A low-affinity, low-molecular-mass endothelin-A receptor in neonatal rat heart

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Endothelin receptors with endothelin-A (ETa) specificity were present in neonatal rat ventricle. However, in both receptor-binding studies and studies of inositol phosphate accumulation, these receptors had lower affinity for endothelin-1 than ETa receptors on isolated neonatal cardiomyocytes or adult left atria. Receptors in the three myocardial preparations were cross-linked to ¹²⁵I-endothelin-1 and their molecular masses measured using SDS/PAGE. Receptors on left atria and neonatal cardio-

myocytes had the expected molecular mass of 48 kDa, whereas the receptors in neonatal ventricle were smaller (38 kDa). Despite this, neonatal ventricles contained ETa receptor mRNA which was not different in size from that in the isolated cells (4.5 kb). Thus the 38 kDa ETa receptor present in neonatal ventricle appears to be transcribed from full-length ETa receptor mRNA and is possibly formed by processing of the 48 kDa receptor.

INTRODUCTION

Endothelin (ET) is a newly described peptide present in the vascular endothelium [1]. It was described initially as a potent vasoconstrictor but has since been shown to initiate a range of different responses including steroidogenesis [2], mitogenesis [3] and fluid transport [4] as well as contractility changes. In addition to the originally described peptide, now termed ET-1, genes for two other endothelin peptides are present in the mammalian genome [5]. The corresponding two peptides, ET-2 and ET-3, have been synthesized and used to characterize ET receptors in different mammalian tissues. Such studies have shown that there are at least two different ET receptors one of which, the endothelin A (ETa) receptor, is selective for ET-1 and ET-2 while the other receptor endothelin B (ETb) does not differentiate between the three peptides [6]. Both of these receptor types are coupled to the stimulation of the phosphatidylinositol (PtdIns) turnover pathway which is probably involved in mediating many of the actions of ET. This pathway involves the release of inositol (1,4,5)-trisphosphate [$\text{Ins}(1,4,5)\text{P}_3$] and *sn*-1,2-diacylglycerol. These two arms of the pathway act in concert by raising cytosolic Ca^{2+} and activating protein kinase C [7].

Both the ETa and ETb receptors have been cloned and shown to be members of the family of receptors which couple to GTP-binding proteins [8,9]. These proteins are highly conserved, all have seven transmembrane-spanning regions and all have predicted molecular masses within the 40–50 kDa range. Despite the fact that multiple genes have not been reported for either the ETa or ETb receptor type, the reported properties of ET receptors appear to vary considerably in terms both of affinity and observed molecular mass. Quoted affinities for ET-1 at ETa receptors vary from 0.1 nM [10] to 2 nM [11] and molecular masses have been reported from 48 kDa [12] to as high as 97 kDa [13]. Some of these differences probably reflect differences in the degree of glycosylation but some degree of receptor heterogeneity also seems likely.

In addition to constrictor effects on the coronary vessels, direct cardiac actions of ET have been reported, including positive

inotropic and chronotropic responses [14], release of atrial natriuretic peptide [15] and initiation of hypertrophy [16]. However, the importance of ET in the control of cardiac function under physiological conditions is not well established. ET is a potent stimulator of release of atrial natriuretic peptide and of the hypertrophic response in isolated neonatal cardiomyocytes [17] but has very low potency when measured in inotropic response studies involving intact neonatal heart tissue [18]. The potency of ET is higher when inotropic responses are measured in adult atria [18]. Such differences might reflect different properties of the receptors in the different preparations. To address this question, we have made a detailed study of ETa receptors present in three different myocardial preparations, adult left atria, intact neonatal heart and isolated neonatal cardiomyocytes. Our findings demonstrate that the receptor present in neonatal heart has a lower molecular mass and a lower affinity for ET-1 than receptors described in other tissues.

MATERIALS AND METHODS

Materials

¹²⁵I-ET-1 and [α -³²P]dATP were supplied by DuPont (Melbourne, Australia). [³H]Inositol was supplied by Amersham (Amersham, Bucks., U.K.). Non-radioactive ET-1 and ET-3 were supplied by Auspep (Melbourne, Australia) and by the Peptide Institute (Osaka, Japan). Bis(sulphosuccinimidyl) suberate (BS³) was obtained from Pierce (Rockford, IL, U.S.A.). Cell culture media and enzymes were supplied by the Commonwealth Serum Laboratories (Parkville, Australia). All other chemicals were AnalaR grade and solutions were prepared using highly purified 'Milli Q' water.

Preparation of neonatal cardiomyocytes

Cardiomyocytes were prepared from 1–4-day-old rats essentially as described elsewhere [19]. Following removal of the atria, great vessels and pericardium, tissue was repeatedly digested in trypsin (0.05 %) containing 0.01 % deoxyribonuclease. Cells were har-

Abbreviations used: ET, endothelin; ETa and ETb, endothelin A and B receptors; PtdIns, phosphatidylinositol; $\text{Ins}(1,4,5)\text{P}_3$, inositol-1,4,5-trisphosphate; BS³, bis(sulphosuccinimidyl) suberate; HBSS, Hepes-buffered salt solution; PMSF, phenylmethanesulphonyl fluoride.

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vested by low-speed centrifugation and resuspended in medium 199 containing 10% fetal calf serum, 2% chick embryo extract and 100 units/ml each of penicillin and streptomycin. The cell preparation was then subjected to preplating for 30 min to remove any fibroblasts present. Bromodeoxyuridine (10 mM) was then added to inhibit fibroblast growth and the cells were plated on to wells in 24-well dishes at a density of 700 cells/mm². Bromodeoxyuridine was deleted from the medium after 2 days in culture. The yield of viable cells averaged 1×10^6 per neonatal heart.

Inositol phosphate generation in isolated neonatal cardiomyocytes

Two days before the experiment, medium was replaced with medium containing 2% BSA and antibiotics. Inositol phospholipids were labelled by incubating with [³H]inositol (10 μ Ci/ml) for 48 h. After the labelling period, cells were washed with non-radioactive medium and subsequently stimulated with ET or noradrenaline in the presence of LiCl (10 mM) for 20 min. Release of inositol phosphates was terminated by adding ice-cold 5% trichloroacetic acid containing 5 mM EDTA and 1 mM phytic acid. The phytic acid was added to protect the labelled inositol phosphates [20]. After centrifugation to remove trichloroacetic acid-insoluble material, excess trichloroacetic acid was removed with freon/tri-*N*-octylamine as described elsewhere [21]. For quantification of inositol phosphates, samples were loaded on 1 ml columns of Dowex-1 anion-exchange resin in the formate form. Inositol phosphates were eluted with ammonium formate/formic acid as described elsewhere [22]. Anion-exchange h.p.l.c. analysis verified the nature of the labelled products as isomers of InsP_1 , InsP_2 and InsP_3 [23].

Inositol phosphate generation in intact neonatal rat hearts

Rats (1–4 days of age) were killed by decapitation. Hearts were removed immediately and transferred to a siliconized organ bath containing Hepes-buffered Krebs' medium containing 0.2% BSA, constantly gassed with O₂/CO₂ (19:1, v/v) at 37 °C. After removal of blood, hearts were incubated in medium containing [³H]inositol (10 μ Ci/ml) for 4 h. After the labelling time, hearts were washed in non-radioactive medium and subsequently stimulated with ET or noradrenaline for 20 min. Inositol phosphate release was terminated by dropping the hearts into liquid N₂. Frozen tissue was extracted with trichloroacetic acid and inositol phosphates quantified as described for the isolated cell experiments.

¹²⁵I-ET-1 binding to intact neonatal cardiomyocytes

¹²⁵I-labelled ET-1 binding was performed essentially as described by Martin et al. [24]. Cells were incubated with ET-1 or ET-3 (10 pM to 0.1 μ M) together with ¹²⁵I-ET-1 (10000 c.p.m./well) in Hepes-buffered salts solution (HBSS). This salts solution contained in mM: NaCl, 137; KCl, 5.4; Na₂HPO₄, 0.33; MgSO₄, 0.4; MgCl₂, 0.5; CaCl₂, 1.25; NaHCO₃, 4; Hepes, 40 (pH 7.4); as well as BSA, 0.2% and penicillin, 100 units/ml. Cells were incubated for 18 h at 4 °C on an orbital shaker rotating at 100 rev./min. After washing, cells were dissolved off the wells using 20 mM Hepes, 10% glycerol, 1% Triton, pH 7.4, and counted in a γ counter at 70% efficiency.

