively.

Endothelins (0.1 nM–1 μM) concentration-dependently increased [^{3}H]-phenylalanine uptake into the neonatal cardiomyocytes (maximal increase at 1 μM ET-1 was 52 ± 4% above basal, $n = 16$); again ET-1 (pEC_{50} value: 8.5 ± 0.1) was much

more potent than ET-3 (Figure 2a); the ET-1-induced increase in [^{3}H]-phenylalanine uptake was antagonized by 1 μM BQ-123 (Figure 2b), and 1 μM bosentan (Table 1), but not at all affected by 1 μM IRL 1038 (Table 1) or 1 μM losartan (data not shown).

We next studied whether a PTX-sensitive G-protein might be involved in ET-1-induced increase in IP-formation and/or [^{3}H]-phenylalanine uptake. For this purpose we treated neonatal rat isolated cardiomyocytes for 16–20 h with 500 ng ml^{-1} PTX or vehicle at 37°C. ADP-ribosylation experiments with fresh PTX and [^{32}P]-NAD in membranes of these cells detected a band with an apparent molecular weight of 41.7 kDa (Figure 3). The radioactivity incorporated into membranes from vehicle-treated cells was 13930±216 c.p.m. mg^{-1} protein. PTX-sensitive G-protein was reduced by 91±2% in membranes from cardiomyocytes which had been treated for 20 h with PTX (Figure 3). In these PTX-pretreated cardiomyocytes ET-1-induced IP-formation was not reduced, but was slightly enhanced (Figure 4a); in contrast, ET-1-induced increase in [^{3}H]-phenylalanine uptake was reduced by about 39±5% in PTX-treated cells (Figure 4b).

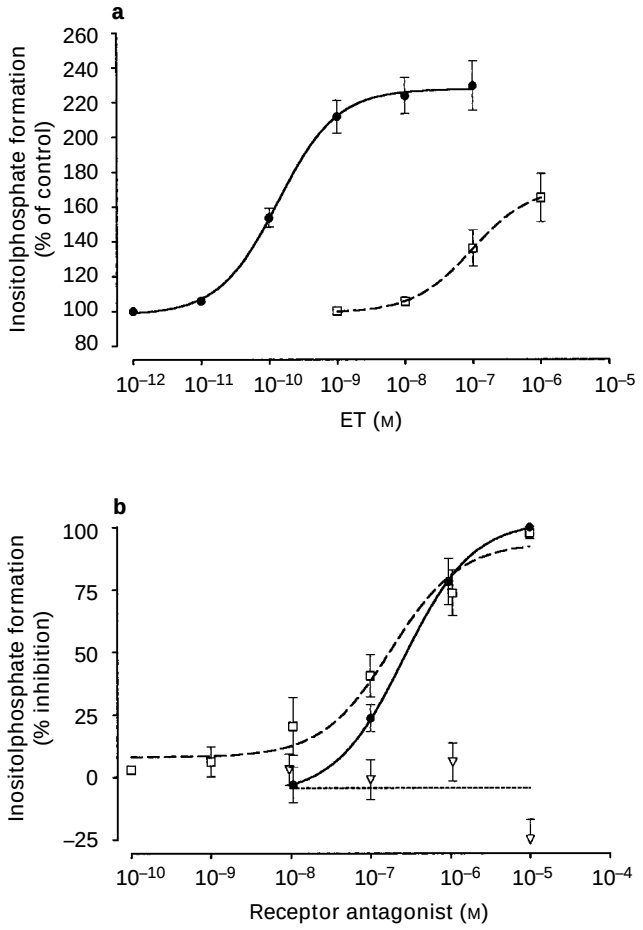

Figure 1 (a) Stimulation by endothelin (ET)-1 and ET-3 of inositol phosphate (IP) generation in neonatal rat ventricular cardiomyocytes. Ordinate scale: [^{3}H]-IP-formation as % of basal formation. Abscissa scale: molar concentrations of ET-1 and ET-3. ET-1 (●, n=25); ET-3 (□, n=4). Basal [^{3}H]-IP-formation was about 1–2% of the incorporated radioactivity and amounted to 2043±261 c.p.m. (n=25). (b) Inhibition of ET-1 (1 nM)-induced IP-formation in neonatal rat ventricular cardiomyocytes by bosentan, BQ-123 and IRL 1038. Ordinate scale: Inhibition of 1 nM ET-1-induced [^{3}H]-IP-formation as %. Abscissa scale: molar concentrations of the antagonists. Bosentan (●, n=3); BQ-123 (□, n=3); IRL 1038 (▽, n=3). In (a) and (b) values are means and vertical lines show s.e.mean.

