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European Journal of Pharmacology 417 (2001) 157–168

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Use of A-192621 to provide evidence for involvement of endothelin ET_B-receptors in endothelin-1-mediated cardiomyocyte hypertrophy

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Received 18 December 2000; received in revised form 26 February 2001; accepted 9 March 2001

Abstract

Increased plasma levels of endothelin-1 correlate with the severity of left ventricular hypertrophy in vivo. The aim of the study was to determine the relative contribution of stimulation of endothelin ET_A and endothelin ET_B receptors, and the associated activation of protein kinase C, to the hypertrophic response initiated by endothelin-1 in adult rat ventricular cardiomyocytes maintained in culture (24 h). Endothelin-1 (10^{-7} M) increased the total mass of protein and the incorporation of [^{14}C] phenylalanine into protein to 26% and 25% greater ($P < 0.05$) than respective basal values. The total content of RNA and the incorporation of 2-[^{14}C] uridine into RNA were increased by 23% and 21%, respectively, by endothelin-1 (10^{-8} M). Actinomycin D (5×10^{-6} M), an inhibitor of transcription, abolished the incorporation of [^{14}C] phenylalanine and the increased protein mass elicited by endothelin-1 (10^{-8} M). The selective agonists at the endothelin ET_B receptor, sarafotoxin 6c (10^{-7} M) and endothelin-3 (10^{-7} M) , increased the incorporation of [^{14}C] phenylalanine to 13% and 13% greater than respective basal values. The incorporation of [^{14}C]phenylalanine in response to endothelin-1 (10^{-7} M) was reduced by 50% ($P < 0.05$) by the selective antagonist at endothelin ET_A receptors, ABT-627 (10^{-9} M), while the response to sarafotoxin 6c was not attenuated. The selective antagonist at endothelin ET_B receptors, A192621 (10^{-10} M), abolished the response to sarafotoxin 6c (10^{-7} M) and attenuated the response to endothelin-1 (10^{-7} M) by 43% ($P < 0.05$). The selective inhibitor of protein kinase C, bisindolylmaleimide (5×10^{-6} M) attenuated the response to sarafotoxin 6c (10^{-7} M) by 78% and that to endothelin-1 (10^{-7} M), elicited in the presence of A192621 (10^{-10} M), by 52%. In conclusion, these data implicate endothelin ET_B receptors, in addition to endothelin ET_A receptors, in endothelin-1-mediated cardiomyocyte hypertrophy and provide evidence for the involvement of protein kinase C, at least in part, in the hypertrophic signalling pathways associated with activation of each receptor subpopulation.

Keywords: Cardiac hypertrophy; Cardiomyocyte; Endothelin

1. Introduction

Endothelin-1 is a potent vasoconstrictor peptide that is expressed in a wide variety of tissues including the myocardium (Yanagisawa et al., 1988). Endothelin-2 and endothelin-3 are isopeptides that differ from endothelin-1 by 2 and 6 amino acids, respectively. Endothelin-1 is the predominant isopeptide expressed in the vasculature (Haynes and Webb, 1993) although endothelin-3 is also present in plasma (Battistini et al., 1993). Increased plasma levels of the endothelins have been found in hypertension (Widimsky et al., 1991; Schriffen, 1995) and heart failure (Cody et al., 1997). Although a causal role for circulating endothelin-1 in these disease states has not been identified, a consistent finding is a correlation between the plasma concentration of the peptide and the severity of left ventricular hypertrophy (Levin, 1995). Endothelin-like immunoreactivity and peptide binding sites are also elevated in cardiac tissue obtained from experimental animal models of left ventricular hypertrophy induced by pressure overload, indicating that the locally derived peptide may make an important contribution to the ventricular remodelling associated with the pathogenesis of left ventricular hypertrophy (Yorikane et al., 1993; Arai et al., 1995; Brown et al., 1995; Sakai et al., 1995; Schunkert et al., 1999). The actions of endothelin-1 are mediated by the endothelin ET_A and endothelin ET_B receptor subtypes, which are both present in the heart; the ET_A:ET_B ratio is approximately 85%:15% in rat ventricular myocardium (Thibault et al., 1995; Fareh et al., 1996). Antagonism of

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0014-2999/01/$ - see front matter
PII: S0014-2999(01)00905-0

the endothelin ET_A receptor has been found to attenuate left ventricular hypertrophy in a number of experimental models in vivo (Ito et al., 1994; Karam et al., 1996; Ehmke et al., 1999) and the mixed endothelin $ET_{A/B}$ receptor antagonist, bosentan, also causes some regression of left ventricular hypertrophy in the absence of an appreciable reduction in blood pressure (Karam et al., 1996). These data indicate that endothelin-1 may exert a local autocrine/paracrine influence on cardiomyocyte hypertrophy independent of the peptide's pressor effects upon the systemic vasculature.

Endothelin-1 induces hypertrophy, characterised by increased cell volume and enhanced synthesis of cellular protein in neonatal rat ventricular cardiomyocytes in vitro (Ito et al., 1991, 1993; Yamazaki et al., 1996; Ponicke et al., 1997); these effects are abolished by the selective antagonist at endothelin ET_A receptors, BQ123 (cyclo[-D-Trp-D-Asp-Pro-D-Val-Leu-]). However, there is also evidence that endothelin ET_B receptor mRNA levels are elevated in hypertrophying neonatal cardiomyocytes (Kanno et al., 1993) and that the endothelin ET_B receptor-selective agonist, endothelin-3, can itself initiate cardiomyocyte hypertrophy (Tamamori et al., 1996). The synthesis and secretion of endothelin-1 from neonatal cardiomyocytes is enhanced in response to mechanical stretch and the peptide, acting through endothelin ET_A receptors, has been implicated in stretch-induced hypertrophy since this response is attenuated by BQ123, but not by the endothelin ET_B receptor-selective antagonist, BQ788 (*N*-[*cis*-(2,6-dimethylpiperizinyl)carbonyl](4Me)L-Leu-(1-methoxycarbonyl)D-Trp-Dnle) (Yamazaki et al., 1996). The role of the endothelin ET_B receptor in cardiomyocyte hypertrophy in response to endothelin-1 remains uncertain.

Significant differences exist between cardiomyocytes isolated from neonatal and adult mammals (Schluter et al., 1995). It is more appropriate that experimental cell models employed in vitro use as a source cardiac tissue obtained from adult mammals since the clinical problems associated with myocardial hypertrophy are related to the adult state. The hypertrophic effect of endothelin-1 has been less extensively investigated in adult cells. Endothelin-1 stimulates de novo protein synthesis in adult rat cardiomyocytes maintained in suspension (Sugden et al., 1993), and increases cellular protein mass and de novo protein synthesis in adult rabbit ventricular cardiomyocytes maintained in short-term culture (Mullan et al., 1997). Activation of the phospholipase C-β signalling cascade has been implicated in the hypertrophic response to endothelin-1 in both rat and rabbit cardiomyocytes (Bogoyevitch et al., 1993; Sugden et al., 1993; Mullan et al., 1997; Bell and McDermott, 1998; Yamazaki et al., 1999). Previously, we provided preliminary evidence for the involvement of both endothelin ET_A and ET_B receptors in the hypertrophic response to the peptide in the rabbit model; this was based on the attenuation by BQ123- and BQ788-selective antagonists at endothelin ET_A and ET_B receptors, respectively. However, significant partial agonist activity was attributed to each compound (Mullan et al., 1997) and it is possible that the mechanism of action of these compounds may not be limited to endothelin receptor antagonism.

