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Agonist Selectivity for Three Species of Natriuretic Peptide Receptor-A

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SUMMARY

We determined the nucleotide sequence of mouse natriuretic peptide receptor-A (NPR-A) cDNA and compared the revised deduced amino acid sequence with those of rat and human NPR-A. The ligand selectivity of these three receptor/guanylyl cyclases was examined by whole-cell stimulation of cGMP production. The 28-amino acid atrial natriuretic peptide (ANP) has only one difference among these three species, i.e., human Met-12 versus rat and mouse Ile-12. However, despite the nearly invariant ANP sequence among these species, ANP analogs have marked differences in ED_{50} values and maximal cGMP responses among the three receptors. With the natriuretic peptide analogs we tested, human NPR-A is less sensitive than rat or mouse NPR-A to changes in the 17-amino acid, disulfide-bonded ring of ANP and to the species differences in brain natriuretic peptide (BNP) but is more sensitive to deletions in the carboxyl tail of ANP. The ANP determinants of agonist potency have therefore changed for different species of NPR-A. This is reflected in the amino acid sequence divergence in the receptor extracellular domains and in the divergence and specificity of BNP among species. Our results suggest that the coevolution of NPR-A and BNP has thus been constrained within the context of the conserved ANP sequence.

In mammals there are three homologous natriuretic peptide hormones, referred to as ANP, BNP, and CNP (1–3). ANP and BNP are both heart-derived vasorelaxants that stimulate natriuresis and diuresis (1, 4). Extensive work suggests that the role of ANP in cardiovascular homeostasis is as an antagonist to vasopressin and endothelin and as a direct and indirect antagonist to the hypertensive renin/angiotensin/aldosterone system (1, 5). *In vivo*, BNP appears to have activities similar to those of ANP. Both ANP and BNP are secreted by atrial myocytes under normal conditions, and BNP expression is markedly induced in the ventricles during cardiac hypertrophy (6). Endothelial cells are the most prominent source of CNP so far identified (7). CNP is a vasodilator like ANP and BNP (3), but it does not appear to have a prominent diuretic or natriuretic effect when injected into the circulation (8, 9).

For many of the biological responses to natriuretic peptides, cGMP produced by receptor guanylyl cyclase has been implicated as the second messenger (1). Molecular cloning has identified two members of the NPR/guanylyl cyclase family. NPR-A, or guanylyl cyclase-A (10, 11), is the target for ANP and BNP. In pharmacological studies this receptor has also been referred to as ANP-R_1 (12). For NPR-B or guanylyl cyclase-B (13, 14), the highest affinity, natural ligand so far identified is CNP (15–17).

A third member of the NPR family is NPR-C (18), also known as ANF-R_2 in pharmacological studies (19). The extracellular domain of this receptor is homologous to those of NPR-A and -B, but it has only a 37-amino acid cytoplasmic domain and is not a guanylyl cyclase. NPR-C plays a role in the internalization and degradation of all three natriuretic peptides and comprises >95% of the binding sites in cultured smooth muscle cells and tissues such as lung and kidney cortex (20, 21). In addition, a receptor with NPR-C-like pharmacology mediates changes in cAMP levels (22) and activation of phospholipase-C (23) in response to hormone binding (24). The role of these responses in the *in vivo* physiology of natriuretic peptides is not known.

All three natriuretic peptides have a conserved, 17-amino acid, disulfide-bonded, ring structure, together with amino tails of varying length and composition. The ANP sequence is highly conserved among a number of species (5), with a single difference of either isoleucine or methionine at position 12. Investigations of ANP structure-activity relationships have been complicated by differing potencies in a variety of test systems (25). Assays in chicken, rat, rabbit, mouse, guinea pig, and dog systems have included relaxation of precontracted tissues, receptor binding in tissues and cell lines, and measurements of

ABBREVIATIONS: ANP, atrial natriuretic peptide; hANP, human atrial natriuretic peptide; rANP, rat atrial natriuretic peptide; BNP, brain natriuretic peptide; hBNP, human brain natriuretic peptide; rBNP, rat brain natriuretic peptide; pBNP, porcine brain natriuretic peptide; CNP, C-type natriuretic peptide; AP, atriopeptin; NPR, natriuretic peptide receptor; hNPR, human natriuretic peptide receptor; rNPR, rat natriuretic peptide receptor; mNPR, mouse natriuretic peptide receptor; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

second messenger cGMP production in cultured cells. With even minor modifications to ANP, such as deletion of residues from the amino or carboxyl tails, there are significant species differences in reactivity (26–28).

In addition to synthetic variants of ANP, species differences in the sequence of BNP have provided naturally occurring peptide variants. pBNP (2), rBNP (29), and hBNP (30, 31) share 47–65% identity over 32 residues. Sequence divergence of BNPs is reflected in different potencies for relaxation of porcine and rat vessels (32) and for stimulation of cGMP production in cell lines (17).

The marked specificity of the BNPs between species and their reduced potency, compared with ANP, in stimulating cGMP from cloned NPR-A (14, 33) suggests that there could be a specific BNP receptor/guanylyl cyclase. However, there has not been a direct analysis of the natriuretic peptide structure-activity relations with cloned NPR-A from different species. To directly establish the role of NPR-A in the species-specific differential responses to natriuretic peptides, we have undertaken a detailed hormone concentration-response study of cloned rNPR-A, mNPR-A, and hNPR-A (10, 11, 34). By cGMP measurements in intact 293 cells expressing recombinant receptors, we show that the ANP structural requirements for agonist potency are different for the three species of NPR-A. These differences have arisen in the context of a nearly invariant ANP structure. We suggest that the ANP structure-activity differences are a consequence of evolved changes in the NPR-A ligand binding site that have occurred in parallel with changes in the BNP sequence, which is significantly different in structure and reactivity among species. