

Natriuretic Peptide Receptor mRNAs in the Rat and Human Heart

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Abstract

Functional studies indicate that atrial natriuretic peptide (ANP), a member of the natriuretic peptide family, has direct effects on cardiac muscle cells. However, conventional ligand-binding studies designed to establish the presence of natriuretic peptide-binding sites in the heart have yielded conflicting results. There are discrepancies also between the latter and the receptor distribution predicted from the pattern of the mRNA transcripts localized by in situ hybridization. Here we have employed the technique of cDNA amplification with the polymerase chain reaction to confirm the presence of natriuretic peptide A, B, and C receptor mRNAs in rat and human cardiac tissue. In the rat heart, the distribution of the A and B receptor transcripts appears to be relatively homogeneous; in contrast, the C type mRNA is concentrated principally in the atria, with no difference between the left and right sides of the heart. A and B receptor DNA products were obtained after amplification of left, but not right, ventricular cDNA from the heart of a 16-yr-old male with cystic fibrosis; the yield of C receptor DNA was similar for both ventricles. If these mRNA transcripts are translated into functional receptors in the rat and human heart, ANP and the other natriuretic peptides may have direct effects on cardiac function, including regulation of natriuretic peptide release via a short feedback loop, modulation of contractility of the heart, or activation of cardiac reflexes. (*J. Clin. Invest.* 1992. 90:1966-1971.) Key words: atrial natriuretic peptide • guanylyl cyclase • cardiac receptors • polymerase chain reaction

Introduction

The natriuretic peptides are a family of cyclic molecules that can be divided broadly into three groups: the atrial natriuretic peptides (ANP)¹ (1), the brain natriuretic peptides (BNP or iso-ANP) (2-4), and the C-type natriuretic peptides (CNP) (5). ANP was the first to be discovered, and its pharmacological and biochemical effects have been characterized most extensively (1, 6). It is a 28-residue peptide that is synthesized predominantly in the cardiac atria and then released into the cir-

ulation to act on specific cell surface receptors (7-16), some of which may transduce its effects (17).

Three subtypes of natriuretic peptide receptor have now been defined by cDNA sequencing. The A and B subtypes contain a domain having guanylyl cyclase activity (10-16), which in the case of the A receptor, appears to mediate many of the effects of ANP and BNP (17). CNP differs from these two peptides in lacking the carboxy-terminal tail beyond the 17-amino acid loop (5) and is a potent and selective activator of the B receptor, with little effect on cyclic GMP (cGMP) levels in cells expressing the A receptor (15). Bennett and colleagues (16) have extended these studies by producing extracellular domain-IgG fusion proteins for the three human receptor subtypes to measure the binding characteristics of ANP, BNP, and CNP. ANP had the highest affinity for the A and C receptors, but CNP was the most potent competitor for the B receptor. This rank order of potency correlated well with the capacity of the ligands to activate the A and B guanylyl cyclases. ANP may also activate the phosphoinositide second messenger system (1, 18). However, it is unclear whether all natriuretic peptide receptors have the capacity for signal transduction by this means. Hirata and colleagues (19) have demonstrated an increase in inositol phosphate turnover, which they believe is mediated by the C receptor, while Berl and coworkers (20) have shown that ANP increases phospholipase C and inositol trisphosphate in cultured RMCT cells, which apparently do not express C receptors (21).

The natriuretic peptide C type receptor has only a short intracellular tail (7-9) and probably no intrinsic capacity to generate GMP, although it may be involved in the clearance of ANP (18) and the modulation of adenylyl cyclase activity via a G_i protein (1, 22-26), in addition to the alteration of phosphoinositide metabolism. Indeed, recent studies demonstrate that the C receptor has biological activity: the antimutagenic effect of atrial peptides may be mediated by this receptor independently of changes in cGMP levels (27) and the specific C receptor ligand, C-ANP₄₋₂₃ (des{Gln¹⁸,Ser¹⁹,Gly²⁰,Leu²¹,Gly²²}-ANP₄₋₂₃), affects neurotransmission in the rabbit vas deferens (28). The complexity of the natriuretic peptide receptor system is increased further by the possibility that signal transducing-binding sites may exist in vivo as large macromolecular complexes (29-31), perhaps containing varying proportions of one or both of the guanylyl cyclase subtypes and the C receptor (C-ANP₄₋₂₃ acts as a partial agonist when applied to cultured rat aortic vascular smooth muscle cells by increasing the synthesis of cGMP and antagonizing the effect of ANP on cGMP production in a dose-dependent manner; reference 32).

