Endocardial localization and characterization of natriuretic peptide binding sites in human fetal and adult heart

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Specific, high affinity binding sites for $^{125}$I-human-α-atrial natriuretic peptide-(1-28) ($^{125}$I-hANP-(1-28)) were identified in human fetal and adult heart and the binding characterized using quantitative in vitro autoradiography. Binding sites were localized to atrial and ventricular endocardium, aorta, pulmonary arteries and epicardial mesothelium. Kinetic studies indicated a $K_d$ value of 32 pM for ventricular endocardial $^{125}$I-hANP-(1-28) binding. The binding was completely inhibited by an excess (1 μM) of unlabelled hANP-(1-28), human brain natriuretic peptide-(1-32) (hBNP-(1-32)) and by the ‘clearance receptor’ specific ring-deleted analogue, C-ANP-(4-23). Competitive inhibition studies indicated a relative inhibitory potency for hBNP-(1-32) and C-ANP-(4-23) of 6% and 3% respectively. The data suggest that a distinct natriuretic peptide receptor subtype is expressed in the endocardium and in addition to a possible clearance function, may represent a site for feedback regulation and peptide interaction.

Autoradiography; Heart; Endocardium; Natriuretic receptors; Natriuretic peptides

1. Introduction

Atrial natriuretic peptide (αANP-(1-28)) is a 28-amino acid peptide involved in the regulation of body fluid and electrolyte balance (Needleman and Greenwald, 1986; Ballerman and Brenner, 1986). Two other related peptides have been identified which display similar biological effects to ANP, the distinct brain natriuretic peptide (BNP-(1-32)), isolated from human heart (Hino et al., 1990) and C-type natriuretic peptide (CNP), isolated from porcine brain (Sudoh et al., 1990). Both display significant sequence homology with αANP-(1-28) and appear, like ANP, to elicit their effects via interaction with specific binding sites. Despite the distinct sequences of ANP, BNP and CNP, these peptides have also been shown to share binding sites on a number of target tissues, including the heart (Oehlenschlager et al., 1989).

At least three distinct ANP receptor subtypes have been identified using molecular cloning and affinity cross-linking techniques (Yip et al., 1985; Leitman et al., 1986; Pandey et al., 1988), two of which (ANP-RA, ANP-RB) are monomeric proteins incorporating a transmembrane guanylate cyclase domain (Chinkers et al., 1989; Chang et al., 1989). CNP has recently been shown to display a selective and distinct affinity for the human ANP-RB receptor subtype inducing activation of guanylate cyclase whereas ANP was ineffective in eliciting any significant guanylate cyclase activity (Koller et al., 1991). The third receptor subtype (ANP-RC) is a dimeric protein composed of two disulphide-linked subunits, not linked to guanylate cyclase and, unlike the other receptor subtypes, does not discriminate between endogenous atrial and brain natriuretic peptides and d-amino-substituted or ring-deleted analogues of ANP-(1-28) such as des-Gln$^{18}$, Ser$^{19}$, Gly$^{20}$, Leu$^{21}$, Gly$^{22}$-rANP-(4-23) (C-ANP-(4-23)) (Rutherford et al., 1991). The C-ANP-(4-23) analogue is considered to be a selective ligand for the ANP-RC receptor subtype, which is ineffective in eliciting the natriuretic, diuretic and vasodilatory responses associated with ANP-(1-28) (Maack et al., 1987). The ANP-RC receptor subtype is thought to have a clearance function, regulating circulating ANP-(1-28) levels (Almeida et al., 1989), however, the receptor may also be linked to other second messenger systems (Anand-Srivastava and Cantin, 1986; Anand-Srivastava et al., 1990) and has...
been shown to mediate functional responses (Johnson et al., 1991).

\(^{125}\)I-ANP-(1-28) binding sites have been identified in the rat heart, but the specific characteristics of the binding were not established (Bianchi et al., 1985; Tjäve and Wilander, 1988; Oehlenschlager et al., 1989), and the presence of similar binding sites in human cardiac tissue has not been investigated. We have previously demonstrated that the ventricular as well as the atrial myocardium in human fetal and failing explanted adult hearts displays ANP-like immunoreactivity (Wharton et al., 1988). In the present study, quantitative in vitro autoradiographic techniques have been employed to both localize and characterize specific binding sites for natriuretic peptides in human fetal and adult heart tissues and the selective ring-deleted analogue C-ANP-(4-23) used to discriminate binding site subpopulations.

