Human atrial natriuretic peptide receptor defines a new paradigm for second messenger signal transduction

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We isolated cDNAs encoding a 115 kd human atrial natriuretic peptide (α ANP) receptor (ANP-A receptor) that possesses guanylate cyclase activity, by low-stringency hybridization with sea urchin *Arbacia punctulata* membrane guanylate cyclase probes. The human ANP-A receptor has a 32 residue signal sequence followed by a 441 residue extracellular domain homologous to the 60 kd ANP-C receptor. A 21 residue transmembrane domain precedes a 568 residue cytoplasmic domain with homology to the protein kinase family and to a subunit of the soluble guanylate cyclase. COS-7 cells transfected with an ANP-A receptor expression vector displayed specific [¹²⁵I] α ANP binding, and exhibited α ANP stimulated cGMP production. These data demonstrate a new paradigm of cellular signal transduction where extracellular ligand binding allosterically regulates cyclic nucleotide second-messenger production by a receptor cytoplasmic catalytic domain.

Key words: Atrial natriuretic peptide receptor/cGMP/membrane guanylate cyclase/second messenger/signal transductions

Introduction

Cardiovascular homeostasis is controlled by several distinct but interrelated regulatory pathways that modulate blood pressure, fluid volume and electrolyte composition. A key regulatory molecule in this system is α atrial natriuretic peptide (α ANP), a 28 amino acid endocrine hormone that is released by ventricular cardiocytes in response to elevated arterial blood pressure and increased intravascular fluid volume (reviewed in Flynn and Davies, 1985; Baxter *et al.*, 1988).

Crosslinking studies with ¹²⁵I-labeled α ANP have identified two subunit mol. wt types of cell surface receptors for α ANP. A low mol. wt receptor (ANP-C receptor) has a 60–70 kd subunit size and forms a disulfide-linked 120–140 kd homodimer (Shenk *et al.*, 1985; Fuller *et al.*, 1988). The high mol. wt α ANP receptor consists of a non-reducible 120–180 kd protein (Misono *et al.*, 1985; Vandlen *et al.*, 1985, 1986; Pandey *et al.*, 1988).

Experiments on the intracellular responses to α ANP have shown that receptor binding results in rapid increases in cyclic 3',5'-guanosine monophosphate (cGMP), suggesting that cGMP may act as a second messenger of α ANP action (Hamet *et al.*, 1984; Waldman *et al.*, 1984; Winquist *et al.*, 1984; Tremblay *et al.*, 1985). Three differentially regulated forms of guanylate cyclase (GC) have been previously identified in eukaryotic cells: a detergent-insoluble particulate enzyme, a detergent-soluble membrane-associated enzyme and a soluble cytoplasmic enzyme which is a heterodimer of 70 and 82 kd subunits (reviewed in Waldman and Murad, 1987). Competition studies with truncated analogs of α ANP have demonstrated that α ANP receptors are pharmacologically distinct, with strict α ANP structural requirements for stimulation of cGMP production by the 120–140 kd receptor, and with less stringent constraints on α ANP structure for binding to the low subunit mol. wt receptor (Leitman *et al.*, 1986; Meloche *et al.*, 1987; Olins *et al.*, 1988). Purification of the 120–140 kd receptor has resulted in co-purification of α ANP binding activity and membrane GC, suggesting that the receptor and enzyme are either in very close apposition or part of the same polypeptide (Kuno *et al.*, 1986; Paul *et al.*, 1987; Takayanagi *et al.*, 1987; Meloche *et al.*, 1988).