Angiotensin

In neonatal rat isolated cardiomyocytes AII (1 nM–1 μM) concentration-dependently increased IP-formation (Figure 5). However, the effects of AII on IP-formation were not con-

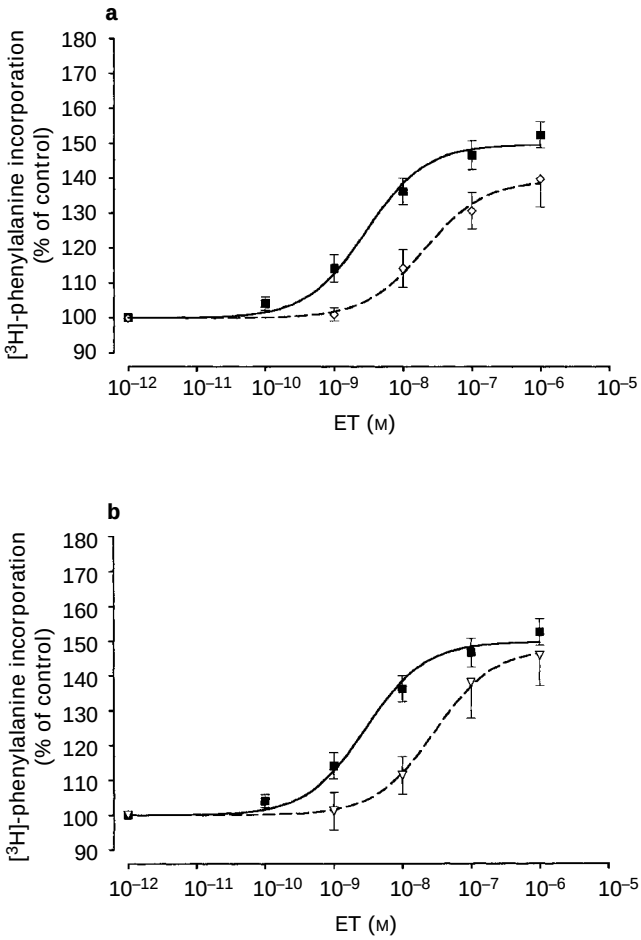

Figure 2 (a) Stimulation by endothelin (ET)-1 and ET-3 of [^{3}H]-phenylalanine incorporation in neonatal rat ventricular cardiomyocytes. Ordinate scale: [^{3}H]-phenylalanine incorporation as % of basal incorporation. Abscissa scale: molar concentrations of ET-1 and ET-3. ET-1 (■, n=16); ET-3 (◇, n=6). Basal [^{3}H]-phenylalanine incorporation amounted to 3985±451 c.p.m. (n=16). (b) Effects of 1 μM BQ-123 on ET-1-induced stimulation of [^{3}H]-phenylalanine incorporation in neonatal rat ventricular cardiomyocytes. Ordinate scale: [^{3}H]-phenylalanine incorporation as % of basal incorporation. Abscissa scale: molar concentrations of ET-1. ET-1 (■, n=16); ET-1+1 μM BQ-123 (▽, n=6). In (a) and (b) values are means and vertical lines show s.e.mean.

Table 1 Effects of 1 μM bosentan and 1 μM IRL 1038 on 100 nM ET-1 induced stimulation of [^{3}H]-phenylalanine incorporation in neonatal rat ventricular cardiomyocytes

ET-1 (100 nM)	*Antagonist* (1 μM)	*Incorporated [^{3}H]-phenylalanine* (% of control)	n	P
+	None	167±12	6	
+	bosentan	135±10	6	<0.05
None	bosentan	98±8	6	n.s.
+	None	150±10	6	
+	IRL 1038	142±7	6	n.s.
None	IRL 1038	101±8	6	n.s.

Values are mean±s.e.mean, n=number of experiments. Basal [^{3}H]-phenylalanine incorporation amounted to 4221±797 c.p.m. (n=12).