The aim of the present study was firstly to confirm that endothelin-1 initiated hypertrophy of adult rat ventricular cardiomyocytes in short-term culture and then to provide evidence for the causal association of de novo synthesis of RNA and of protein with the increased cellular protein mass obtained in response to the peptide. Secondly, ABT-627 (2-(4-methoxyphenyl)-4-(1,3-benzodioxol-5-yl)-1-(*N*,*N*-di(*n*-butyl)amino carbonylmethyl)-pyrrolidine-3-carboxylic acid) and A-192621, (2-(4-propoxyphenyl)-4-(1,3-benzodioxol-5-yl)-1-(2,5-ethylphenyl)amino carbonylmethyl)-pyrrolidine-3-carboxylic acid), novel nonpeptide antagonists at endothelin ET_A and endothelin ET_B receptors, respectively, were used to determine the contribution of each receptor subtype to the response to endothelin-1: these compounds, developed by Abbott Laboratories (Illinois, USA), demonstrate 1400-fold selectivity for their respective receptors (Douglas, 1997). Finally, the possibility that differences existed in regard to the signal transduction cascade associated with each endothelin receptor subtype, namely the requirement for the involvement of protein kinase C, was investigated.

2. Materials and methods

2.1. Solutions

Serum-free 'creatinine–carnitine–taurine' (CCT) medium for the culture of cardiomyocytes consisted of modified glutamine-free Medium M199 supplemented with Earle's salts, HEPES (15 mM), creatinine (5 mM), L-carnitine (2 mM), taurine (5 mM), ascorbic acid (100 μM), penicillin (100 IU ml^{-1}) and streptomycin (100 μg ml^{-1}). Medium was also supplemented with cytosine β-D arabinofuranoside (10 μM) to prevent growth of nonmyocytes. The composition of the Ca^{2+}-free Krebs–Ringer solution used in the isolation of cardiomyocytes was as follows: NaCl (110 mM); KCl (2.6 mM); $NaHCO_3$ (25 mM); $MgSO_4$ (1.2 mM); KH_2PO_4 (1.2 mM); glucose (11 mM). This solution was gassed with 95% O_2/5% CO_2 and maintained at a pH of 7.4 at 37°C. The composition of the phosphate-buffered saline (PBS) was as follows: NaCl (137mM); KH_2PO_4 (1.5 mM); Na_2PO_4 (1.0 mM), pH 7.4. The composition of DNA assay solution was NaCl (1.985 M), Na_2HPO_4 (25 mM), at pH 7.4. Bisbenzamide was dissolved in water (0.2 mg ml^{-1}). This stock solution was diluted 1:200 with DNA assay solution to give a working concentration of 1 μg ml^{-1}. Solutions of bisbenzamide are very sensitive to light and were, therefore, kept in the dark prior to use. The stock solution was stable for 6 months in the dark at 4°C. Dilute solutions were prepared daily.

2.2. Isolation and culture of cardiomyocytes

All work was performed in accordance with the Home Office *Guidance on the operation of the Animals (Scientific Procedures) Act 1986*, published by Her Majesty's Stationary Office, London. Ventricular cardiomyocytes were isolated as described previously (Bell et al., 1995). Briefly, two 12-week-old male Sprague–Dawley rats were subjected to deep isoflurane anaesthesia and their hearts excised. The excised hearts were perfused using a Langendorff apparatus with Ca^{2+}-free Krebs Ringer solution containing collagenase (0.4 mg/ml) until they became flaccid. The two hearts were chopped finely and the mince was pooled and agitated gently in the same medium to dissociate individual cells. The resulting cell suspension was filtered to remove undigested material and the cells were sedimented at 750 rpm for 4 min. Ca^{2+} tolerance of the cells was restored gently by resuspending the sediment in Krebs–Ringer solution containing a progressively higher concentration of Ca^{2+} to a final concentration of 1 mM. The cell suspension (3–4 ml) was then layered gently onto a 4% (w/v) albumin solution (12.5 ml), contained in a tube of length 20 cm and internal diameter 1 cm, in order to sediment viable cardiomyocytes and effectively remove nonmuscle cells and cell debris. The resultant sediment was resuspended in serum-free CCT medium. Cells derived from the two hearts were pooled, mixed thoroughly and resuspended at a concentration of 1.5×10^5 viable cardiomyocytes ml^{-1}. Petri dishes were preincubated for 2 h with foetal calf serum (4% v/v) in M199. For experiments to quantify RNA content, 10 ml of cell suspension was pipetted gently onto Petri dishes of 100-mm diameter. For all other experiments, aliquots of cell suspension (1 ml) were pipetted gently onto Petri dishes of 35-mm diameter. After 1 h, viable cardiomyocytes had attached to the surface of the dish. The dishes were washed with fresh CCT medium to remove nonattached cells and cell debris and the attached cells, employed as an experimental model for investigation of the initiation of cardiomyocyte hypertrophy, were incubated at 37°C for the times indicated in CCT medium (1 ml) containing the appropriate concentrations of the various hypertrophic stimuli and/or inhibitors as specified in the experimental protocols. Under all experimental conditions, cardiomyocytes remained mechanically quiescent.

2.3. Incorporation of L-U-[^{14}C]phenylalanine and total mass of cellular protein and total content of cellular DNA

The extent of de novo synthesis of protein in the cell cultures was estimated by measuring uptake of radiolabelled amino acid into cellular protein. The cells were exposed for 24 h to L-U-[^{14}C]phenylalanine (0.1 μC_i ml^{-1} culture medium). Incorporation of radioactivity into the acid-insoluble cell fraction was determined. At the end of the chosen period of incubation, experiments were terminated by removal of the supernatant medium from the dishes. The attached cells were washed with an aliquot (1 ml) of ice-cold PBS, prior to the addition of an aliquot (1 ml) of ice-cold trichloroacetic acid (10% w/v). After storage overnight at 4°C, the acid containing the intracellular precursor pool was removed from the dishes and the attached cells were washed with an aliquot (1ml) of PBS. The precipitate remaining on the culture dishes was dissolved in an aliquot (1 ml) of NaOH (0.1 M)/sodium dodecyl sulphate (0.01% w/v) by overnight incubation at 37°C. In these samples, concentration of protein was determined by the colorimetric method of Lowry, the concentration of DNA in the neutralized sample was determined by a spectrophotometric method in which bisbenzamide dye was incorporated into DNA (Mullan et al., 1997), and the radioactivity was counted. The ratio of protein to DNA per culture served as the parameter of cell mass and the ratio of L-U-[^{14}C]phenylalanine incorporated to DNA per culture served as a measure of de novo synthesis of protein. The concentration of DNA is a reliable measure of cell number as adult cardiomyocytes do not proliferate in culture (Jacobson and Piper, 1986); also nonmyocytic cells are virtually absent from these fresh cultures, and the very few present cannot proliferate, since they would be killed by the presence of cytosine b-D-arabinofuranoside (Piper and Volz, 1990).