We recently described the structure of the membrane form of GC from the sea urchin *Arbacia punctulata* (Singh *et al.*, 1988). We reasoned that portions of the cytoplasmic domain of the sea urchin GC might have significant amino acid sequence similarity to the mammalian membrane GC, based on biochemical evidence; the membrane form of GC from sea urchin spermatozoa resembles mammalian membrane GC with respect to kinetic properties (Waldman and Murad, 1987), and antibodies to the sea urchin sperm enzyme will immunoprecipitate rat membrane GC (Garbers, 1978). Here we describe the use of sea urchin membrane GC cDNA

Fig. 1. Sea urchin membrane guanylate cyclase. A diagram of the structural domains of the sea urchin *Arbacia punctulata* membrane guanylate cyclase (Singh *et al.*, 1988) is shown flanked by a line denoting cloned regions of 5'- and 3'-untranslated sequence. At the 5' end the coding region begins with an amino-terminal signal sequence followed by an extracellular domain. Two regions of sequence homology in the cytoplasmic catalytic domain are indicated to the right of the transmembrane domain. Probe A and probe B under the cytoplasmic domain indicate the segments of cloned sea urchin cDNA used as hybridization probes on a human kidney cDNA library. The scale is shown at the bottom of the figure in kilobase pairs (kb).

probes to isolate cDNAs encoding a 115 kd human α ANP receptor with GC activity.

Results

Cloning of human cDNAs homologous to sea urchin guanylate cyclase

Given the proposed identity of the high mol. wt α ANP receptor and particulate GC, together with the kidney as a site of α ANP action, we chose to screen a human kidney cDNA library in λ gt10 at low stringency with two probes from the *A. punctulata* sperm membrane GC (AP-GC) cytoplasmic domain (Figure 1, probes A and B). Of 15 clones identified by hybridization for sequence analysis, three were found to overlap and encode part of a protein with 46% sequence similarity beginning at AP-GC amino acid 568 and extending to the carboxyl terminus. A complete coding region for this AP-GC related human protein was determined from cloned kidney and placenta cDNAs, and from a genomic clone (see Materials and methods).

Based on our data for α ANP hormone binding to the protein expressed from the complete coding region of this mRNA (discussed below) we have designated this protein the ANP-A receptor. The nucleotide and predicted amino acid sequence of the ANP-A receptor is shown in Figure 2 with 43 nucleotides (nt) of 5'-flanking sequence, and 576 nt of 3'-flanking sequence. A translation initiation codon at position 44 defines the beginning of a 1061 codon open reading frame (ORF). Upstream of the initiator codon at position 44 there is an out-of-frame ATG at position 3 followed by a termination codon at position 147. The N-terminal amino acid sequence encoded by the ORF is hydrophobic, and probably constitutes a signal sequence of 32 amino acids. Based on the consensus sequence for signal peptidase cleavage (Von Heijne, 1983) we predict the mature protein to begin with glycine at position +1 followed by a 441 amino acid extracellular domain with seven potential N-linked glycosylation sites and seven cysteine residues (Figure 2). A 21 amino acid transmembrane domain from residues 442 to 462 is followed by the stop-transfer sequence Arg-Lys (Sabatini *et al.*, 1982). The cytoplasmic domain has two potential N-linked glycosylation sites, 10 cysteine residues and extends for 568 residues to the carboxyl terminus. The putative mature ANP-A receptor protein has 1029 amino acids with a M_r of 115 426. After the translation termination codon at position 3227 there are 573 bases of 3'-untranslated sequence with a potential polyadenylation signal (Proudfoot and Brownlee, 1976) 21 nt upstream of the poly(A) tail (Figure 2).

Domain structure and homologies of the ANP-A receptor

There is 33% amino acid sequence similarity between the human ANP-A receptor and the bovine ANP-C receptor (Fuller *et al.*, 1988) (Figure 3). This similarity is distributed throughout the extracellular domain of the ANP-A receptor and extends to the Arg-Lys stop-transfer sequence after the transmembrane domain. Of the remaining 35 amino acids

of the ANP-C receptor cytoplasmic domain, only four are identical to the ANP-A sequence.