¹²⁵I-ET-1 binding to membranes from neonatal rat hearts or adult left atria

Hearts from 1–4-day-old rats or left atria from adult rats were homogenized in ice-cold HBSS using a Potter–Elvehjem homo-

genizer. The homogenate was centrifuged at 5000 *g* for 5 min and the supernatant at 30000 *g* for 30 min. The final pellet was washed twice with HBSS and filtered before use in receptor binding assays. Incubates contained heart membranes (150–200 μ g of protein) together with ET-1 or ET-3 (10 pM to 0.1 μ M) plus ¹²⁵I-ET-1 (10000 c.p.m.) and soybean trypsin inhibitor (1 mg/ml) in polypropylene tubes. Tubes were capped and incubated for 18 h at 4 °C on an orbital shaker rotating at 100 rev./min. Bound ligand was separated by vacuum filtration through glass-fibre filters (Whatman GF/C) coated with 4% BSA, followed by washing with 10 ml of HBSS. Filters were counted in a γ counter.

Analysis of binding data

All binding data were analysed using LIGAND to provide statistically based estimates of receptor concentration and affinity. A single affinity binding site was assumed unless a two-site model provided a statistically better fit for the data.

Cross-linking of ¹²⁵I-ET-1 to receptors on intact heart membranes or on isolated cardiomyocytes

Binding of ¹²⁵I-ET-1 to receptors on isolated cells or on membranes prepared from intact tissue was performed as described above except that the ¹²⁵I-ET-1 concentration was 0.4 nM. To ensure specificity of the binding, parallel experiments were performed which contained 0.1 μ M ET-1 or ET-3. At this concentration ET-1 competes for binding but ET-3 does not.

After washing, bound ¹²⁵I-ET-1 was covalently bound to sites on the isolated cells in HBSS buffer (0.25 ml/well) using the membrane-impermeable homobifunctional cross-linking reagent BS³ at a final concentration of 0.2 mM [25]. After 10 min at 22 °C, the reaction was terminated by making the samples 35 mM in Tris buffer, pH 7.4, for a further 20 min. Following aspiration of the reaction buffer, cells from two wells (80–100 μ g of protein) were solubilized with a total of 0.25 ml of 1% Triton X-100 in HBSS containing 0.2 mM phenylmethanesulphonyl fluoride (PMSF) and 0.1 mg/ml soybean trypsin inhibitor.

¹²⁵I-ET-1 was cross-linked to receptors on membranes prepared from intact neonatal heart (0.8–1.0 mg of protein) or left atria (200–300 μ g of protein) using similar methods, except that membranes bound to ¹²⁵I-ET-1 were washed four times by centrifugation (1 min, 15000 *g*, 4 °C) and resuspended in 0.25 ml of HBSS buffer before addition of BS³ to 0.2 mM. After cross-linking for 10 min at 22 °C and quenching with 35 mM Tris, pH 7.4, for a further 20 min, the membranes were solubilized and analysed exactly as described for the cardiomyocytes.

Samples were analysed by SDS/5–20% exponential gradient polyacrylamide gel electrophoresis under reducing conditions as described elsewhere [25,26].

Estimation of the size of the ETa receptor mRNA

Total RNA was isolated from neonatal rat cardiomyocytes, intact neonatal hearts and adult atria by homogenizing in 4 M guanidine isothiocyanate using a 'Polytron' homogenizer at half maximum speed for 15 s, followed by centrifugation through 5.7 M CsCl [27]. Oligo(dT)–cellulose chromatography was used to isolate poly(A)⁺ RNA where required [28]. The RNA was size-fractionated by agarose gel electrophoresis in the presence of formaldehyde [28] and transferred to a Zeta Probe GT nylon membrane (Bio-Rad) by capillary transfer using 5 mM NaOH as the transfer solvent.

A cDNA clone for the rat ETa receptor was provided by Dr.