2.4. Incorporation of 2-[^{14}C]uridine into cellular RNA

The extent of de novo synthesis of RNA in the cell cultures was estimated by measuring uptake of radiolabelled nucleotide into cellular RNA. The cells were exposed for < 8 h to 2-[^{14}C] uridine (0.2 μC_i ml^{-1} culture medium). At the end of the chosen period of incubation, experiments were terminated according to the protocol described in Section 2.3. The incorporation of radioactivity into the acid-insoluble cell fraction was determined. The ratio of 2-[^{14}C]uridine incorporated to DNA per culture served as a measure of de novo synthesis of RNA.

2.5. Total mass of cellular RNA

Nucleic acids were isolated using a modified guanidine thiocyanate extraction method according to Chomczynski and Sacchi (1987). Briefly, RNA was extracted using chloroform, precipitated using isopropanol and the resulting precipitate was dissolved in diethylpyrocarbonate-treated water. DNA present in the isolates was extracted using chloroform, precipitated using ethanol and the resulting precipitate was dried and dissolved in NaOH (40 mM). The concentrations of RNA and DNA were determined using a standard spectrophotometric method based on the absorbance of the samples at 260 nm. The ratio of RNA:DNA per culture served as a measure of cell RNA content.

2.6. Data analysis

In each experiment, the total population of cells contained in culture plates was obtained from a pooled suspension prepared from two hearts. Under each condition (in the absence/presence of peptide at various concentrations, with or without inhibitor), the average value measured in three culture plates was calculated for each parameter ([^{14}C]phenylalanine incorporation or protein/DNA content). Replicate data were obtained for n preparations ($3 \leq n \leq 13$) and the mean value ± S.E.M. was calculated. Data were analysed statistically using a one or two factor repeated measures analysis of variance (SPSS-PC, version 8.0). In the experiments to examine peptide concentration–response relationships in the presence of the different antagonists or inhibitors, average tests of significance for within-subjects effects (concentration and the concentration–antagonist/inhibitor interaction) were adjusted if necessary to accommodate lack of constant variance using the Huynh–Feldt epsilon. If $P < 0.05$ for the overall effect of concentration under a particular condition, differences between the mean values at a particular concentration (x_1) and at baseline (x_0) were tested by calculation of the *t*-statistic as $(x_1 - x_0)/\sqrt{\text{residual mean square } (2/n)}$.

2.7. Drugs

Endothelin-1, endothelin-3 and sarafotoxin 6c were supplied by the American Peptide (Sunnyvale, CA, USA). The endothelin receptor antagonists, ABT-627 and A-192621 were gifts from Abbott Laboratories. Bovine serum albumin (Cat. No. A7030), L-carnitine, creatine, taurine, cytosine-β-arabinofuranoside, DNA (sodium salt, from calf thymus), actinomycin D (Cat. No. A9415), assay kits for the determination of microprotein, diethylpyrocarbonate and Tri-Reagent for the isolation of RNA were obtained from Sigma Chemical (Poole, Dorset, UK). Phenylephrine hydrochloride was supplied by Research Biochemicals International (MA, USA). Liquid scintillation fluid was obtained from BDH Chemicals (UK). Bisindolylmaleimide and collagenase B were purchased from Boehringer Mannheim (Mannheim, Germany). Insulin (porcine, highly purified) was purchased from Novo Nordisk Wellcome (England). Medium M199 (glutamine-free with Earle's salts), foetal bovine serum and penicillin (5000 IU)/streptomycin (5 mg ml^{-1}) were supplied by GIBCO (UK). Bisbenzamide (H 33258) was purchased from Riedel-de-Haen (Germany). Plastic Petri dishes were obtained from Falcon (Becton-Dickinson, UK). L-U-[^{14}C]phenylalanine and 2-[^{14}C]uridine were supplied by Amersham International (Buckinghamshire, UK). All chemicals used in the extraction and purification of RNA were of molecular biology grade and purchased from Sigma. All other chemicals were of analytical grade and purchased from BDH Chemicals.

3. Results

3.1. DNA content of cultures

The DNA content of cultures incubated for 24 h in the presence of the concentrations of the various agonists, antagonists and inhibitors of signal transduction employed in the study did not differ significantly from that of cultures maintained in the absence of these agents for a similar period of time (Table 1). Therefore, since there was no evidence of cell death or of cell proliferation in response to any of these agents, the DNA content of the cultures was accepted as a reliable index of cell number.

3.2. Total mass of cellular protein and de novo protein synthesis

Insulin (1 unit ml^{-1}) employed as a positive control for cardiomyocyte hypertrophy, increased the total mass of cellular protein after 24 h to 37% greater than the basal value (53.1 ± 5.7 μg μg DNA^{-1}, $n = 6$). Endothelin-1 increased the total mass of cellular protein significantly and in a concentration-dependent manner (Fig. 1A). The maximum increase, obtained at 10^{-7} M, was 26% greater than the basal value (53.1 ± 5.7 μg μg DNA^{-1}, $n = 6$). The incorporation of L-U-[^{14}C]phenylalanine into de novo protein was increased to 52% greater than the basal value (733.3 ± 85.4 dpm μg DNA^{-1}, $n = 13$) in the presence of insulin (1 unit ml^{-1}). Endothelin-1 increased the incorporation of L-U-[^{14}C]phenylalanine significantly and in a concentration-dependent manner (Fig. 1B). The maximum increase, obtained at 10^{-7} M, was 25% greater than the

Table 1
DNA contents of cardiomyocyte cultures maintained in short-term (24 h) primary culture in the absence and presence of the various agonists, antagonists and inhibitors employed in the study. DNA contents are expressed as μg culture^{-1} and are the means of *n* experiments

	Basal DNA (μg culture^{-1})	+ Pharmacological agent (μg culture^{-1})	*n*
Endothelin-1 (10^{-7} M)	4.55 ± 0.32	4.82 ± 0.35	20
Endothelin-3 (10^{-7} M)	3.86 ± 0.57	3.81 ± 0.41	5
Sarafoxin 6c (10^{-7} M)	4.59 ± 0.36	4.76 ± 0.33	19
ABT-627 (10^{-10} M)	4.88 ± 0.49	4.95 ± 0.47	5
A192621 (10^{-10} M)	4.53 ± 0.46	4.52 ± 0.51	12
Bisindolylmalimide (5×10^{-6} M)	3.87 ± 0.41	3.66 ± 0.42	13
Actinomycin D (5×10^{-6} M)	6.50 ± 0.95	6.47 ± 0.81	5
Insulin (1 unit ml^{-1})	5.89 ± 0.26	6.04 ± 0.33	60
Phenylephrine (5×10^{-6} M)	5.41 ± 0.36	5.37 ± 0.27	24