Alignment of the sea urchin AP-GC sequence with the human ANP-A receptor is shown starting 27 amino acids before the ANP-A transmembrane domain (Figure 3). The extracellular domains of these proteins are <20% identical (data not shown) but a segment of 14 similar amino acids exists before the transmembrane domain, the transmembrane domains are unrelated, then there is significant homology between these proteins beginning with the stop transfer sequence and extending into the cytoplasmic domain (Figure 3). There is ~46% sequence similarity between AP-GC and ANP-A receptor amino acids 463–869, at which point the remaining 45 residues of AP-GC show little similarity to ANP-A.

There is 23–25% sequence similarity between members of the protein kinase family and a region of the AP-GC cytoplasmic domain (Singh *et al.*, 1988). Homology between both the human ANP-A receptor and sea urchin AP-GC with protein kinases is illustrated in Figure 3 by alignment with the *c-fes* proto-oncogene protein sequence (Roebroek *et al.*, 1985). Starting with *c-fes* residue 567 and extending to the C terminus at residue 825, there is ~30% sequence similarity to the ANP-A receptor from amino acids 503 to 774: the kinase homology region. We have highlighted 33 amino acids that are highly conserved among members of the protein kinase family (Figure 3) (Hanks *et al.*, 1988). Of these 33 residues, 22 are conserved in the ANP-A receptor kinase homology region. The protein kinase nucleotide binding domain consensus sequence Gly-X-Gly-X-X-Gly, *c-fes* residues 571–576, is changed to Gly-X-Gly-X-X-X-Gly in ANP-A (Figure 3, ANP-A residues 503–509). However, there is no apparent similarity to this consensus sequence in the corresponding region of AP-GC. The invariant Lys residue of protein kinase catalytic domains (Figure 3, *c-fes* residue 593) is changed to Gln in ANP-A, but is conserved in the AP-GC sequence.

There is ~42% similarity between the ANP-A receptor and the bovine lung 70 kd SolGC subunit (Koesling *et al.*, 1988) over a 253 amino acid region extending to the C terminus of each protein (Figure 3). The homology between SolGC and the ANP-A receptor begins just C-terminal to the kinase homology region. The SolGC sequence is also homologous to a 59 amino acid segment of AP-GC (40% identity) C-terminal to the AP-GC kinase homology region (Figures 1 and 3) (Garbers *et al.*, 1988). The homology between the membrane GC sequences with SolGC defines two different degrees of similarity based on the extent to which the homology encompasses the C-terminal region of SolGC.

Expression of ANP-A receptor mRNA in tissues

To examine the organ-specific expression of ANP-A receptor mRNA, we used a 2603 bp fragment of ANP-A cDNA (Figure 2, nucleotides 1204–3807) to probe a Northern blot of poly(A)⁺ RNA from a variety of human tissues. As shown in Figure 4A, this probe detects an ~3900 base mRNA at equivalent abundance in kidney (lane 1), adrenal

Fig. 2. Nucleotide and deduced amino acid sequence of the ANP-A receptor. Nucleotides are numbered at the beginning of each line and amino acids are numbered above the sequence. The predicted first residue of the mature protein is indicated by +1. Before this the 32 residue signal sequence is overlined with a thin line. The transmembrane domain is indicated by a thick black line over the sequence. Potential N-linked sites of carbohydrate addition are shown by a box around Asn-X-Ser/Thr tripeptides, where X denotes any amino acid. Cysteine residues are shown by a solid dot above the sequence. A potential polyadenylation signal is overlined by a black bar.

Fig. 3. Homologies of the ANP-A receptor. The amino acid sequence of the mature ANP-A receptor (ANP-A) is aligned with the bovine ANP-C receptor sequence (Fuller *et al.*, 1988), the sea urchin AP-GC membrane guanylate cyclase (Singh *et al.*, 1988), human proto-oncogene *c-fes* (Roebroek *et al.*, 1985) and a subunit of the bovine soluble guanylate cyclase (SoIGC) (Koesling *et al.*, 1988). Gaps introduced for an optimal alignment are shown by dashes and amino acids identical in two or more of the aligned sequences are boxed. The transmembrane domain of ANP-A is shown by a hatched overline. The transmembrane domains from AP-GC and ANP-C end at the same position as the ANP-A transmembrane domain. In the kinase homology region defined by alignment of ANP-A and AP-GC with *c-fes*, amino acids that are invariant in 62 of 65 kinases are shown by a dot below the *c-fes* sequence and amino acids conserved in 63 of 65 kinases are shown by an open circle (Hanks *et al.*, 1988).

Fig. 4. Steady-state level of ANP-A receptor mRNA. Approximately 4 μ g of poly(A)⁺ RNA from human kidney (lane 1), adrenal (lane 2), spleen (lane 3), terminal ileum (lane 4), adipose tissue (lane 5), placenta (lane 6) and from the human cell lines TE671 medullablastoma (lane 7), SK-N-MC neuroblastoma (lane 8) and 1321-N1 astrocytoma (lane 9) was electrophoresed through a 6% formaldehyde–1.2% agarose gel and blotted to nitrocellulose. The filter was probed with ³²P-labeled ANP-A receptor cDNA (panel A), where the position of 28S and 18S rRNAs is shown on the left. The relative amount of β -actin mRNA in each sample was determined by rehybridization of the filter in panel A with an actin specific oligonucleotide (Panel B).

(lane 2) and adipose tissue (lane 5), and at relatively lower levels in terminal ileum (lane 4). We do not detect any hybridization to mRNA from spleen and placenta (lanes 3 and 6 respectively) or from the TE671 medullablastoma, SK-N-MC neuroblastoma or 1321-N1 astrocytoma cell lines (Figure 4A, lanes 7–9). The lack of hybridization to mRNA from several tissues suggests that ANP-A receptor mRNA in these tissues is at a very low level or absent. However, low amounts are present in human placenta, as we obtained cloned cDNAs for the ANP-A receptor from this tissue. Hybridization of an actin probe to the same filter illustrates the relative levels of β -actin mRNA in these samples (Figure 4B).

ANP-A receptor is guanylate cyclase

To confirm that the cDNA clones we have obtained indeed encode a receptor for α ANP, we constructed the expression vector pRKANP-AR in which the putative ANP-A receptor coding sequence is under the transcriptional control of the cytomegalovirus long terminal repeat in the vector pRK (see Materials and methods). The ligand binding properties of the receptor encoded by the pRKANP-AR expression vector were determined by equilibrium binding (Figure 5) of [¹²⁵I] α ANP in the presence of unlabeled α ANP (1–28) or

Fig. 5. Competitive binding of [¹²⁵I]αANP to transfected cells. [¹²⁵I]αANP (1–28) (500 pM) was incubated with pRKANP-AR (closed symbols), or control pRK (open symbols), transfected COS-7 cells and increasing concentrations of unlabeled αANP (1–28) (■, □) or unlabeled rat atriopentin I (5–25) (●, ○). Data are expressed as the percentage of specific [¹²⁵I]αANP binding and values are the means of duplicate determinations.

the truncated αANP analog rat atriopentin I (5–25) (Currie *et al.*, 1984). Competition for [¹²⁵I]αANP binding with unlabeled αANP gave an EC₅₀ in the range of 3 nM, and the less potent atriopentin I had an EC₅₀ in the range of 300 nM. In addition, competition by αANP for [¹²⁵I]αANP binding to control pRK-transfected cells indicates the presence of endogenous high-affinity receptors on COS-7 cells (Figure 5). These binding data establish the identity of the cloned human cDNA as encoding an αANP receptor, and we have thus designated this protein the ANP-A receptor.