S. Nakanishi [29]. The 1055 bp *Hpa*I fragment from this pRIA9 clone was isolated using NA45 paper (Schleicher and Schull). This fragment was labelled by random primer synthesis using [α^{32} P]dATP (DuPont) and denatured with 0.4 M NaOH. Hybridization was carried out in 50 % freshly deionized formaldehyde, 0.5 % Blotto (Diplomat non-fat milk powder), 1 % polyethylene glycol, 8 % SDS and 2 $\times$ SSPE (SSPE: 0.15 M NaCl, 10 mM sodium phosphate, 1 mM EDTA, pH 7.4) at 50 °C overnight. The filters were washed twice at room temperature in 2 $\times$ SSC (SSC: 0.15 M NaCl, 0.015 M sodium citrate)/0.1 % SDS for 10 min followed by a single wash at 50 °C in 0.2 $\times$ SSC/1 % SDS for a further 10 min. Autoradiography was carried out at -70 °C for between 48 and 65 h using Kodak XAR-5 film.

RESULTS

Effects of ET peptides on inositol phosphate accumulation in intact neonatal rat hearts

Effects of ET-1 and ET-3 on the accumulation of inositol

Figure 1 Inositol phosphate accumulation in intact neonatal rat hearts stimulated with ET-1 (●) or ET-3 (○)

Hearts were labelled with [3 H]inositol and subsequently stimulated with the indicated concentration of ET peptide for 20 min in the presence of 10 mM LiCl. Inositol phosphates were extracted and quantified as described in the Materials and methods section. Values shown are c.p.m./mg of tissue; mean $\pm$ S.E.M. of values obtained from four different hearts.

Figure 2 Inositol phosphate accumulation in isolated neonatal cardiomyocytes stimulated with ET-1 (●) and ET-3 (○)

Cells were labelled with [3 H]inositol and subsequently stimulated with peptide for 20 min in the presence of 10 mM LiCl. Inositol phosphates were extracted and quantified as described in the Materials and methods section. Results shown are c.p.m./well of cells; mean $\pm$ S.E.M. of four different experiments.

phosphates were investigated in intact neonatal rat hearts. [3 H]Inositol-labelled hearts were stimulated with peptide in the presence of LiCl for 20 min. Inositol phosphates were extracted and quantified. Both peptides caused a stimulation of inositol phosphate accumulation but were effective only at high concentrations. The response to ET-1 had not reached plateau level at 1 μ M. The response to ET-3 was lower than that to ET-1 at all concentrations, but since plateau levels were not reached with either peptide, it was not possible to estimate relative potencies (Figure 1).

ET-1 had higher potency in stimulating inositol phosphate accumulation when studies were performed using isolated, adult rat atria [30].

Effects of ET peptides on inositol phosphate accumulation in isolated neonatal cardiomyocytes

The effects of ET-1 and ET-3 on the accumulation of inositol phosphates were examined in isolated neonatal cardiomyocytes in culture. [3 H]Inositol-labelled cells were incubated with ET-1 or ET-3 for 20 min in the presence of LiCl and the accumulated inositol phosphates extracted and quantified. Both peptides produced a stimulation of inositol phosphate accumulation. The potency of ET-3 was low and plateau levels were not reached at 1 μ M, similar to the response observed in intact hearts. In contrast, ET-1 produced a potent stimulation of inositol phosphate accumulation in cardiomyocytes with an average EC_{50} value of 3.2 ± 0.5 nM (mean $\pm$ S.E.M., $n = 4$) (Figure 2).

Effects of noradrenaline on inositol phosphate accumulation in intact hearts and isolated cells

The observed difference between responses in intact tissue and in isolated cells suggested either altered properties of the ET receptors or some alteration in the responsiveness of the PtdIns turnover pathway. To discriminate between these possibilities, studies were performed using noradrenaline which stimulates cardiac PtdIns turnover through α_1 -adrenoceptors. [3 H]Inositol-labelled neonatal hearts were stimulated with noradrenaline in the presence of propranolol and LiCl for 20 min and the inositol phosphates extracted and quantified. Noradrenaline produced a similar maximal stimulation of inositol phosphate accumulation to that observed with ET-1. The EC_{50} value calculated from a dose-response curve constructed from the average values from four hearts at each noradrenaline concentration was 0.125 μ M.

Addition of noradrenaline to [3 H]inositol-labelled cardiomyocytes also caused a stimulation of inositol phosphate accumulation and the maximum stimulation reached was similar to that with ET-1. However, unlike the response to ET-1, the potency of noradrenaline was similar in intact tissue and in isolated cells ($EC_{50} = 0.19 \pm 0.02$ μ M; mean $\pm$ S.E.M., $n = 3$) (Figure 3).