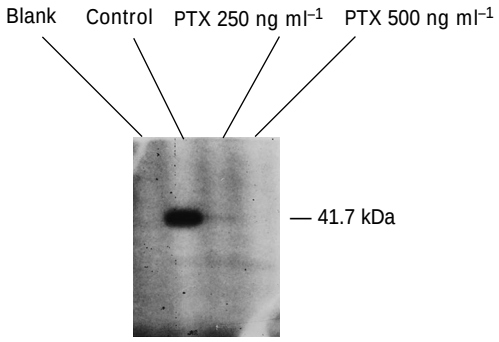

Figure 3 Effect of pertussis toxin (PTX) treatment of neonatal rat isolated ventricular cardiomyocytes on PTX-catalysed ADP-ribosylation of membranes derived from these cardiomyocytes. Cells were treated with 250 ng or 500 ng PTX ml^{-1} culture medium for 20 h at 37°C. Following washout membranes were prepared and incubated with fresh PTX and [^{32}P]-NAD for 1 h at 30°C. The figure shows an autoradiogram of the SDS-polyacrylamide gel analysis of the reaction product. Lanes were loaded with membranes from vehicle pretreated cells (control) and with membranes from PTX pretreated cells (250 ng PTX ml^{-1} or 500 ng PTX ml^{-1}).

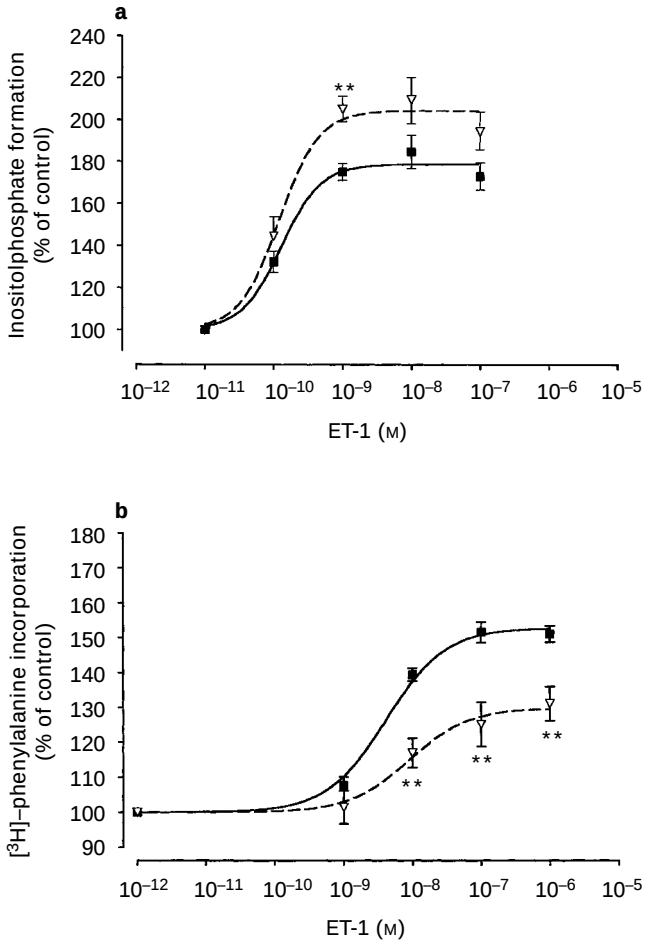

Figure 4 Effects of pertussis toxin (PTX, 500 ng ml^{-1}) treatment overnight on endothelin-1 (ET-1)-induced inositol phosphate (IP) generation (a) and [^{3}H]-phenylalanine incorporation (b) in neonatal rat ventricular cardiomyocytes. For details see legend of Figures 1 and 2. Basal [^{3}H]-IP-formation in control cells was 3402 ± 416 c.p.m., in PTX-pretreated cells 2999 ± 283 c.p.m. Basal [^{3}H]-phenylalanine incorporation in control cells was 4223 ± 983 c.p.m. in PTX-pretreated cells 4159 ± 938 c.p.m. Values are means and vertical lines show s.e.mean. ET-1, control cells (■, $n=5$); ET-1, PTX-pretreated cells (▽, $n=5$). $^{**}P<0.01$ vs the corresponding values in non-PTX-treated cells.