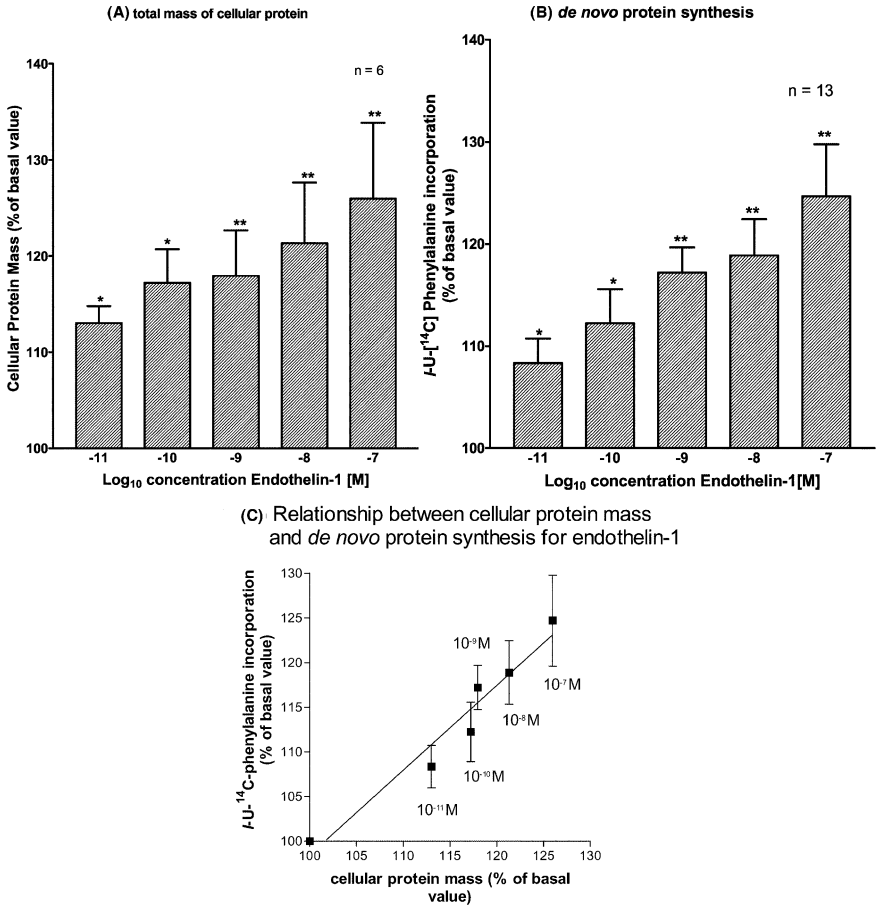


Fig. 1. Concentration dependence of the effect of endothelin-1 on (A) total mass of cellular protein and (B) incorporation of *l*-U-[^{14}C] phenylalanine into cellular protein of ventricular cardiomyocytes, isolated from the hearts of adult rats, and maintained in short-term (24 h) serum-free culture. Data are expressed as percentage differences from basal values and are the means ± S.E.M. of 6–13 experiments. Significant variation between responses elicited with and without endothelin-1 ($^{*}P < 0.05$, $^{**}P < 0.01$); (C) relationship between increases in cellular protein mass and in de novo protein synthesis elicited in response to endothelin-1.

basal value (733.3 ± 85.4 dpm μg DNA^{-1}, $n = 13$). At each concentration of endothelin-1, similar increases were obtained for de novo protein synthesis as for cellular protein mass, implying a causal relationship (Fig. 1C).

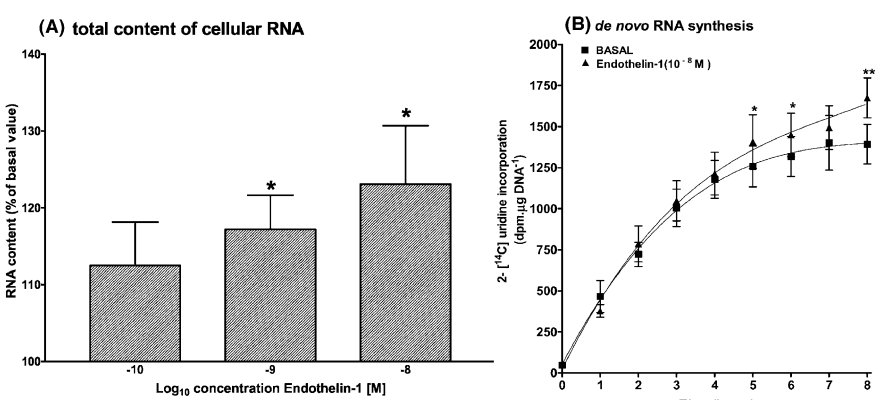


Fig. 2. Effect (A) of endothelin-1 (10^{-8} M) on total mass of cellular RNA of cardiomyocytes maintained in short-term (24 h) serum-free primary culture. Data are expressed as percentage differences from basal values and are the means ± S.E.M. of six experiments; (B) temporal dependence of the effect of endothelin-1 (10^{-8} M) on the incorporation of 2-[^{14}C]uridine into cellular RNA. Data are expressed as dpm μg DNA^{-1} and are the means ± S.E.M. of six experiments. Significant variation between responses elicited with and without endothelin-1 ($^{*}P < 0.05$, $^{**}P < 0.01$).

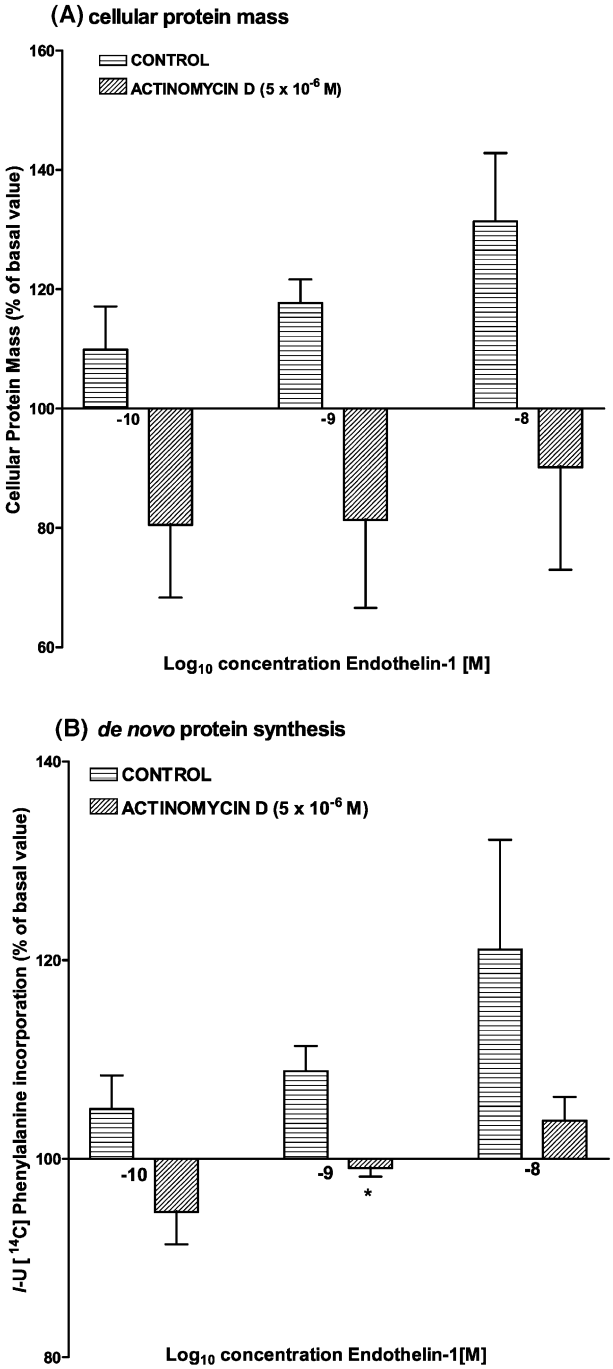


Fig. 3. Inhibition by actinomycin D (5×10^{-6} M) of the effect of endothelin-1 (10^{-10} M–10^{-8} M) on (A) the incorporation of *l*-U-[^{14}C]phenylalanine into cellular protein and (B) total mass of cellular protein of cardiomyocytes maintained in short-term (24 h) serum-free primary culture. Data are expressed as percentage differences from basal values (±actinomycin D) and are the means±S.E.M. of four experiments. Significant variation between paired data ($^{*}P < 0.05$).

3.3. Total content of cellular RNA and de novo synthesis of RNA

In the presence of insulin (1 unit ml^{-1}), the total content of cellular RNA was 31% greater than the basal value (0.65 ± 0.06 μg μg DNA^{-1}, $n = 12$). Endothelin-1 increased the total content of cellular RNA in a concentration-dependent manner (Fig. 2A). The maximum increase, obtained at 10^{-8} M, was 23% greater than the basal value (0.56 ± 0.09 μg μg DNA^{-1}, $n = 4$). The incorporation of 2-[^{14}C]uridine into cellular RNA in response to endothelin-1 (10^{-8} M) became progressively greater than control values over a period of 8 h when the increase of 21% was

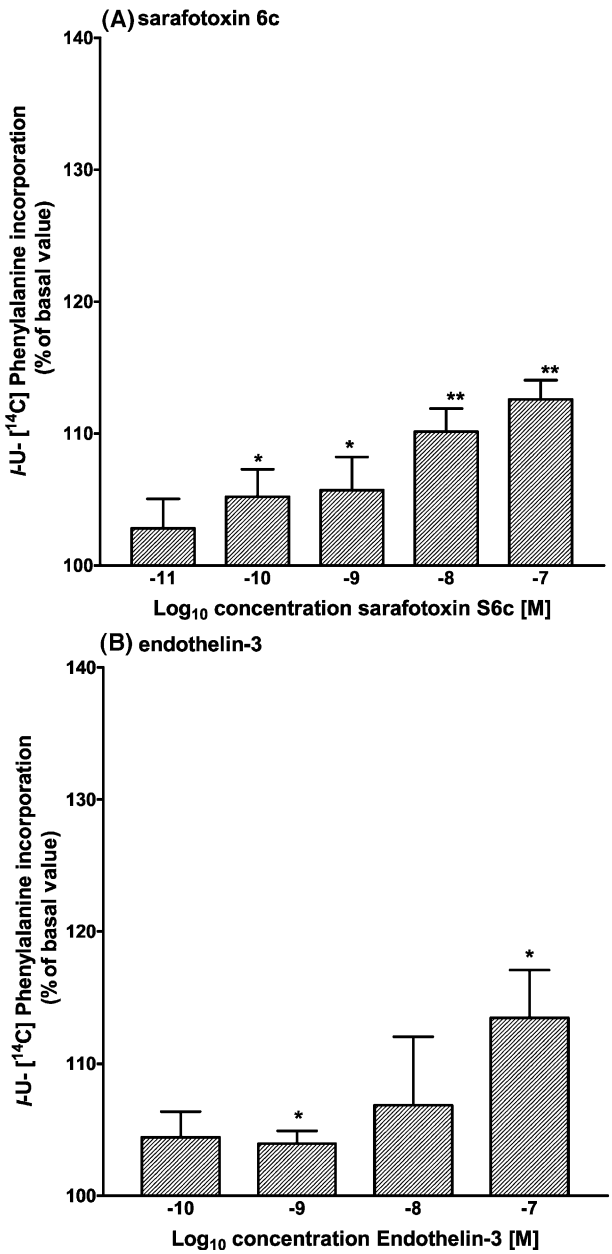


Fig. 4. Concentration dependencies of the effects of (A) sarafotoxin 6c and (B) endothelin-3 on the incorporation of *l*-U-[^{14}C]phenylalanine into cellular protein of ventricular cardiomyocytes, isolated from the hearts of adult rats, and maintained in short-term (24 h) serum-free culture. Data are expressed as percentage differences from basal values and are the means±S.E.M. of 6–10 experiments. Significant variation between responses elicited with and without peptide ($^{*}P < 0.05$, $^{**}P < 0.01$).

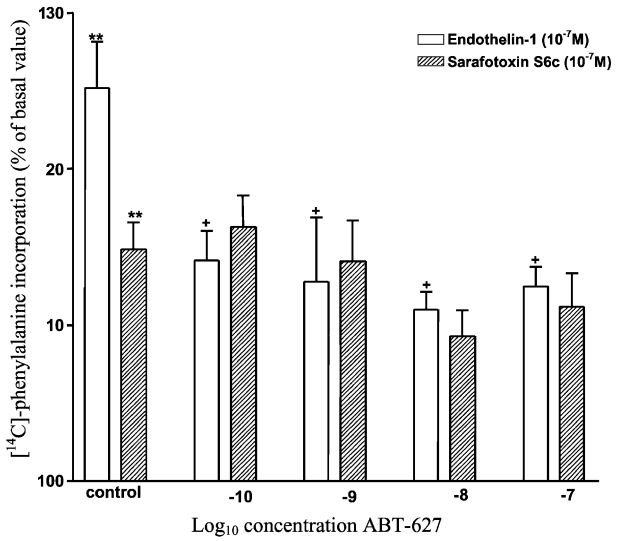


Fig. 5. Effects of the endothelin ET_A receptor-selective antagonist, ABT-627, on the effect of endothelin-1 (10^{-7} M) and of sarafotoxin 6c (10^{-7} M) on the incorporation of *l*-U-[^{14}C]phenylalanine into cellular protein of cardiomyocytes maintained in short-term (24 h) serum-free primary culture. Data are expressed as percentage differences from basal values (± antagonist) and are the means ± S.E.M. of five experiments. * Denotes a significant difference from basal response (* $0.01 < P \leq 0.05$; * * $0.005 < P \leq 0.01$). Denotes a significant difference with respect to the absence of ABT-627. (* $P < 0.05$).

significantly greater than the basal value (1392 ± 120 dpm μg DNA^{-1}, $n = 6$) (Fig. 2B).

3.4. Effect of actinomycin D

In the presence of actinomycin D (5×10^{-6} M), an inhibitor of transcription, the basal mass of cellular protein

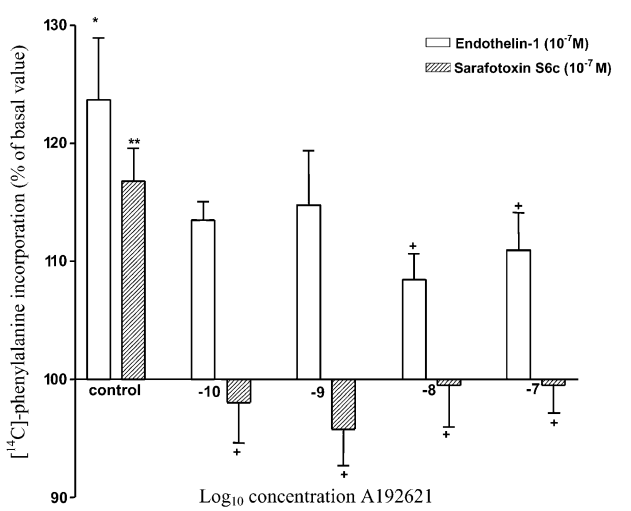
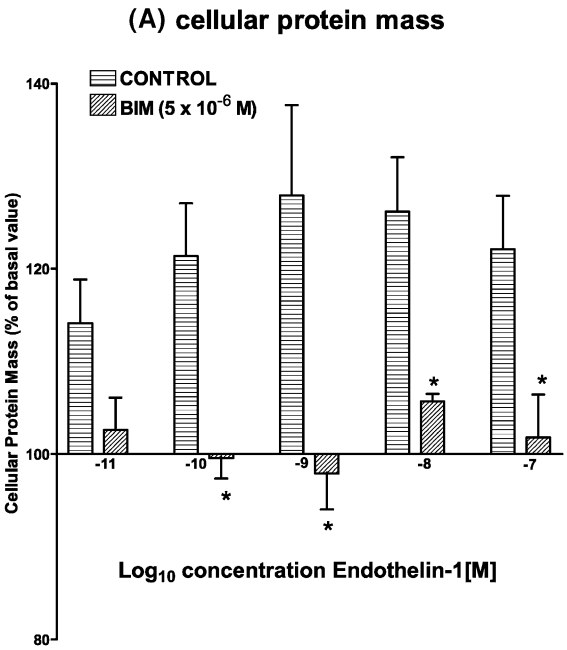


Fig. 6. Effects of the endothelin ET_B receptor-selective antagonist, A192621, on the effect of endothelin-1 (10^{-7} M) and of sarafotoxin 6c (10^{-7} M) on the incorporation of *l*-U-[^{14}C]phenylalanine into cellular protein of cardiomyocytes maintained in short-term (24 h) serum-free primary culture. Data are expressed as percentage differences from basal values (± antagonist) and are the means ± S.E.M. of six experiments. * Denotes a significant difference from basal response (* $0.01 < P \leq 0.05$; * * $0.005 < P \leq 0.01$). + Denotes a significant difference with respect to the absence of A192621 (* $P < 0.05$).



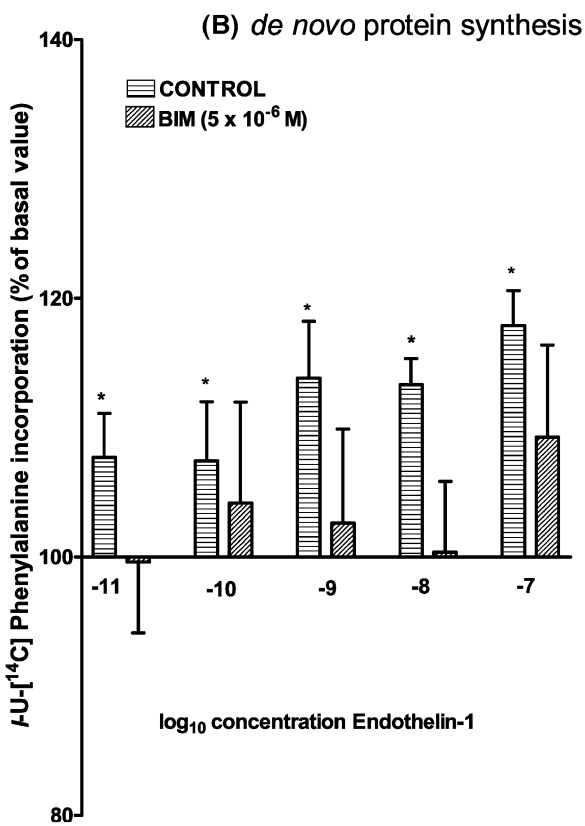


Fig. 7. Inhibition by the protein kinase C inhibitor, bisindolylmaleimide (BIM, 5×10^{-6} M), of the effect of endothelin-1 (10^{-11} M–10^{-7} M) on (A) total mass of cellular protein and (B) the incorporation of *l*-U-[^{14}C]phenylalanine into cellular protein of cardiomyocytes maintained in short-term (24 h) serum-free primary culture. Data are expressed as percentage differences from basal values (± BIM) and are the means ± S.E.M. of six experiments. Significant variation between paired data (* $P < 0.05$).

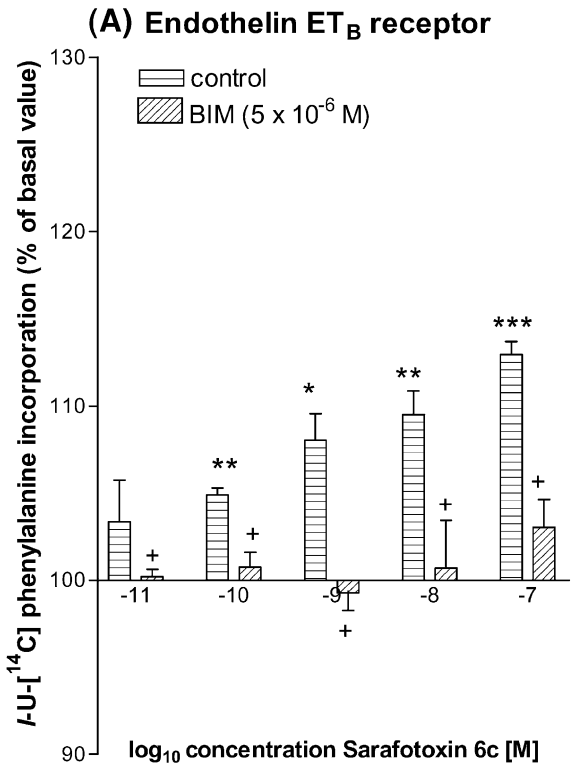
and the basal incorporation of L-U-[^{14}C]phenylalanine were attenuated by 9% and 15%, respectively; therefore, responses to trophic stimuli were expressed as a percentage of the basal response obtained in the absence of stimulus with and without actinomycin D. Actinomycin D abolished the increased incorporation of L-U-[^{14}C]phenylalanine and the increased cellular protein mass elicited by foetal bovine serum (10% v/v), employed as a positive control. Actinomycin D (5×10^{-6} M) also abolished the increases in both parameters elicited in response to endothelin-1 (10^{-10} M–10^{-8} M) (Fig. 3A,B).

3.5. Effects of endothelin receptor subtype-selective agonists and antagonists

The selective agonists at the endothelin ET_B receptor, sarafotoxin 6c (Fig. 4A) and endothelin-3 (Fig. 4B), increased the incorporation of L-U-[^{14}C]phenylalanine maximally at 10^{-7} M to 13.0% greater than respective basal values (504.0 ± 44.8 dpm μg DNA^{-1}, $n = 10$, and 518.9 ± 27.0 dpm μg DNA^{-1}, $n = 6$). In the presence of the endothelin receptor antagonists, A192621 and ABT-627, the basal incorporation of L-U-[^{14}C]phenylalanine was increased by 11% and 5%, respectively; therefore, responses to receptor agonists were expressed as percentages of the basal response obtained in the absence of agonist with and without the respective antagonist. The incorporation of L-U-[^{14}C]phenylalanine in response to endothelin-1 (10^{-7} M) was significantly decreased by 50% by the selective antagonist at endothelin ET_A receptors, ABT-627 (10^{-9} M), while the response to the selective agonist at endothelin ET_B receptors, sarafotoxin 6c, was not attenuated by this concentration of the antagonist (Fig. 5). Conversely, the selective antagonist at endothelin ET_B receptors, A192621 (10^{-10} M), abolished the response to sarafotoxin 6c (10^{-7} M) and attenuated the response to endothelin-1 (10^{-7} M) by 43.0% (Fig. 6). The amount of L-U-[^{14}C]-phenylalanine incorporated in response to the structurally unrelated peptide, insulin (1 unit ml^{-1}) was not attenuated by the addition of either endothelin receptor antagonist ($\leq 10^{-7}$ M).

3.6. Effect of inhibition of protein kinase C

In the presence of bisindolylmaleimide (5×10^{-6} M), a selective inhibitor of protein kinase C, the basal incorporation of L-U-[^{14}C]phenylalanine was attenuated by 12%; therefore, responses to trophic stimuli were expressed as



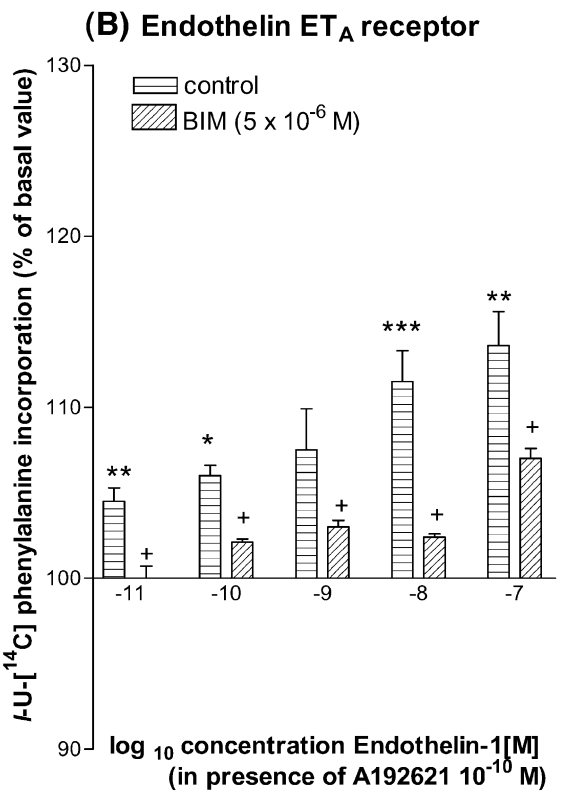


Fig. 8. Inhibition by the protein kinase C inhibitor, bisindolylmaleimide (BIM, 5×10^{-6} M), of the effect of (A) endothelin ET_B receptor stimulation by sarafotoxin 6c and (B) endothelin ET_A receptor stimulation by endothelin-1 in the presence of selective antagonist at endothelin ET_B receptors, A192621 (10^{-10} M) on the incorporation of *l*-U-[^{14}C]phenylalanine into cellular protein of cardiomyocytes maintained in short-term (24 h) serum-free primary culture. Data are expressed as percentage differences from basal values (±BIM) and are the means ± S.E.M. of four experiments. * Denotes a significant difference from basal response (* $0.01 < P \leq 0.05$; * * $0.005 < P \leq 0.01$). + Denotes a significant difference with respect to the absence of BIM (* $P < 0.05$).

percentages of the basal response obtained in the absence of stimulus with and without bisindolylmaleimide. The basal mass of cellular protein was unaltered in the presence of the inhibitor. The increased protein mass and incorporation of L-U-[^{14}C]phenylalanine in response to the alpha adrenoceptor agonist, phenylephrine (5×10^{-6} M), employed as a positive control for protein kinase C-dependence, were attenuated by 71% and 86%, respectively, by bisindolylmaleimide, while the responses to insulin (1 unit ml^{-1}), which acts via a protein kinase C-independent mechanism, were not reduced.

The increased protein mass (Fig. 7A) and incorporation of L-U-[^{14}C]phenylalanine (Fig. 7B) in response to endothelin-1 (10^{-11} M–10^{-7} M) were significantly attenuated at all concentrations in the presence of bisindolylmaleimide (5×10^{-6} M). Bisindolylmaleimide abolished the incorporation of L-U-[^{14}C]phenylalanine in response to endothelin ET_B receptor stimulation by sarafotoxin 6c (10^{-11} M–10^{-8} M) and attenuated the response to sarafotoxin 6c (10^{-7} M) significantly by 78% (Fig. 8A). Similarly, bisindolylmaleimide attenuated the incorporation of L-U-[^{14}C]phenylalanine in response to endothelin ET_A receptor stimulation by endothelin-1 (10^{-7} M), elicited in the presence of endothelin ET_B receptor antagonism by A192621 (10^{-10} M), by 52% (Fig. 8B).

4. Discussion

Endothelin-1 initiated a hypertrophic effect, as evidenced by increased protein mass, directly in adult rat ventricular cardiomyocytes maintained in short-term culture (24 h). This response was associated with an increased cellular content of total RNA, the majority of which is thought to represent ribsomal RNA (Schluter et al., 1995). Increased synthesis of de novo RNA and of protein was also demonstrated in response to the peptide. Actinomycin D, an inhibitor of transcription, attenuated endothelin-1-mediated increase in protein mass. Taken together, these data provide evidence for a causal association between de novo synthesis of (ribsomal) RNA, indicative of increased protein synthesising capacity, and increased cellular protein mass in response to endothelin-1.

This conclusion is supported by the linear relationship observed in regard to upregulation of protein mass and protein synthesis in response to endothelin-1 (Fig. 1C). These data are in agreement with previous studies by Sugden et al. (1993) who found that endothelin-1 increased protein synthesis in adult rat ventricular cardiomyocytes in suspension, and also with data obtained within our own laboratory (Mullan et al., 1997) utilising rabbit ventricular cardiomyocytes, but now establish conclusively the causal association between endothelin-1-mediated protein synthesis and the increased cell mass characteristic of cardiomyocyte hypertrophy. The actions of endothelin-1 are mediated by the endothelin ET_A and endothelin ET_B receptor subtypes, which are both present in the heart; the ET_A:ET_B ratio is approximately 85%:15% in adult rat ventricular cardiomyocytes (Thibault et al., 1995; Fareh et al., 1996). However, receptor number does not necessarily relate to the efficacy of a response: for example, although endothelin receptors are expressed more abundantly than angiotensin receptors in neonatal cardiomyocytes, endothelin-1 and angiotensin II have similar abilities to activate mitogen-activated protein kinase in these cells, indicating poorer coupling of endothelin receptors to activation of the signalling pathway (Booz and Baker, 1996). Although endothelin ET_B receptors are found less abundantly than endothelin ET_A receptors, stimulation of endothelin ET_A and ET_B receptors each accounted for approximately half of the hypertrophic response to endothelin-1 obtained in adult rat ventricular cardiomyocytes. This observation is in contrast to results obtained in neonatal cardiomyocytes in which the hypertrophic effect of endothelin-1 was completely inhibited by the selective antagonist at endothelin ET_A receptors, BQ123 (Ito et al., 1993; Ponicke et al., 1997). Indeed, Hilal-Davidan et al. (1997) have concluded that the endothelin ET_B receptor is not expressed in neonatal rat cardiomyocytes and that only endothelin ET_A receptors are present on these cells. However, the endothelin ET_B-selective agonist, endothelin-3, did initiate protein synthesis in neonatal cardiomyocytes (Tamamori et al., 1996), as in the present study. It has been proposed that endothelin-3, acting via endothelin ET_B receptors in neonatal cells, may stimulate production and release of endothelin-1 in an autocrine fashion, and that the secreted peptide may act via endothelin ET_A receptors to provide amplification of the response initiated by endothelin ET_B receptor stimulation (Tamamori et al., 1996). It is also important to note that endothelin ET_B receptor mRNA is upregulated in hypertrophied neonatal cardiomyocytes; this change has been attributed to the action of angiotensin II (Kanno et al., 1993). Endothelin ET_A and ET_B receptors have each been implicated also in the hypertrophic response initiated by endothelin-1 in adult rabbit ventricular cardiomyocytes on the basis of antagonism of the actions of the peptide by BQ123- and BQ788-selective antagonists at endothelin ET_A and ET_B receptors, respectively (Mullan et al., 1997). However, it is possible that the single high concentration (10^{-7} M) of each antagonist employed in that study might have exerted some antagonism of the reciprocal receptor. In addition, appreciable partial agonist activity was ascribed to BQ123 and to BQ788 in that model and it is possible that the effects of each compound cannot be attributed exclusively to selective antagonism at their respective receptors. Conclusive evidence has been provided in the present study regarding the concentration dependence of the selectivity of the compounds ABT-627 and A192621 for each receptor subtype. In addition, partial agonist activity was greatly reduced relative to that of BQ123 and BQ788 (Mullan and Bell, unpublished observation).