The highest concentrations of ANP and BNP are found in the heart (1, 33), where pharmacological and biochemical studies have suggested the existence of natriuretic peptide receptors (1, 23, 34-39). However, the interpretation of these data has been complicated by the conflicting results obtained with [¹²⁵I]ANP to demonstrate specific cardiac-binding sites

1. Abbreviations used in this paper: ANP, atrial natriuretic peptide; BNP, brain natriuretic peptide; CNP, C-type natriuretic peptide; cGMP, cyclic GMP; PCR, polymerase chain reaction.

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(37, 40–47), even though the latter are easily detectable in many tissues by autoradiographical and ligand-binding techniques (48).

The cloning and sequencing of the natriuretic peptide receptor cDNAs has made it possible to reexamine the question of the presence of these receptors in the heart using techniques that focus on the expression of their mRNAs rather than on the binding of radioiodinated ligands. Wilcox and colleagues (49) have employed *in situ* hybridization to determine the localization of the three natriuretic peptide receptor mRNAs in a macaque monkey. In our studies, we also have adopted an approach that focuses on the expression of the receptor mRNAs. In our experience, Northern blotting has been insufficiently sensitive to detect reliably the receptor mRNAs, and therefore, we have used cDNA amplification with the polymerase chain reaction (PCR) (50) to investigate the expression of the transcripts for the different subtypes of natriuretic receptor.

Methods

Tissues. The hearts were obtained from adult male Porton Wistar rats (weighing ~ 300 g). Right and left atrial appendages and blocks of left and right ventricle from 10 animals were pooled for isolation of RNA. The kidneys were also removed for RNA extraction.

Recipient left and right ventricular tissue was obtained at the time of heart–lung transplantation from a 16-yr-old male with cystic fibrosis.

cDNA amplification. Total RNA was extracted using a modified guanidinium isothiocyanate method and treated with RNAase-free DNAase (40 U for 1 h at 37°C; Pharmacia Inc., Piscataway, NJ). 50 µg of total RNA were reverse transcribed using oligo-dT or 12-mer randomers (10 µM) as previously described (51). One twentieth of the cDNA was amplified by the PCR for 30–40 cycles (93°C, 20 s; 55°C 30 s; 73°C 1 min; final extension: 73°C, 10 min) using pairs of oligodeoxynucleotide primers specific for each of the three receptor subtypes (1 µM each primer), 2 U of Taq DNA polymerase (Promega or Cambio, Cambridge, UK), 200 µM deoxynucleotides in the supplier's buffer containing 10 mM Tris HCl pH 8.3, 50 mM KCl, 1.5 mM MgCl₂, 0.01% gelatin, as well as unspecified detergents. The receptor primers were designed from published sequences (7, 10, 14) to permit the differentiation by size of the PCR products derived from each natriuretic peptide receptor subtype cDNA (451 bp for the A, 692 bp for the B and ~ 573 bp for the C subtype). In some experiments, forward and reverse primers (1 µM) specific for β -actin cDNA (816 bp from cDNA; 52, 53) were included to permit coamplification of β -actin and natriuretic peptide receptor cDNAs, we have used this as a simple but approximate marker for variation in the efficiency of RNA extraction, reverse transcription, and cDNA amplification.

Oligodeoxynucleotides used for PCR amplification. A receptor (from rat sequence; reference 10): forward: 5'-AAGAGCCTGATAATCCTGAGTACT; reverse: 5'-TTGCAGGCTGGGTCTCAT-TGTCA. B receptor (from rat sequence; reference 14): forward: 5'-AACGGGCGCATTGTGTATATCTGCGGC; reverse: 5'-TTAT-CACAGGATGGGTCTGCCAAGTCA. C receptor (from bovine sequence; reference 7): forward: 5'-ATCGTGCGCCACATCCAGGC-CAGT; reverse: 5'-TCCAAAGTAATACCAATAACCTCCTGGG-TACCCGC. β -actin (rat/human consensus sequences; references 52, 53) forward: 5'-CACCTTCTACAATGAGCTGCGTGTGGC; reverse: 5'-TGTTTGCTGATCCACATCTGCTGGAAGGTGGA.

Southern analysis. After amplification and separation by electrophoresis through a 1.8% agarose gel, the PCR products were denatured, blotted onto Hybond-N membranes (Amersham International, Amersham, UK), and hybridized overnight at 37–42°C in a solution containing 5× SSPE (0.9 M NaCl; 0.05 M sodium phosphate, pH 7.7; and 0.005 M EDTA), 50% formamide, 0.5% SDS, 5× Denhardt's solution (1 g BSA, 1 g Ficoll 400, and 1 g polyvinyl pyrrolidone/1000 ml), 250

µg/ml denatured herring sperm DNA, and 2.5–25 pmol/25 ml of labeled oligonucleotides complementary to sequences within the cDNAs flanked by the PCR primers (A receptor probe: 5'-TGACA-CAGCCATTAGCTCCTGGGAAGT; B receptor probe: 5'-GAT-ATCTTCGTCCTGCATCTTTTCCAC; C receptor probe (consensus sequence; see below): 5'-GGCATCTATGGAGACCTGCC-CGGCGATACCTTC). These probes were designed to exhibit minimal cross-receptor hybridization. They were labeled at the 3' end with terminal transferase and digoxigenin-dUTP (Boehringer Mannheim, Mannheim, FRG) (54). After stringent washing (down to 20 mM Na⁺ + 0.1% SDS at 37–42°C), the membranes were processed for localization of digoxigenin.

To detect the digoxigenin-labeled oligomers, after washing, the membrane was incubated for 30 min at room temperature with a 0.5% solution of blocking agent (Boehringer Mannheim) in buffer 1 (100 mM Tris HCl pH 7.4, 150 mM NaCl), and then, for 30 min at room temperature, in the same solution containing 1:5000 dilution of a polyclonal antidigoxigenin sheep antibody Fab fragment conjugated to alkaline phosphatase (Boehringer Mannheim). The membrane was washed in buffer 1 for 30 min at room temperature with one change of solution, and then equilibrated in buffer 3 (100 mM Tris HCl, pH 9.5; 100 mM NaCl, 5 mM MgCl₂) for 2 min. Alkaline phosphatase activity was localized by incubating the membrane at room temperature or 37°C with the substrates nitroblue tetrazolium (340 µg/ml of buffer 3) and 5-bromo-4-chloro-3-indolyl phosphate (170 µg/ml of buffer 3) to produce an insoluble purple/brown precipitate. When a suitable signal was obtained, the membrane was transferred into 10 mM Tris HCl pH 7.4, 1 mM EDTA buffer to stop the enzymatic reaction.