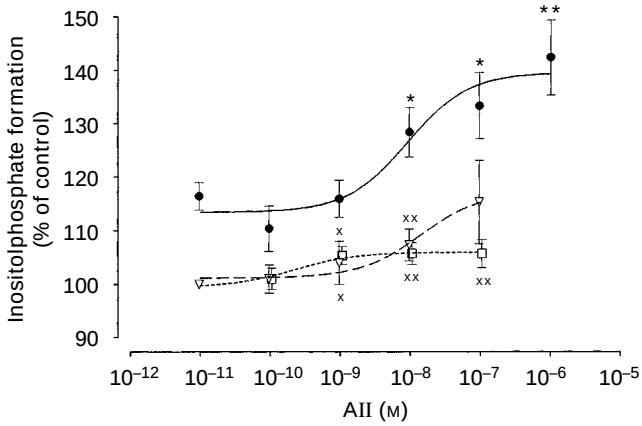

Figure 5 Stimulation by angiotensin II (AII) of inositol phosphate (IP) generation in neonatal rat ventricular cardiomyocytes in the absence and presence of 1 μM losartan or 1 μM BQ-123. Ordinate scale: [^{3}H]-IP-formation as % of basal formation. Abscissa scale: molar concentrations of AII. Values are means and vertical lines show s.e.mean. AII (●, $n=16$); AII + 1 μM losartan (▽, $n=3$); AII + 1 μM BQ-123 (□, $n=3$). Basal [^{3}H]-IP-formation was about 1–2% of the incorporated radioactivity and amounted to 1536 ± 282 c.p.m. ($n=16$). $^{**}P<0.01$, $^{*}P<0.05$ vs basal [^{3}H]-IP-formation; $^{xx}P<0.01$, $^{x}P<0.05$ vs the corresponding AII-values.

sistent and rather weak; significant effects could only be obtained at the very high unphysiological concentrations of 10 nM – 1 μM. The AII-induced increase in IP-formation was suppressed by 1 μM losartan, but also by 1 μM BQ-123 (Figure 5).

AII (10 nM – 1 μM) concentration-dependently increased [^{3}H]-phenylalanine uptake (Figure 6); similar to AII-induced IP-formation [^{3}H]-phenylalanine uptake was weak and significant effects were only obtained at the very high concentrations of AII, 0.1 μM and 1 μM. The AII-induced increase in [^{3}H]-phenylalanine uptake was antagonized by 1 μM losartan, but also by the endothelin-receptor antagonists BQ-123 (1 μM) and bosentan (1 μM) (Figure 6).

According to these results AII-induced IP-formation and [^{3}H]-phenylalanine uptake were inhibited by endothelin-receptor antagonists indicating that endothelin might be involved in the AII action. We, therefore, next tried to find out what might be the source of this AII-induced release of endothelins. For this purpose we studied neonatal rat cardiomyocytes that had been prepared by the continuous use of cytosine-β-D-arabinofuranoside (10 μM) to prevent almost completely contaminations with non-myocyte cells (see Methods). Under these conditions AII failed to increase [^{3}H]-phenylalanine uptake significantly (Figure 7) while endothelin-induced [^{3}H]-phenylalanine uptake was not at all affected (data not shown). However, when we then added non-myocyte cells to the highly purified cardiomyocytes, the ability of AII to increase [^{3}H]-phenylalanine uptake was restored (Figure 7).

Discussion

In the present study, in neonatal rat isolated cardiomyocytes, endothelins caused concentration-dependent increases in IP-formation and in incorporation of [^{3}H]-phenylalanine (as a measure of protein synthesis); for both, IP-formation and [^{3}H]-phenylalanine incorporation, the order of potency was ET-1 > > ET-3 indicating the involvement of ET_A-receptors. This assumption is supported by antagonist experiments, which showed that these ET-1 effects could be inhibited by the non-selective ET_A/ET_B-receptor antagonist bosentan and the selective ET_A-receptor antagonist BQ-123, while the ET_B-receptor antagonist IRL 1038 and the AT_1-receptor antagonist losartan were without any effects. From these results we can