The phospholipase C-β signalling cascade has been implicated in induction of cardiomyocyte hypertrophy by endothelin-1: Sugden et al. (1993) reported a correlation between phosphatidyinositol turnover and protein synthesis in response to endothelin-1; evidence was obtained in our own laboratory that endothelin-1-mediated hypertrophy of adult rabbit ventricular cardiomyocytes was attenuated by pp56, a novel inositol 1,4,5 trisphosphate antagonist (Bell and McDermott, 1998) and by the selective inhibitor of protein kinase C, bisindolylmaleimide (Mullan et al., 1997). In the present study, bisindolylmaleimide attenuated the responses to endothelin ET_A and to ET_B receptor stimulation. These data indicate that both receptors are coupled to hypertrophy, at least in part, via the activation of the phospholipase C-β signalling cascade. However, this pathway cannot account for all of the response elicited by stimulation of either receptor since 48% of the response to endothelin ET_A receptor stimulation and 24% of the response to endothelin ET_B receptor stimulation remained in the presence of bisindolylmaleimide. Protein kinase C-ε is the predominant isoform of the enzyme activated in response to endothelin-1 both in adult (Bogoyevitch et al., 1993) and neonatal (Eskildsen-Helmond et al., 1997) rat ventricular cardiomyocytes although the translocation of protein kinase C-β to the perinuclear region in response to α_1-adrenoceptor activation indicates that this isoform is also important in growth responses (Long et al., 1992). Although bisindolylmaleimide, at the concentration employed in the present study, is a selective inhibitor of protein kinase C-α, β and γ in vivo and in vitro (Toullec et al., 1991), it's inhibitory action on other isoforms, including protein kinase C-δ and ε has not been firmly established. The activation of these isoforms in response to endothelin-1 may account for the component of the response insensitive to inhibition by bisinsolylmaleimide. The involvement of additional, nonprotein kinase C-dependent mechanisms cannot be discounted particularly in regard to the endothelin ET_A receptor subtype. Although both receptor subtypes have been linked to the same fundamental signalling pathway, namely the activation of protein kinase C, the selective recruitment of specific isoforms of this enzyme in response to the activation of a specific endothelin receptor subtype and subsequent translocation to discrete target regions within the cell or activation of the expression of a specific subset of genes, should be considered.

The normal plasma levels of endothelin-1, 10^{-13} M–10^{-12} M, (Ito et al., 1991) are lower than the concentrations at which endothelin-1 induced hypertrophy, 10^{-11} M–10^{-7} M, in the present study. However, endothelin-1 has been detected at concentrations of up to 10^{-11} M in the plasma of patients with myocardial infarction (Omland et al., 1994), diabetes (Takahashi et al., 1990), congestive heart failure and hypertension (Lerman et al., 1991), conditions associated with the development of left ventricular hypertrophy. It is also possible that local concentrations of the peptide in the vicinity of the cardiomyocytes may be significantly higher than those found in plasma. Indeed, cardiomyocytes themselves can secrete endothelin-1, and this release is enhanced in response to other hypertrophic stimuli including endothelin-3 (Tamamori et al., 1996), angiotensin II (Ponicke et al., 1997; Herizi et al., 1998). In neonatal cells, secretion of endothelin-1 has been demonstrated in response to the mechanical stretch that occurs as a consequence of pressure overload (Yamazaki et al., 1996) although release of the peptide from adult cells in response to mechanical stretch has not been established (Schluter and Piper, 1999). Cardiac fibroblasts provide an additional source of intra-cardiac ET-1 (Harada et al., 1997; Gray et al., 1998). It is very probable, therefore, that the direct hypertrophic effect of endothelin-1 observed in the present study would also occur in vivo in response to sustained hypertension.

The findings of the present study support the involvement of endothelin ET_B receptors, in addition to endothelin ET_A receptors, in the hypertrophic response to endothelin-1. It is possible that the contribution of endothelin ET_B receptors would assume an even greater significance in man since endothelin ET_B receptors account for up to 40% of the total endothelin receptor population present in human ventricular myocardium (Molenaar et al., 1993) compared to less than 20% in rat myocardium. Endothelin ET_A receptor antagonism, but not endothelin ET_B receptor antagonism, partially attenuates the hypertrophic response induced by mechanical stretch in neonatal cardiomyocytes in vitro (Yamazaki et al., 1996), decreases the ratio of left ventricular weight to body weight and the area of cardiomyocytes isolated from the left ventricle of rats with pressure overload induced by aortic banding (Ito et al., 1993) and attenuates cardiomyocyte hypertrophy in renovascular hypertensive rats (Ehmke et al., 1999). Chronic intervention with endothelin receptor antagonists significantly attenuates hypertension only in those circumstances where marked overexpression of endothelin-1 in blood vessel walls is clearly demonstrable. For this reason, such treatment is particularly effective in more malignant models of hypertension, for example, the stroke-prone or deoxycorticosterone acetate-salt treated spontaneously hypertensive rat in which significant enhancement of prepro-endothelin-1 mRNA is evident (Nishikibe et al., 1993; Okada et al., 1994). The failure of some experimental models of hypertension to respond to treatment with mixed endothelin $ET_{A/B}$ receptor antagonists may reflect the unwanted effect of blockade of endothelin ET_B receptor-mediated vasodilatation (Schriffin, 1999; Schriffen et al., 1997). There is evidence, however, to indicate that the mixed endothelin $ET_{A/B}$ receptor antagonist, bosentan, causes some regression of left ventricular hypertrophy in the absence of an appreciable reduction in blood pressure (Karam et al., 1996); these data provide further evidence to support the conclusion that endothelin-1 exerts a local autocrine/paracrine influence on cardiomyocyte hypertro-

phy independently of the peptide's pressor effects upon the systemic vasculature. For this reason, further investigation of the therapeutic potential of endothelin ET_B receptor antagonists in prevention and regression of left ventricular hypertrophy in man is warranted. The limitations associated with this approach as a consequence of concurrent inhibition of endothelin ET_B receptor-mediated vasodilatation and the resultant increase in the mechanical loading of the myocardium could potentially be overcome by the coadministration of a direct acting vasodilator drug, such as hydralazine, alongside an endothelin ET_B receptor antagonist.

Acknowledgements

This work was supported by a grant from the British Heart Foundation (FS:95063). The authors express their thanks to Dr. J. Wessale and colleagues (Abbott Laboratories) for the generous gift of the endothelin receptor antagonists ABT-627 and A192621.

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