Binding of ET peptides to receptors on cells and intact tissue

The differences in the inositol phosphate responses to ET-1 in intact neonatal hearts and in myocytes suggested some differences in the properties of the ET receptors. Accordingly, experiments were performed to examine the binding affinities of ET-1 at receptors in the different myocardial preparations.

Membranes were prepared from neonatal hearts and the binding of 125 I-ET-1 examined using increasing concentrations of the unlabelled peptide as described in the Materials and methods section. ET-1 bound to a single class of sites with an average affinity of 3.9 ± 0.7 nM (mean $\pm$ S.E.M., $n = 4$) and a capacity

Figure 3 Inositol phosphate accumulation in intact neonatal rat hearts (lower panels) and isolated neonatal cardiomyocytes (upper panels) stimulated with noradrenaline

Hearts or cells were labelled with [3 H]inositol and subsequently stimulated with noradrenaline for 20 min in the presence of 10 mM LiCl. Inositol phosphates were extracted and quantified as described in the Materials and methods section. Results shown are c.p.m./well of cells or c.p.m./mg of tissue; mean $\pm$ S.E.M. of three experiments.

of 487 ± 182 fmol/mg of protein (Figure 4). ET-3 did not compete for [125 I]-ET-1 binding at concentrations below $0.1 \mu\text{M}$.

[125 I]-ET-1 also bound to a single class of receptors on isolated neonatal cardiomyocytes (Figure 5). The receptor concentration averaged 346 ± 18 fmol/mg of protein and the affinity of the sites was 72 ± 16 pM (mean $\pm$ S.E.M., $n = 3$). ET-3 did not compete for binding at concentrations below $0.1 \mu\text{M}$, showing that the bulk of the receptors present on the isolated cells, like those in intact tissue, were of the ETa class. Binding of ET-1 was also examined in membranes prepared from isolated cardiomyocytes after scraping from the culture dishes. The binding affinity of ET-1 was 368 ± 30 pM (mean $\pm$ S.E.M., $n = 3$), lower than the affinity measured on intact cells but higher than that of the receptor on the intact tissue. The affinity of receptors present on membranes prepared from adult left atria was similar to values obtained using membranes prepared from isolated cells (440 ± 33 pM; mean $\pm$ S.E.M., $n = 3$). However, the receptor concentration was higher (1257 ± 424 fmol/mg of protein).

To ensure that the observed difference between binding profiles in membranes from intact neonatal heart and in cell preparations was not due to degradation of the receptor on the intact tissue during preparation of the membranes, cells were incubated with homogenate of intact neonatal rat heart (approx. $300 \mu\text{g}$ of protein/well) for 3 h at 37°C and their binding affinity assessed subsequently. Such treatment did not alter the binding affinity or capacity of the receptors present on isolated cardiomyocytes. In addition, experiments were performed in which protease inhibitors (0.1 mM PMSF and 1 mg/ml soybean trypsin inhibitor)

were included during membrane preparation. Inclusion of protease inhibitors did not alter the affinities of the receptors on any of the membrane preparations.

The possibility that different degrees of glycosylation were responsible for the different properties of the receptors was examined. N-glycosidase-F has been used to remove N-linked glycosyl residues from β -adrenoceptors [31]. Neonatal cardiomyocytes were incubated with N-glycosidase-F (0.2 units/ml) for up to 3 h at 37°C . No effect on receptor affinity was observed, indicating that N-linked glycosyl residues were unlikely to be responsible for the observed differences in receptor affinity.

Molecular mass of receptors in intact heart and isolated cardiomyocytes

The different binding properties of the receptors in the three different preparations suggest some difference in the ET receptors themselves. ET receptors in the three preparations were cross-linked with [125 I]-ET-1 and the molecular masses analysed using gradient SDS/PAGE, as described in the Materials and methods section. ETa receptors were identified by running parallel experiments containing $0.1 \mu\text{M}$ ET-1 and ET-3. At this concentration, unlabelled ET-1 competes for [125 I]-ET-1 binding but unlabelled ET-3 does not. Results of typical experiments are shown in Figure 6.