DNA sequencing of the rat C receptor PCR products. The amplification products obtained using rat kidney cDNA and C receptor primers were separated by electrophoresis. A major DNA band of the size predicted from the position of the primers along the bovine C receptor cDNA sequence was extracted from the agarose gel using glassmilk (GeneClean; BIO 101 Inc., Vista, CA) as directed by the supplier and was digested with the restriction enzyme, Kpn 1 (Bethesda Research Laboratories, Gaithersburg, MD). This endonuclease recognizes a specific cleavage site only within the downstream reverse primer. The digested DNA was ligated to Kpn 1-linearized Bluescribe vector (Stratagene, La Jolla, CA) using 2.5 U of T₄ DNA ligase (Bethesda Research Laboratories) to form a linear construct. After phenol/chloroform purification, the construct was blunt-ended with 1.5 U of T₄ DNA polymerase to fill in overhangs produced either by incomplete extension during amplification or the 3' terminal transferase activity of Taq DNA polymerase (55). The constructs were circularized with 2.5 U of T₄ DNA ligase and introduced into TG1 cells made competent with CaCl₂ (56). DNA from white colonies was purified and sequenced by the dideoxy chain-termination method (57) using Sequenase (US Biochemical Corp., Cleveland, OH). The sequences obtained were analyzed using University of Wisconsin programs (58).

Results and Discussion

This study was designed to circumvent the problems of detecting binding sites for [¹²⁵I]ANP in cardiac tissues by focusing on the distribution of the mRNAs coding for the natriuretic peptide A, B, and C receptors.

Amplification of rat kidney cDNA with primers complementary to the bovine C receptor cDNA sequence yielded a major product similar in size to that predicted from the positions of the primers along the bovine receptor cDNA (results not shown). After cloning into Bluescribe vector, this DNA was sequenced to confirm the origin of the PCR product and also to enable the synthesis of an oligomer probe that would detect amplified rat C receptor cDNAs on Southern blots. In the 87-bp sequence of the amplified rat DNA shown in Fig. 1, there is 86% similarity with both bovine (7) and human (8, 9) C receptor cDNAs. The C receptor probe used for Southern

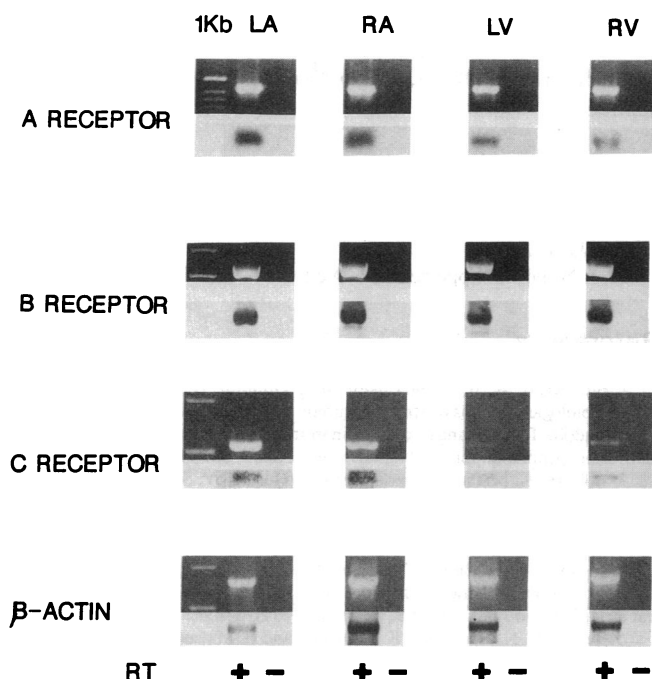


Figure 2. Amplification of rat cardiac cDNAs with natriuretic peptide A, B, and C receptor and β -actin primers. Left (LA) and right (RA) atrial, left (LV) and right (RV) ventricular cDNAs from the rat were amplified with the PCR using primers specific for the A, B, and C receptor and β -actin cDNAs. All RNA samples were pretreated with RNAase-free DNAase before reverse transcription and PCR. The reverse transcriptase was omitted in control (RT-) RNA samples during the cDNA synthesis step. After separation by electrophoresis, DNA bands of the sizes predicted (451-bp for the A receptor, 692-bp for the B, ~ 573-bp for the C subtype, and 816-bp for β -actin) hybridized to the appropriate probes. The presence of the specific receptor cDNA bands depended on prior reverse transcription. There appeared to be little regional difference in the amounts of A or B receptor products obtained. For the C receptor, the bands obtained from atrial cDNA were consistently greater than those generated from ventricular cDNA. This difference was not due to large variation in the amount of total cDNA before PCR, as shown by the similar intensity of the β -actin bands. 1 kb: Bands from a 1-kb DNA ladder (Bethesda Research Laboratories), A receptor: 517/506 bp, 396 bp, 344 bp, 298 bp; B and C receptor and β -actin: 1,018 bp, 517/506 bp.