2. Materials and methods

2.1. Tissue preparation

Human fetal hearts (n = 9, 9-14 weeks gestation) were obtained following legal abortion by uterine evacuation for medical reasons other than suspected cardiac abnormality. Transmural samples of left and right ventricle were also obtained from explanted hearts (n = 5) during transplantation. Tissues were mounted on cork mats, surrounded in mounting medium (Tissue-Tek, Miles Inc., Elkhart, Indiana, U.S.A.) and snap frozen in melting dichlorodifluoromethane (Arcton-12, ICI, Cheshire, U.K.) suspended in liquid nitrogen. Cryostat sections (10 \(\mu\)m) were cut at -25°C, mounted on Vectabond (Vector Laboratories, Peterborough, U.K.) treated slides, dried for 1 h at 4°C and stored at -20°C.

2.2. Binding studies

Sections were preincubated at room temperature (19-22°C) for 15 min in phosphate buffer (comprising 10 mM NaH\(_2\)PO\(_4\)(2H\(_2\)O), 20 mM Na\(_2\)HPO\(_4\), 123 mM NaCl, pH 7.4), containing 40 mg/l bacitracin, and then in fresh buffer containing 1% bovine serum albumen (Fraction V, Sigma) and 250 pM \(^{125}\)I\(\alpha\)-tyrosyl human \(\alpha\)-ANP-(1-28) (\(^{125}\)I-hANP-(1-28), specific activity = 2000 Ci/mmol, Amersham U.K.) for up to 120 min. The slides were then rinsed twice in phosphate buffer for 5 min, once in distilled water at 4°C and dried under a stream of cold air. Under these incubation conditions the optimum specific/non-specific binding ratio was reached within 30 min. Competitive inhibition of specific \(^{125}\)I-hANP-(1-28) binding was examined in the presence of increasing concentrations (10 \(^{12}\) - 10 \(^{-6}\) M) of unlabelled \(\alpha\)-hANP-(1-28), hBNP-(1-32) and C-ANP-(4-23). Ligand integrity was assessed by re-applying the incubation medium to fresh sections, under identical conditions and using computer assisted microdensitometry to compare the subsequent autoradiographic images. The specificity of binding was further investigated by co-incubating sections with \(^{125}\)I-hANP-(1-28) and an excess of unrelated peptide including vasoactive intestinal polypeptide (VIP), calcitonin gene-related peptide (CGRP), endothelin-1 (ET-1), neuropeptide tyrosine (NPY) and gastrin.

2.3. Autoradiography

Dried labelled sections were apposed to Hyperfilm-\(^{3}\)H (Amersham, U.K.) together with sections of \(^{14}\)C-labelled polymer standards (American Radiolabelled Chemicals Inc., St. Louis, U.S.A.) which previous studies have shown can be used for calibration of \(^{125}\)I-labelled ligands in quantitative in vitro autoradiography, for 4 days at 4°C (Miller and Zahniser, 1987; Baskin and Wimpy, 1989). The resulting autoradiographic film was then developed for 5 min in filtered Kodak D-19 developer and fixed in Amfix for 5 min at 20°C. Greater anatomical resolution of the binding sites was obtained when the labelled sections were subsequently fixed in Bouin’s solution for 1 h at 20°C, washed twice in phosphate buffer (2-3 min each) at 4°C, rinsed in distilled water (1 min) at 4°C, dried under a stream of cold air and dipped in LM-1 emulsion (Amersham U.K.) at 43°C. Slides were then stored in the dark at 4°C for up to 10 days, developed in Kodak D-19 for 2.5 min at 20°C, fixed, stained with haematoxylin and eosin, and mounted in DPX synthetic mounting medium.