Guanylate cyclase activity in the membrane fraction from pRKANP-AR and control transfected cells was measured by radioimmunoassay of cGMP. Membranes from cells transfected with pRK ANP-AR had a guanylate cyclase specific activity of 2.25 ± 0.04 pmol cGMP/min/mg protein, compared to membranes from control pRK transfected cells which had 0.27 ± 0.06 pmol cGMP/min/mg protein. Stimulation of intracellular cGMP production by αANP was tested on intact COS-7 cells expressing the ANP-A receptor. Before stimulation, experimental and control cells had 0.22 ± 0.05 and 0.18 ± 0.10 pmol cGMP respectively. One minute after stimulation with αANP there was 0.61 ± 0.08 and 0.22 ± 0.03 pmol cGMP in experimental and control cells respectively.

Discussion

ANP-A receptor cloning

Using fragments of the sea urchin AP-GC cloned cDNA (Singh *et al.*, 1988) as probes in a low stringency hybridization experiment, we isolated human cDNAs encoding the ANP-A receptor/guanylate cyclase. Several lines of evidence

support our conclusion that the 1029 amino acid mature ANP-A protein is a cell surface receptor for αANP with ligand-stimulated intrinsic guanylate cyclase activity in the cytoplasmic catalytic domain. The expression of recombinant protein on the surface of COS-7 cells with specific binding of [¹²⁵I]αANP identifies this molecule as a receptor for αANP. This receptor displays a 100-fold higher affinity for αANP over a truncated αANP analog, which is consistent with the previously described ligand pharmacology of the GC-coupled high mol. wt αANP receptor (Leitman *et al.*, 1986; Olins *et al.*, 1988).

We recently described the structure of AP-GC, the sperm membrane guanylate cyclase (Singh *et al.*, 1988), which had been proposed to be the receptor for the egg peptide resact (Shimomura *et al.*, 1986). However, heterologous expression of functional AP-GC in either cell-free systems or in cultured cells was unsuccessful (S.Singh *et al.*, unpublished data), forcing us to rely on correlative data for the establishment of both ligand-binding and guanylate cyclase activities in the same molecule (Singh *et al.*, 1988). For the ANP-A receptor we have demonstrated a direct correlation between expression vector transfection, increased membrane guanylate cyclase activity and αANP stimulated cGMP production in intact cells. These results are consistent with the catalytic activity being part of the ANP-A receptor protein. Previous data on the co-purification of the 120–180 kd αANP receptor and GC activity (Kuno *et al.*, 1986; Paul *et al.*, 1987; Takayanagi *et al.*, 1987; Meloche *et al.*, 1988) further support this conclusion.

Structural and functional domains of the ANP-A receptor

The human ANP-A receptor is 90% identical throughout to a rat brain αANP receptor/guanylate cyclase (Chinkers *et al.*, 1989) which we assume to be the rat homolog of the human ANP-A receptor described here. Amino acid sequence homology between the ANP-C receptor (Fuller *et al.*, 1988) and the extracellular domain of ANP-A receptor reflects a common function shared between these two proteins: αANP binding. The sequence similarity between these receptors may define structural features important for αANP binding, and conversely the sequence differences would define the basis for the distinct ligand specificities of these two receptors (Leitman *et al.*, 1986; Meloche *et al.*, 1987; Olins *et al.*, 1988). The functional and structural homology between the extracellular domains of these molecules define a new, albeit yet small, protein family.

Nucleotide sequence homology between the sea urchin AP-GC cytoplasmic domain and the human ANP-A receptor was significant enough to allow us to clone the ANP-A receptor using AP-GC as a probe. The similarity between the nucleotide sequences encoding the cytoplasmic domains of these proteins is a reflection of structural and functional homology. Our original experiment was based on the prediction of significant conservation through evolutionary time of the structure of membrane guanylate cyclase. Given that the region of sequence similarity between AP-GC and ANP-A receptors is in the cytoplasmic domain, and that transfection of the ANP-A receptor expression vector into mammalian cells results in an 8-fold increase in membrane-GC specific activity, we conclude that at least part of the homologous region comprises the catalytic domain of these two proteins.