When [125 I]-ET-1 was cross-linked to cultured cardiomyocytes, a major cross-linked species was observed with an apparent molecular mass of 48 kDa under reducing conditions. The

Figure 4 Binding of ET peptides to membranes prepared from intact neonatal rat hearts

Membranes were incubated with ^{125}I -ET-1 (10000 c.p.m.) together with increasing concentrations of non-radioactive ET-1 (●) or ET-3 (○). Values shown are mean $\pm$ S.E.M. of four experiments.

Figure 5 Binding of ET peptides to isolated neonatal cardiomyocytes

Cells or membranes were incubated with ^{125}I -ET-1 (10000 c.p.m.) together with the indicated concentration of non-radioactive ET-1 or ET-3. Values shown are mean $\pm$ S.E.M. of three experiments. Key to symbols: ●, ET-1 binding to intact cells; □, ET-3 to intact cells; ▽, ET-1 binding to membranes prepared from isolated cells; ○, ET-1 binding to membranes prepared from adult left atria.

formation of this species was strongly inhibited by unlabelled ET-1 (Figure 6, lane 2) but was unaffected by the same concentration of unlabelled ET-3 (Figure 6, lane 3), indicating that

Figure 6 Autoradiograms of ^{125}I -ET-1 cross-linked with BS^3 to receptors on isolated neonatal cardiomyocytes (a), membranes prepared from intact neonatal hearts (b) and left atrial membranes (c)

Cross-linked proteins were analysed on SDS/5–20% exponential gradient polyacrylamide gels under reducing conditions. Binding of ^{125}I -ET-1 (0.4 nM) was performed without unlabelled ligand (lanes 1, 4 and 7) or in the presence of 0.1 μM ET-1 (lanes 2, 5 and 8) or ET-3 (lanes 3, 6 and 9) before addition of BS^3 . The major cross-linked species involving ^{125}I -ET-1, which satisfies the criteria for an ETa receptor, is indicated by the open triangle. Molecular-mass markers (arrows) in order of decreasing molecular mass are myosin (200 kDa), β -galactosidase (130 kDa), phosphorylase B (94 kDa), BSA (68 kDa), ovalbumin (43 kDa), carbonic anhydrase (29 kDa), soybean trypsin inhibitor (21 kDa) and lysozyme (14.4 kDa). The experiment was performed three times with cardiomyocytes and adult atria and five times with neonatal cardiac membrane preparations.

this band corresponds to ^{125}I -ET-1 bound to an ETa receptor. Cross-linking of ^{125}I -ET-1 to membranes prepared from adult left atria revealed a similar profile of labelled species. The major cross-linked species had an apparent molecular mass, under reducing conditions, of 48 kDa (Figure 6, lane 7). Labelling was inhibited by ET-1 but not by ET-3 (Figure 6, lanes 8 and 9), demonstrating it to represent an ETa receptor. Labelled material with higher molecular mass was apparent also and probably represents glycosylated ETa receptors. The ETa receptor has two N-glycosylation sites on the N-terminal extracellular tail [8].

Cross-linking of ^{125}I -ET-1 to membranes prepared from intact neonatal hearts revealed a different pattern. In contrast with the profiles obtained from cardiomyocytes and adult atria, there was no species with appropriate specificity present at 48 kDa (Figure 6, lane 4). Instead, one major band with an apparent molecular mass of 38 kDa under reducing conditions was diminished in the presence of excess ET-1 but not ET-3 (Figure 6, lanes 5 and 6). Furthermore, treatment of the ^{125}I -ET-1–receptor complex with acid (0.2 M acetic acid, 0.5 M NaCl) prior to cross-linking dissociated the ligand from the 38 kDa protein. This procedure dissociates ET-1 from ETa receptors but not from ETb receptors [12].