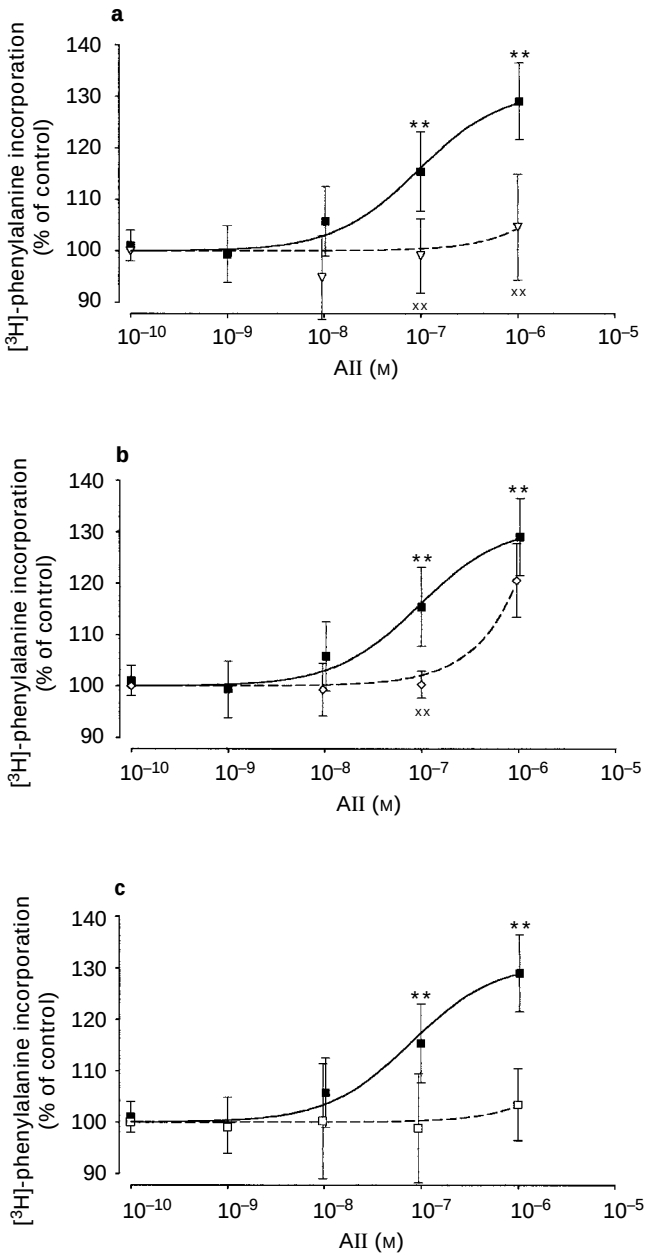

Figure 6 Stimulation by angiotensin II (AII) of [^{3}H]-phenylalanine incorporation in neonatal rat ventricular cardiomyocytes in the presence or absence of 1 μM losartan [a], 1 μM bosentan [b] or 1 μM BQ-123 [c]. Ordinate scales: [^{3}H]-phenylalanine incorporation as % of basal incorporation. Abscissa scale: molar concentrations of AII. Values are means and vertical lines show s.e.mean. AII (■, $n=9$); AII + 1 μM losartan (▽, $n=5$); AII + 1 μM bosentan (◇, $n=5$), AII + 1 μM BQ-123 (□, $n=5$). Basal [^{3}H]-phenylalanine incorporation amounted to 3664 ± 577 c.p.m. ($n=9$). $^{**}P<0.01$ vs basal [^{3}H]-phenylalanine incorporation; $^{xx}P<0.01$ vs the corresponding AII-values.