The cytoplasmic domain of membrane guanylate cyclases, the AP-GC and ANP-A receptor, is homologous to the large protein kinase family (Singh *et al.*, 1988; Figure 3). Although the functional significance of this homology is presently unknown, it is non-random and is limited to the catalytic region shared in common among all known or suspected protein kinases (Hanks *et al.*, 1988). We note that the invariant lysine residue of protein kinase catalytic domains which is essential for kinase activity and may be directly involved in the phosphotransferase reaction (reviewed in Hanks *et al.*, 1988), is changed to Gln-517 in the ANP-A receptor. Although this amino acid substitution likely eliminates any possible intrinsic phosphotransferase activity of the kinase homology region, this domain may still function as an ANP-A receptor nucleotide binding site.

Carboxyl-terminal to the kinase homology region, an additional region of sequence homology was found between the ANP-A receptor and a subunit of the soluble form of guanylate cyclase (Koesling *et al.*, 1988). The variable extent of similarity between the sea urchin and human forms of membrane GC and the SolGC subunit suggests that this region (ANP-A amino acids 790–1029) may not be directly involved in the catalytic function of these enzymes.

The three distinct and non-overlapping domains of sequence similarity between the ANP-A receptor and three classes of proteins that are unrelated has interesting evolutionary and functional implications. We assume that these sequence similarities represent true homology through divergence from common ancestral genes. The most parsimonious explanation for this is the assembly of the ANP-A receptor gene from exons that are shared in common with genes for other proteins. This type of domain homology through exon shuffling (Gilbert, 1985) has been observed in the structure of the LDL receptor gene, which is built of exons homologous to those found in several supergene families (Sudhof *et al.*, 1985a,b).

ANP-A receptor signal transduction: a new paradigm

Two general classes of cell surface receptors involved in cellular regulation have been identified: those with a single transmembrane domain and those with multiple membrane-spanning domains. The receptor tyrosine kinases epitomize the first class of receptor in which ligand-stimulated intracellular tyrosine kinase activity is necessary for a biological response (reviewed by Yarden and Ullrich, 1988).

The multiple membrane-spanning receptors may be divided into two subclasses: the oligomeric ligand-gated ion channels involved in neuronal excitation (Stevens, 1987), and the seven transmembrane-domain receptors. This latter class of receptors is involved in the widely studied G-protein-mediated regulation of intracellular signal transduction. In this classic paradigm of sensory transduction, ligand binding to a receptor regulates the activity of an intracellular effector protein, for example an ion channel or adenylyl cyclase production of the second messenger cAMP, through an intermediary coupling protein, a G-protein (Neer and Clapham, 1988).

The direct production of the cyclic nucleotide second messenger cGMP from the ANP-A receptor represents a profound departure from the previously recognized paradigms of intracellular signal transduction. As shown in our model for α ANP stimulation of cGMP production through the ANP-A receptor (Figure 6), ligand binding to

Fig. 6. Model for ANP-A receptor function. The unoccupied ANP-A receptor is shown on the left with a basal rate of cGMP synthesis indicated by a thin arrow. The effect of ligand binding to the amino-terminal extracellular domain is shown on the right. Proposed allosteric modulation of guanylate cyclase by α ANP binding is schematically illustrated by a change in shape of the intracellular catalytic domain, and a thicker arrow to denote an increase in guanylate cyclase specific activity with greater production of the second messenger cGMP.