2.4. Quantification

Autoradiographic images were quantified by using an IBAS 2000 Image Analysis System (Kontron Electronic Ltd., Watford, U.K.). Specific binding was calculated by subtracting the non-specific binding, obtained by co-incubating the sections with \(^{125}\)I-hANP-(1-28) and an excess (1 \(\mu\)M) of the unlabeled peptide, from the total binding obtained by incubating sections with \(^{125}\)I-hANP alone. The non-linear curve fitting program, GraphPAD INPLOT Version 3.1 (GraphPAD Software, San Diego, U.S.A.) was used to analyse binding data. A four parameter logistic equation was used to describe the relationship between the percentage of \(^{125}\)I-hANP-(1-28) bound and the concentration of hANP-(1-28), hBNP-(1-32) and C-ANP-(4-23) in competitive inhibition experiments. The concentration of unlabelled peptide which inhibited 50% of the specific ligand binding (IC\(_{50}\)) and the pseudo-Hill coeffi-
cient were calculated for each competing peptide. The dissociation constant ($K_i$) of each competing peptide was estimated using the equation of Cheng and Prusoff (1973), $K_i = IC_{50}/(1 + [L]/K_d)$, where $[L]$ is the ligand concentration and $K_d$ the equilibrium dissociation constant derived from kinetic studies. The calculated values are expressed as geometric means with 95% confidence intervals (95% CI), being derived from logarithmically transformed data.

2.5. Statistics

Since each of the fetal hearts was examined for all three competing peptides, the competitive inhibition data was analysed as a randomised block experiment, using analysis of variance (ANOVA), treating the fetal hearts as a blocking factor and the competing peptides as a within subject factor (Armitage and Berry, 1987). The residuals were checked for Normal distribution using the Shapiro-Francia W-test (Royston J.P., 1983) and for equal variances within the peptide groups using the Bartlett test (Snedecor and Cochran, 1989).

3. Results

3.1. Localization of binding sites

Film autoradiographs demonstrated specific $^{125}$I-hANP-(1-28) binding to sections of human fetal heart, localized to the trabecular region of both ventricles, the aorta and pulmonary artery (fig. 1). Sections which included lung tissue exhibited binding associated with intra-pulmonary blood vessels. High density $^{125}$I-hANP-(1-28) binding sites were also identified on the endocardium of explanted adult ventricular tissues (fig. 2). No significant difference ($P = 0.752$) was found between the relative density of endocardial binding in sections of fetal (33.86 amol mm$^{-2}$; 95% CI, 29.30–38.42) and adult ventricle (32.75 amol mm$^{-2}$; 95% CI, 24.52–40.98), incubated with 250 pM $^{125}$I-hANP-(1-28) for 30 min. Microscopic examination of emulsion coated sections showed that $^{125}$I-hANP-(1-28) binding sites were localized to the endocardial layer of fetal and adult ventricle (fig. 3). The atrial endocardium exhibited a lower density of binding than the ventricles and silver grains were not detected overlying the endothelial lining of valve cusps. Binding to the fetal aorta and pulmonary arteries was localized to the vessel walls, with silver grains overlying the media and adventitia. In contrast, binding associated with intra-pulmonary arteries was distributed over the adventitia and adventitial-medial junction, rather than the media and intima (fig. 4). Silver grains were also observed overlying mesothelial cells in the ventricular epicardium. Fixation of labelled sections in Bouin’s solution and rapid
washing at 4°C, prior to the dipping of dried sections in emulsion, resulted in an optimum specific/ non-specific binding ratio, retention of regional differences in ligand binding and preservation of tissue morphology. The dissociation of ligand from specific binding sites was inhibited following this procedure, but not completely prevented, as prolonged washing at 20°C led to a reduction in binding density.

Co-incubation of 125I-hANP-(1-28) and an excess (1 μM) of either unlabelled hANP-(1-28), hBNP-(1-32) or C-ANP-(4-23) completely inhibited binding associated with the endocardium, epicardium and intrapulmonary blood vessels (figs. 3, 4). Binding to the aorta and extra-pulmonary arteries was also completely inhibited by hANP-(1-28) and hBNP-(1-32), but only partially inhibited by 1 μM C-ANP-(4-23) (fig. 1). 125I-hANP-(1-28) binding was not inhibited by co-incubation with either VIP, CGRP, ET-1, NPY or gastrin.