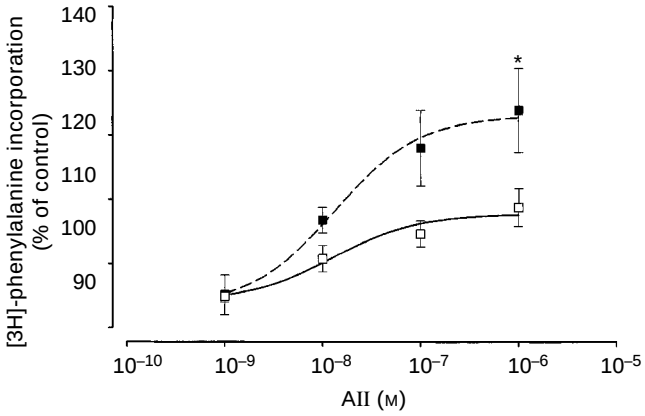

Figure 7 Stimulation by angiotensin II (AII) of [^{3}H]-phenylalanine incorporation in neonatal rat ventricular cardiomyocytes (CM, not contaminated by non-myocyte cells) and in cardiomyocytes co-cultured with non-myocyte cells (NMC). For details see Methods section. Ordinate scale: [^{3}H]-phenylalanine incorporation as % of basal. Abscissa scale: molar concentration of AII. Values are means and vertical lines show s.e.mean. (□) 10^6 CM/well ($n=4$); (■) 10^6 CM/well + 10^5 NMC/cell ($n=4$). Basal [^{3}H]-phenylalanine incorporation amounted in CM to 3893 ± 102 c.p.m. ($n=4$), in NMC + CM to 7783 ± 508 c.p.m. $^*P<0.05$ vs the corresponding value in CM.

conclude that, in neonatal rat cardiomyocytes, ET_A-receptors mediate increases in IP-formation and protein synthesis exclusively, in accordance with published data from the literature (Shubeita *et al*., 1990; Suzuki *et al*., 1990; Sugden *et al*., 1993; Ito *et al*., 1993; Hilal-Dandan *et al*., 1994).

It has been postulated that stimulation of IP-formation and increased rate of protein synthesis are causally linked via activation of protein kinase C (PKC) (for review see Chien *et al*., 1991; Sugden & Bogoyevitch, 1995). In support of this hypothesis is the fact that in cardiomyocytes stimulation of receptors that couple via $G_{q/11}$ to IP-formation, such as ET_A- (Suzuki *et al*., 1990; Shubeita *et al*., 1990; Ito *et al*., 1991) or α_1-adrenoceptors (Simpson, 1983; Lee *et al*., 1988; Knowlton *et al*., 1993) induce cell growth; moreover, direct activation of PKC by phorbol esters caused increased rate in protein synthesis in rat cardiomyocytes (Dunnmon *et al*., 1990; Allo *et al*., 1992). However, the present results clearly demonstrate that, in neonatal rat cardiomyocytes, an additional signalling pathway must be included in the ET-1 induced increases in rate of protein synthesis. As shown in Figure 3, pretreatment of the cells with PTX (500 ng ml^{-1}) resulted in a nearly complete inactivation of G_i. This PTX-pretreatment did not cause any attenuation of ET-1-induced IP-formation; in contrast after PTX treatment ET-1 induced IP-formation tended to be slightly increased. We have recently also found that in rat isolated renal cells inactivation of G_i by PTX caused an increase in ET-1-induced IP-formation (Becker *et al*., 1996); and similar results of an enhanced IP-formation following activation with stimulating hormones have been recently published in mouse cultured F9 teratocarcinoma stem cells and ROS 17/2.8 cells in which G_i expression was suppressed by antisense RNA (Watkins *et al*., 1994). Thus, it might be that in certain tissues and/or cells (including neonatal rat cardiomyocytes) phospholipase C (PLC) activity is under tonic inhibitory control of G_i.

In contrast to IP-formation, in the present study ET_A-receptor mediated increase in [^{3}H]-phenylalanine incorporation was in PTX-pretreated cardiomyocytes significantly reduced by about 39%. Similar results of a PTX-insensitive IP-formation and a (at least partly) PTX-sensitive protein synthesis induced by ET-1 has been also demonstrated in the adult rat (Hilal-Dandan *et al*., 1994) and feline cardiomyocytes (Jones *et al*., 1996). Thus, from the present results and those from the literature it can be concluded that the increase in rate of protein synthesis induced by ET_A-receptor activation involves at least two signalling pathways, one via a PTX-insensitive G-protein (most likely $G_{q/11}$, Takigawa *et al*., 1995) coupled to IP-formation and subsequent PKC-activation, and the other one via a PTX-sensitive G-protein (for recent review see Sugden & Bogoyevitch, 1995).