the extracellular domain would allosterically regulate an increase in the specific activity of the cytoplasmic catalytic domain. Although this represents a new model for receptor-mediated signal transduction, this idea had been recognized as a formal possibility for the hormonal regulation of adenylyl cyclase (Robison *et al.*, 1967). Our model is based on observations of a rapid increase in intracellular cGMP in response to α ANP for cells transfected with the ANP-A receptor, and is consistent with previous observations on the intracellular response to α ANP stimulation (Hamet *et al.*, 1984; Waldman *et al.*, 1984; Winquist *et al.*, 1984; Tremblay *et al.*, 1985). The enzyme activation mechanism is presently obscure. In crude membrane preparations particulate guanylate cyclase exhibits positive co-operativity with respect to GTP binding with at least two nucleotide binding sites (Waldman and Murad, 1987). Solubilization of the protein with detergents leads to a loss of ligand stimulation and co-operative GTP binding, together with activation of the guanylate cyclase (Kuno *et al.*, 1986; Paul *et al.*, 1987; Takayanagi *et al.*, 1987; Waldman and Murad, 1987; Meloche *et al.*, 1988). These results suggest the presence of an unstable conformation of the enzyme such as an oligomeric state, or the presence of associated molecules necessary for guanylate cyclase activity or regulation of the enzyme.

The ubiquity of the ANP-A receptor/cGMP second messenger paradigm with respect to other hormones and their receptors is unknown. As a member of the single transmembrane domain class of receptors with a unique signaling function, the ANP-A receptor defines a previously unrecognized signal transduction paradigm.

Materials and methods

Molecular cloning

Approximately 10^6 recombinant phage from an oligo(dT)-primed human kidney cDNA library (Pennica *et al.*, 1987) were screened with 32 P-labeled (Feinberg and Vogelstein, 1983) sea urchin guanylate cyclase cDNA probes A and B (Figure 1) at 42°C in 20% formamide, $5 \times$ SSC, 0.1% $\text{Na}_4\text{P}_2\text{O}_7$, 50 mM NaPO_4 (pH 6.5), $5 \times$ Denhardt's solution, 50 $\mu\text{g}/\text{ml}$ of sonicated

denatured salmon DNA and 10% dextran sulfate for 18 h. Nitrocellulose filters were rinsed at room temperature in $5 \times \text{SSC}$ and washed at 56°C in $5 \times \text{SSC}$, 0.1% SDS. Autoradiography was for 5 h at 80°C with Kodak XAR-5 film and intensifying screens. Of 19 clones picked on the first screen, 15 rehybridized on plaque purification. *EcoRI* cDNA insert fragments were subcloned into M13 mp vectors (Yanisch-Perron *et al.*, 1985) for dideoxynucleotide sequence analysis (Sanger *et al.*, 1977) and three clones (2A, 3A and 9A) were found to be homologous to the sea urchin membrane guanylate cyclase cDNA probes. Clone 9A cDNA fragment was used to screen $\sim 10^6$ phage from a human placenta cDNA library in $\lambda\text{gt}10$ (Ullrich *et al.*, 1985). This screen yielded clone 7A extending from position 831 to the poly(A) tail in Figure 2.

To obtain cloned cDNAs extending further 5' we isolated human kidney and placenta ANP-A receptor cDNAs in an iterative experiment using specifically primed and homopolymer tailed cDNA in a modification (M.-S. Chang and D.V. Goeddel, unpublished data) of the polymerase chain reaction (PCR) procedure (Mullis and Faloona, 1987). Cloned PCR fragments were screened by oligonucleotide hybridization, sequenced, and the experiment was repeated using a further 5' primer based on the PCR-cDNA clone sequence. In this way we obtained the entire cDNA structure of the ANP-A receptor shown in Figure 2. To confirm the cDNA sequence obtained by PCR cloning we isolated a human genomic clone, $\lambda\text{MT1.1}$, from a human genomic library in phage Charon 4A (Lawn *et al.*, 1978) using a 5'-PCR clone as a hybridization probe. $\lambda\text{MT1.1}$ contains the gene encoding the ANP-A receptor and 43 bp of 5'-flanking sequence.