amplification cannot provide direct evidence for the translation of these transcripts and the distribution of the receptor proteins in the rat heart, it avoids potential problems such as "blockade" of binding sites by large amounts of ANP (and BNP) released during tissue preparation, and its ability to define specific receptor subtypes is currently unattainable with conventional ligand-binding or autoradiographical techniques using ^{125}I -labeled compounds. One group has reported the existence of binding sites for ^{125}I ANP in ventricular membrane preparations (37), but they have now published some contradictory data (40). Others have reported the presence of ANP-binding sites on ventricular endocardium (41–44) and on cultured mesenchymal nonmyocardial cells from the rat heart (46). Anand-Srivastava et al. (47) employed receptor autoradiography to detect displaceable binding of ^{125}I ANP in conducting tissue in the rabbit heart, but a similar distribution of binding has not been observed in the rat heart by others (reference 45 and D. J. Nunez, unpublished observations). The buffers used in many binding studies permit very extensive

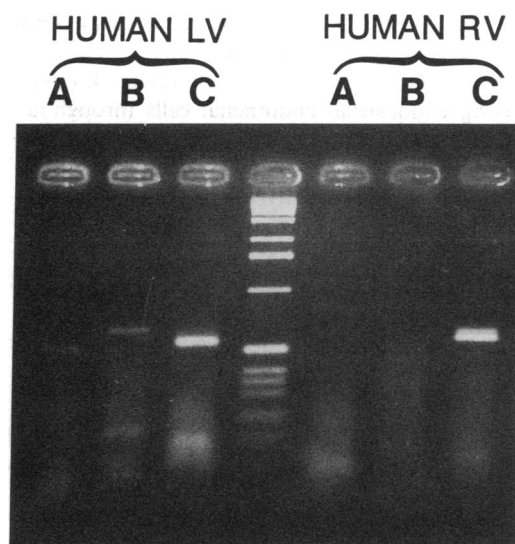


Figure 3. The PCR products obtained after amplification of human ventricular cDNAs with the natriuretic peptide receptor primers. The PCR was employed to amplify human right and left ventricular cDNA derived from the heart of a 16-yr-old male who had undergone cardiac transplantation for cystic fibrosis. Amplification products specific for the three receptor subtypes were detected. There appeared to be reproducible differences in the intensities of the A and B receptor cDNA products between left and right ventricular samples; similar yields of the C receptor product were obtained from both ventricles. The 1-kb DNA ladder (Bethesda Research Laboratories) is included for size comparison.

degradation of ^{125}I ANP, which would reduce the apparent prevalence of binding sites and alter the binding kinetics (64), but we have been unable to detect "covert" binding sites after the inclusion of phosphoramidon ($2\ \mu\text{M}$) in the reaction mixture to prevent ANP metabolism by neutral endopeptidase (E.C. 3.4.24.11) (D. J. Nunez, unpublished results).

Although there are discrepancies in the detailed distribution of cardiac natriuretic peptide receptors, the functional data discussed above suggests that cardiac myocytes themselves express ANP receptors, but our results cannot exclude a contribution from other cell types, such as smooth muscle and endothelial cells and neurons, which are distributed throughout the heart. Wilcox and colleagues (49) have reported the results of