Data obtained in several animal models as well as in isolated cardiomyocytes appear to support a potential role of AII in the development of cardiac hypertrophy (Lindpaintner & Ganten, 1991; Baker *et al*., 1992). Thus, constant infusion of AII in rats led to cardiac hypertrophy (Tan *et al*., 1991) and administration of angiotensin converting enzyme (ACE)-inhibitors in spontaneously hypertensive rats (SHR) can prevent

or reverse cardiac hypertrophy (Sen *et al*., 1980; Brilla *et al*., 1991). The mechanism of these trophic effects of AII is still a matter of debate since studies in isolated cardiomyocytes have led to divergent results. Thus, in rat (Sadoshima & Izumo, 1993; Miyata & Haneda, 1994; Kinugawa *et al*., 1995) and chicken isolated cardiomyocytes (Baker & Aceto, 1990) AII caused increased rate of protein synthesis via direct activation of AT_1-receptors. On the other hand, Ito *et al*. (1993) in neonatal rat isolated cardiomyocytes, found that the AII-induced protein synthesis could be inhibited by the AT_1-receptor antagonist, losartan, but could be also inhibited by the ET_A-receptor antagonist, BQ-123; from these results they concluded that ET-1 locally generated and secreted from the cardiomyocytes may contribute to AII-induced cardiac hypertrophy. And recently, Kim *et al*. (1995) presented data demonstrating that the trophic effect of AII in neonatal rat cardiomyocytes appears to be mediated by activation of AT_1-receptors on cardiac fibroblasts. They showed that in well-defined cultures of neonatal cardiomyocytes (that were nearly free of contaminating non-myocyte (mainly cardiac fibroblasts) cells) there exists only a very low number of AT_1-receptors: in addition, in these cells AII failed to increase [^{3}H]-leucine incorporation (as a measure of protein synthesis). On the other hand, cultures of non-myocyte cells (mainly cardiac fibroblasts) exhibited a large number of AT_1-receptors and, in the presence of non-myocyte cells, AII significantly increased [^{3}H]-leucine incorporation in the cardiomyocytes.

In the present study, AII caused inconsistent and weak increases in IP-formation and [^{3}H]-phenylalanine incorporation, and the data showed a large experimental scatter. These AII effects that were by far less pronounced than those induced by ET-1 (see above) could be inhibited by the AT_1-receptor antagonist losartan demonstrating that they were mediated by AT_1-receptor stimulation. However, the AII effects on IP-formation and protein synthesis could be also inhibited by the endothelin-receptor antagonists bosentan and BQ-123 in ET-receptor selective concentrations, suggesting that ET-1 might be involved in this AII effect. On the other hand, if we prepared the cardiomyocytes under conditions that nearly completely prevented contamination with non-myocyte cells (see Methods) AII failed to increase the rate of protein synthesis significantly (see Figure 7). These findings that are comparable with those of Kim *et al*. (1995) discussed above, support the idea that non-myocyte cells might significantly contribute to the effects of AII on the rate of protein synthesis in neonatal rat cardiomyocytes. Our co-culture experiments support this view. As shown in Figure 7, after addition of non-myocyte cells to the neonatal rat cardiomyocytes AII now caused significant increases in rate of protein synthesis; this again could be inhibited by the ET-receptor antagonists bosentan and BQ-123 (data not shown). These results are compatible with the view that the trophic effect of AII on neonatal rat cardiomyocytes is brought about via ET-1 secretion upon AT_1-receptor stimulation in cardiac non-myocyte cells.

In conclusion; in neonatal rat ventricular cardiomyocytes the ET-1-induced increase (through ET_A-receptor stimulation) in rate of protein synthesis involves at least two signalling pathways: one via a PTX-insensitive G-protein coupled to IP-formation, and the other one via a PTX-sensitive G-protein. The trophic effects of AII are brought about via local ET-1 secretion upon AT_1-receptor stimulation on neonatal rat ventricular non-myocyte cells.

The skilful technical assistance of I. Adler, A. Dunemann, A. Hauser, H. Katzer, P. Matthes, I. Michalke, M. Niebisch is gratefully acknowledged. The authors are thankful to Dr Clozel and Dr Timmermans for generous gifts of bosentan and losartan, respectively. This work was supported by a grant of the Deutsche Forschungsgemeinschaft (DFG Br 526/3-3).

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(Received October 11, 1996
Revised January 18, 1997
Accepted January 21, 1997)