ANP-A receptor expression

Expression of the ANP-A receptor coding sequence was placed under the transcriptional control of the cytomegalovirus long terminal repeat in the expression vector pRK5 (R.Klein and D.V. Goeddel, unpublished data). Coding sequence from the *EcoRI* cloning linker to *EcoRV* (position 863, Figure 2) was from the genomic clone $\lambda\text{MT1.1}$ and further 3' sequences were derived from clone 7A to generate pRKANP-AR.

COS-7 cells maintained in Dulbecco's modified Eagle's medium supplemented with F-12 nutrient mixture, 20 mM Hepes, pH 7.4, 100 U/ml of penicillin/streptomycin and 10% fetal bovine serum, were transfected with plasmid DNA using DEAE-dextran as described by Cullen (1987). Cells were seeded at 6×10^5 cells/60 mm dish or 1×10^6 cells/100 mm plate. Five micrograms and 2 μg of DNA were used for transfecting 100 mM and 60 mM plates respectively. One day after transfection, cells from the 100 mM plates were harvested, and 2×10^5 cells/well were seeded into 12-well Costar cell culture plates, or 5×10^5 cell/well of a 6-well Costar culture plate.

Competition binding assay

Binding assays were performed in 12-well dishes 48 h after transfection with control plasmid pRK or ANP-A expression vector pRKANP-AR. Prior to incubation with [^{125}I]ANP (1-28), confluent monolayers were washed three times with 2 ml of binding buffer [DMEM containing 2 mg/ml bovine serum albumin (BSA) at room temperature]. [^{125}I]ANP (0.5 μCi , sp. act. 2000 Ci/mmol, Amersham) in 0.5 ml of binding buffer was added to the wells in the presence of various concentrations (10^{-11} – 10^{-6} M) of human αANP or the truncated analog rat atriopeptin I and incubated for 30 min at 37°C . Following five 1-ml washes in Hank's balanced salt solution containing 2 mg/ml BSA, cells were dissolved in 1 ml of 0.5 M NaOH and counted in a Beckman gamma 7000 counter. All determinants were in duplicate. Results are expressed as the percentage of specific binding where total binding of [^{125}I]ANP was 13 400 c.p.m. and background binding in the presence of $1 \mu\text{M}$ αANP was 4500 c.p.m.

Guanylate cyclase assay

Cells in 60 mm culture dishes were washed twice with 5 ml cold Hepes-buffered saline, scraped into 3 ml cold 20 mM Hepes, pH 7.4, 50 mM NaCl, 5 mM EDTA, 1 mM dithiothreitol, and homogenized with 40 strokes of a Dounce homogenizer at 0°C . Nuclei were pelleted and the supernatant was centrifuged at 100 000 g in a SW55Ti rotor to obtain a membrane pellet. Membrane proteins were solubilized by incubation on ice for 30 min in solution containing 20 mM Hepes, pH 7.4, 100 mM NaCl, 10% glycerol, 1% Triton X-100 and 1 mM dithiothreitol. The reaction mixture for the guanylate cyclase assay consisted of 20 mM Hepes, pH 7.4, and 1 mM each GTP, MnCl_2 and 0.1 mM isobutyl methylxanthine (IBMX), in a total reaction volume of 250 μl . The incubations were carried out at 37°C for 15 min and were stopped by adding 500 μl of 50 mM acetic acid, pH 6.2, and boiled for 3 min. Samples were analyzed for cGMP by radioimmunoassay.

For measurement of intracellular levels of cGMP, cell monolayers in 6 well Costar plates were washed with DMEM containing 25 mM Hepes

(pH 7.3), and pre-incubated with DMEM containing 25 mM Hepes, 0.1 mM IBMX for 10 min at 37°C . Medium was replaced with DMEM containing 25 mM Hepes, 0.1 mM IBMX and 0.5 μM αANP and the cells incubated for various times. A zero time-point for αANP stimulation was taken at the end of the 10 min pre-incubation. Sample wells were aspirated and cells lysed with 0.5 M perchloric acid. The amount of cGMP in the samples were determined by radioimmunoassay. Results presented are the average of triplicate determinations.

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