Estimation of the size of the ETa receptor mRNA

One possible explanation for the low molecular mass of the ETa receptor in intact neonatal heart is that it is the product of an mRNA species in which part of the coding sequence has been deleted as a result of alternate gene splicing. To investigate this possibility, the molecular mass of the ETa receptor mRNA in neonatal rat heart was measured and compared with molecular masses of the mRNA in cardiomyocytes. RNA was extracted and subjected to Northern-blot analysis as described in the Materials and methods section. In some experiments, poly(A)⁺ RNA was separated from the total RNA. Profiles obtained with poly(A)⁺ RNA were not different from those obtained using total RNA and subsequent experiments used total RNA. ETa receptor mRNA in the two preparations showed similar profiles with the

Figure 7 Northern-blot analysis of RNA isolated from neonatal rat cardiomyocytes and intact neonatal heart and adult left atria

RNA blot hybridization analysis (10 µg of total RNA per lane) was carried out as described in the Materials and methods section using the 1055 bp *Hpa*I fragment excised from the pRIA9 clone as probe. The experiment was performed twice with poly(A)⁺RNA and three times with total RNA. Lane A, neonatal heart; lane B, isolated cardiomyocytes.

major labelled band corresponding to 4.4 kb (Figure 7). This value is similar to estimates reported in adult rat heart as well as in other tissues [8,11,29,32].

DISCUSSION

Neonatal rat ventricles contain a novel form of the ETa receptor which has a low affinity for ET-1. Low affinity was evident both in receptor-binding studies and in studies of inositol phosphate release. Furthermore, unusually low potency of ET-1 in eliciting an inotropic response in neonatal heart has been reported previously [18]. Compared with receptors in neonatal ventricles, receptors present both in adult left atria and in isolated neonatal cardiomyocytes had higher affinities for ET-1 and higher molecular masses. This suggests a relationship between the low molecular mass of the receptor on neonatal ventricle and its low affinity for ET-1.

The receptor on adult atria resembled the receptor present on the isolated cells rather than the receptors present on intact neonatal tissue, even though the methods used for the study of neonatal hearts and adult atria were identical. This eliminates procedural differences as causing apparent differences in receptor properties. Furthermore, ETa receptors derived from neonatal heart showed unusual properties in intact tissue preparations, in membrane binding studies and in cross-linking experiments, which argues against such trivial explanations as differences in [³H]inositol-labelling and poor access of ET-1 to its receptor in intact tissue preparations. The possibility that other cell types present in the intact hearts are the source of ET receptors can be eliminated because the profile of inositol phosphate release in intact heart is different from that in other tissues [30,33] and the metabolism of inositol phosphates following ET stimulation had the characteristics of the cardiac pathway. The possibility of contamination of the isolated cardiomyocyte preparations was eliminated by the isolation procedure and, as shown previously, by investigating the specificity of the stimulation [2].

ETa receptors have been cloned from a number of different tissues obtained from rat, bovine and human sources [8,11]. The cloned receptors are highly homologous members of the G-protein-associated class of receptors characterized by seven transmembrane-spanning regions. The predicted molecular mass on the basis of DNA sequence data is 48 kDa, similar to that of

the receptor on adult atria and on isolated neonatal cells and larger than the molecular mass of the receptor present in intact neonatal tissue. Thus the receptor present on neonatal ventricle is smaller than predicted from the sequence of the ETa receptor gene. This might suggest that the neonatal receptor is the product of a different gene. However, two factors argue against this. First, to date, only one ETa receptor gene has been identified in mammalian tissues [32] and secondly, mRNA derived from the ETa receptor gene was clearly present in neonatal heart. The possibility of alternate gene splicing was discounted by the finding that the ETa receptor mRNA in neonatal rat heart was similar in size to the mRNA in isolated cardiomyocytes and similar to the molecular mass reported previously in adult rat heart [29]. Thus the neonatal ETa receptors appear to be transcribed from full-length ETa mRNA. This means that the most likely explanation for the observed low molecular mass is that the receptors are formed by proteolytic processing of the 48 kDa receptor. However, the data argue that the low-molecular-mass form of the ETa receptor present in neonatal heart is unlikely to be the product of non-specific proteolysis occurring during the preparation of membranes for the following reasons. First, the 38 kDa receptor was observed only in neonatal tissue even though identical methods were used for the preparation of membranes from adult tissue. Secondly, the receptor on neonatal heart had low affinity for ET-1 when measured in intact tissue preparations (inositol phosphate studies and inotropic studies [18]), as well as when measured in membranes. Thirdly, incubation of isolated cardiomyocytes with homogenate of neonatal heart did not generate the low-affinity form of the receptor. Fourthly, inclusion of protease inhibitors during membrane preparation did not alter the properties of the receptors. Thus, if the 38 kDa ETa receptor is formed by proteolytic cleavage of the 48 kDa form, then this is likely to be a specific process which occurs following synthesis of the receptor.