3.2. Binding studies

The characteristics of 125I-hANP-(1-28) binding to fetal ventricular endocardium were examined by association and dissociation kinetic studies and competitive inhibition. Analysis of the time course of association for 250 pM 125I-hANP-(1-28) at 19-22°C gave a rate constant (k+1) of 4.44 × 10⁸ M⁻¹ min⁻¹ with apparent
equilibrium binding achieved within 30 min. Eighty percent of bound $^{125}$I-hANP-(1-28) was displaced within 120 min of adding 1 µM unlabelled hANP-(1-28) to sections at equilibrium (fig. 5). The rate constant for dissociation ($k_{-1}$) was $1.3 \times 10^{-3}$ min$^{-1}$ and the apparent equilibrium dissociation constant ($K_d$) was 32.1 pM.

Competitive inhibition studies (fig. 6) demonstrated that hANP-(1-28) had a significantly greater inhibitory capacity ($P < 0.001$) for $^{125}$I-hANP-(1-28) endocardial binding sites than either hBNP-(1-32) or C-ANP-(4-23). Binding was completely inhibited by an excess (1 µM) of all three peptides, but compared to hANP-(1-28), hBNP-(1-32) and C-ANP-(4-23) displayed a relative inhibitory potency of 6 and 3% respectively (table 1). Whilst the competition curves for hBNP-(1-32) and C-ANP-(4-23) corresponded to 1, the estimated Hill coefficient for hBNP-(1-32) was less than unity.

### 4. Discussion

The binding data indicate the presence of high affinity binding sites for $^{125}$I-hANP-(1-28) on the endocardium of the human fetal and adult heart. Specific binding to these sites was completely inhibited by co-incubation with excess of unlabelled hANP-(1-28) or hBNP-(1-32), an observation consistent with previous studies suggesting that the similar physiological activity of these two peptides may be mediated via common receptor sites (Hirata et al., 1988; Oehlenschlager et al., 1989). The apparent affinity of the binding sites for hANP-(1-28) was, however, significantly greater than that observed for hBNP-(1-32). The binding characteristics indicate that the endocardial sites probably represent ANP clearance receptors (ANP-RC), recognising both endogenous natriuretic peptide sequences and the ring deleted analogue C-ANP-(4-23), but the possibility of receptor heterogeneity can not be excluded.

Natriuretic peptide sequences have been shown to bind to at least three distinct receptor populations, which are distinguished by their intrinsic guanylate cyclase activity (Waldman et al., 1984; Hamet et al., 1984; Chinkers et al., 1989; Chang et al., 1989), affinity for the recently described C-type natriuretic peptide (Koller et al., 1991) and affinity for ring-deleted ANP analogues such as C-ANP-(4-23) (Maack et al., 1987). In contrast to guanylate cyclase linked ANP-RA and ANP-RB receptors, ANP-RC receptors may be influenced by body fluid volume (Martin et al., 1989; Kolenda et al., 1990) and are thought to possess a clearance function, modulating the plasma concentration of ANP-(1-28) (Maack et al., 1987; Almeida et al., 1989). There is, however, evidence indicating that at least some ANP-RC receptors may be coupled to other second messenger systems and that C-ANP-(4-23) can elicit physiological responses. For example, the interaction of C-ANP-(4-23) with ANP receptors has previously been shown to inhibit adenylate cyclase activity in rat brain, aorta and adrenal cortical membranes (Anand-Srivastava et al., 1990) and in cultured atrial and ventricular cardiocytes ANP receptors may be negatively coupled to the adenylate cyclase/cyclic AMP signal transduction system through an inhibitory guanine nucleotide regulatory protein (Anand-Srivastava and Cantin, 1986). In addition, C-ANP-(4-23) has been shown to inhibit electrically induced purinergic and adrenergic contractile force generation in rat vas deferens via an inhibition of neurotransmission (Johnson et al., 1991).