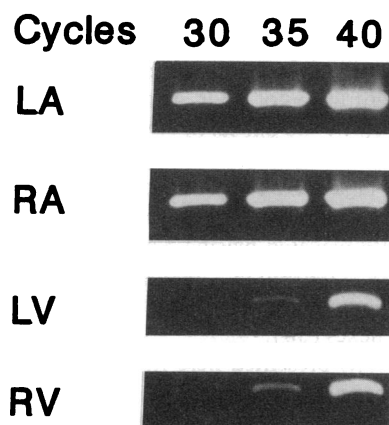


Figure 4. The effect of altering the number of PCR cycles on the yield of C receptor product from rat heart cDNAs. Left (LA) and right (RA) atrial, left (LV) and right (RV) ventricular cDNAs from the rat were amplified with C receptor primers for 30, 35, and 40 cycles. The specific C receptor product was detectable in the atrial samples at lower levels of amplification, indicating that

the initial prevalence of C receptor mRNA transcripts in the atrial samples is greater than that in the ventricular ones.

an extensive in situ hybridization histochemical study on a single rhesus monkey using cRNA probes to the A, B, and C receptor mRNAs. A and C receptor probe hybridization was observed overlying endocardial endothelial cells throughout the heart, except for the left ventricle in the case of the A receptor. High levels of C receptor cRNA hybridization were seen associated with many right atrial myocytes. B receptor transcripts were not detectable. The differences between these results and our data may represent species variation. However, their C receptor antisense cRNA hybridization experiments cannot be assessed by comparison with formal controls. Although they observed anatomical localization, which was apparently discrete, this is not a sufficient criterion of hybridization specificity, since we have found indistinguishable regionally localized hybridization of antisense and sense oligonucleotides specific for the A and C receptor mRNAs within the rat kidney (D. J. Nunez, unpublished results). Furthermore, the fact that the templates used to synthesize the control sense A and B receptor cRNAs were 1,000 bases longer than the antisense ones may have affected probe size and penetration into the tissue sections in an unpredictable manner (65, 66), producing an artifactual reduction in the "nonspecific" background. Furthermore, there appear to be some discrepancies between the detailed localization of receptors predicted from the pattern of expression of the transcripts and the binding sites defined using [125 I]ANP (41–44, 47).

If the natriuretic peptide receptor mRNAs we and others have detected in cardiac tissue are translated and the proteins expressed appropriately, it still remains to be established whether the effects of ANP on cardiac tissue discussed above are mediated by the known receptor types or whether there are others still to be defined, either of the "functional" guanylyl cyclase type or of a cGMP-independent form, which appear to mediate, for instance, the stimulation of amiloride-sensitive $^{22}\text{Na}^+$ uptake into cells in the rabbit aorta (67) and the inhibition of aldosterone secretion from adrenal cortical cells (68, 69). However, MacFarland and colleagues (70) have cast some doubt on the latter results by demonstrating that 8-bromo-cGMP can inhibit ACTH-induced aldosterone secretion by lowering cAMP levels via a cGMP-dependent increase in phosphodiesterase activity.

Our data showing the presence of natriuretic peptide receptor mRNAs in the rat and human heart and the evidence reviewed above that indicates that these transcripts are translated into functional receptors raises the question of the physiological roles of these receptors. One possibility is that they are involved in the regulation of ANP (and BNP) synthesis/release by myocytes via a short feedback loop, allowing these cells to "sense" intracardiac concentrations of ANP. Another function of the receptors is suggested by the effects of ANP on cardiac contractility and second messenger systems. Indeed, the enhancement by ANP of the positive inotropic effect of angiotensin II (34) may be beneficial in cardiac failure, a pathophysiological state associated with elevated plasma ANP concentrations (1). Alternatively, natriuretic peptide receptors synthesized within cardiac nerve terminals may function to modulate cardio-renal reflexes (38).

In conclusion, the results presented here indicate that ligand-binding techniques are not reliable in demonstrating cardiac natriuretic peptide receptors and that other methods, perhaps based on specific antireceptor antibodies, are required to study the expression of the receptor molecules themselves. The evidence in favor of the existence of these receptors in the heart

requires a reevaluation of the mechanisms by which ANP and BNP alter renal function, since some of these may be mediated by cardio-renal reflexes, rather than by direct actions of the peptide.

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