The formation of a low-molecular-mass (32 kDa) form of the ETb receptor has been reported previously, in a number of different tissues. Unlike the low-molecular-mass form of the ETa receptor, the small ETb receptor has similar affinity to the native receptor molecule and appears to be formed by non-specific proteolysis [34,35]. Sensitivity to such proteolysis is a feature of the ETb receptor and similar low-molecular-mass forms are not formed from the ETa receptor under similar conditions [12]. There are a number of reasons why the 38 kDa receptor in neonatal heart is unlikely to be derived from an ETb receptor. First, the receptor had ETa specificity in all studies involving both intact tissue and membrane preparations. Receptors on intact tissue preparations would not be expected to be accessible to proteases. Secondly, the 38 kDa receptor had low affinity for ET-1 whereas the low-molecular-mass form of the ETb receptor has similar affinity to the native 48 kDa receptor. Thirdly, no cross-linked species with a molecular mass of 48 kDa was present in neonatal heart membranes, indicating a loss of this form of the ETa receptor. The ETb receptor content of heart tissue has been reported to be less than 10% of the content of ETa receptors [36,37]. Fourthly, the ¹²⁵I-ET-1-receptor complex was sensitive to acid treatment whereas ETb receptors are insensitive [12].

There are a number of reports of ETa receptors with molecular masses in the vicinity of 48 kDa as measured in cross-linking studies [38,39]. Some studies have reported higher molecular masses [13], possibly due to dimerization or to glycosylation at one or both of the N-glycosylation sites present on the N-terminal tail [40]. To date, ETa receptors with molecular masses as low as 38 kDa have not been reported. This might mean that the low-molecular-mass form of the receptor is present only in neonatal rat heart. However, given the low affinity of the 38 kDa

form of the receptor, it would be unlikely to be detected in cross-linking studies in the presence of the high-affinity form. Two different forms of ETa receptor mRNA have been identified in a number of tissues. In rat, mRNA species of 5.2 kb and 4.2 kb have been reported in a number of different tissues [29]. Monkey testis contained two species of 4.2 and 3.3 kb but other monkey tissues contained only the larger species [11]. Several bovine tissues also contained the 4.2 and 3.3 kb species [8] but human tissues contained only one species of 4.3 kb [32] except for placenta which contained a larger species [11]. The reason for the multiple bands in some preparations is not clear but they do not appear to relate to the different forms of the ETa receptor described here. The ETa receptor is unusual among the G-protein-coupled receptors in having an apparently hydrophobic leader sequence which is not present in most receptors of this class and more importantly, an unusually long N-terminal tail extending extracellularly [8,11,40]. While most of the ETa receptor sequence is highly conserved, this N-terminal sequence shows considerable diversity between different species [41]. Given that the binding profiles of the receptors from different species are similar, the N-terminal region is unlikely to be a major ET-binding region. The functional significance of the N-terminal tail is not obvious and it has been reported to be deleted under some (undefined) circumstances [11]. While direct evidence is lacking, it is possible that deletion of most of the N-terminal tail is the basis for the observed difference between the two forms of the rat cardiac ETa receptor. Deletion of most of the N-terminal tail, a total of 80 residues would account for the difference in molecular mass of the two forms.

Our studies show that the properties of the cardiac ETa receptors change as the rats mature and this explains previously reported differences in ET-1 potency in rats of different ages [18]. The finding that isolated, cultured neonatal cardiomyocytes contain the 48 kDa form, apparently exclusively, suggests a loss of specific processing. We have previously reported that the isolation of neonatal cardiomyocytes causes a change in the pattern of inositol phosphate metabolism [2]. The loss of the 38 kDa neonatal ETa receptor may represent a related de-differentiation phenomenon. ET has been suggested as a potentially damaging agent for the myocardium by virtue of its ability to induce arrhythmias following Ca^{2+} release from intracellular stores [42] and by its hypertrophic action [43]. ET-1 can be released from the coronary endothelium but, to date, there is little evidence for direct cardiac actions of ET under physiological conditions, although it may contribute to ischaemic damage [44]. The change to a low-affinity form of the ETa receptor might protect the heart from ET-induced damage but it is not clear why such a mechanism should apply only to the neonatal animal.

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