The localization of $^{125}$I-hANP-(1-28) binding sites to the endothelial lining of the endocardium correlates both with recent receptor cloning studies where the mRNA encoding the ANP-C receptor was detected in the human fetal heart (Porter et al., 1990) and with our own in situ hybridization studies where the localization of ANP-C receptor mRNA expression corresponds closely with the distribution of binding sites demonstrated by in vitro autoradiography (unpublished observations). The results also appear consistent with previous in vitro and whole body autoradiographic studies performed on rat heart (Bianchi et al., 1985; Tjälke and Wilander, 1988; Oehlenschlager et al., 1989). Autoradiographic studies with dissociated cell culture preparations from rat and guinea-pig atria and ventricles have also identified $^{125}$I-hANP-(1-28) binding sites on a number of cell types including endothelial cells, glial cells and fibroblasts (James et al., 1990), although their localization was not determined in situ. The present findings indicate that binding in the human heart is mainly localized to the endocardial lining.

The endocardium is continuously exposed to the superfusing blood in the ventricles and this has led to the proposal that just as vascular endothelium influences the function of the underlying smooth muscle, so the endothelial lining of the endocardium may have a similar role in the regulation of the underlying my-

### TABLE 1

Competitive inhibition of specific $^{125}$I-hANP-(1-28) binding to human fetal endocardium by unlabelled peptide sequences.

<table>
<thead>
<tr>
<th>Peptide</th>
<th>$K_i$ (nM)</th>
<th>95% CI</th>
<th>$nH$</th>
<th>95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>hANP-(1-28)</td>
<td>0.07</td>
<td>(0.03-0.11)</td>
<td>0.94</td>
<td>(0.81-1.07)</td>
</tr>
<tr>
<td>hBNP-(1-32)</td>
<td>1.26</td>
<td>(0.35-3.61)</td>
<td>0.70</td>
<td>(0.58-0.81)</td>
</tr>
<tr>
<td>C-ANP-(4-23)</td>
<td>2.64</td>
<td>(1.68-4.72)</td>
<td>1.20</td>
<td>(0.96-1.45)</td>
</tr>
</tbody>
</table>

$^a$ Significant difference ($P < 0.001$) between the inhibitory potency of hANP-(1-28) and the other competing peptides.
ocardium through release of vasoactive factors (Henderson, 1991). This proposal is supported by work on isolated cat cardiac muscle where ANP has been shown to exert an effect mediated by the endothelial lining of the endocardium, which can be abolished by previous impairment of the endocardial surface (Meulemans et al., 1988). The apparent lack of binding of $^{125}$I-hANP-(1-28) to the vascular endothelium of the aorta, coronary arteries and pulmonary arteries, and the binding to the endothelium lining the atria and ventricles, but not the valve cusps indicates a heterogeneity in cardiac endothelial cell receptor expression. A similar phenomenon has also been demonstrated in the distribution of endothelin binding sites with non-selective (ET$_B$) binding sites specifically localized to the endothelial lining of valve cusps and selective (ET$_A$) binding sites localized to myocardium and vascular smooth muscle (Wharton et al., 1991). ANP and BNP have also been found to exert an inhibitory effect on the release of ET-1 from cultured human endothelial cells, a peptide which itself stimulates ANP and BNP secretion from the heart, raising the possibility of a feedback loop regulating the release of these peptides (Kohno et al., 1991).

The aorta and extrapulmonary arteries exhibited specific binding of $^{125}$I-hANP-(1-28) which was completely inhibited by co-incubation with hANP-(1-28) and hBNP-(1-32), but only partially inhibited by C-ANP-(4-23), suggesting the presence of distinct binding sites on these tissues. The reason for the presence of the ANP-RC receptor subtype on vascular smooth muscle is as yet unclear, since the direct vasodilatory effects of ANP are thought to be mediated through activation of guanylate cyclase (Ohishi et al., 1988). Intra-pulmonary artery binding sites appear to be exclusively of the ANP-RC subtype, however the binding was localized to the adventitia and adventitial-medial junction, rather than the media, and appears to be associated with cell types other than smooth muscle cells, such as fibroblasts and perivascular nerves.

In conclusion, we have demonstrated the localization of $^{125}$I-hANP-(1-28) binding sites in the human heart using the in vitro autoradiographic technique. The affinity of the binding is characteristic of the ANP-RC receptor subtype and the endocardial localization may indicate a clearance role for circulating natriuretic peptides and a possible site of regulatory peptide interaction in the heart.

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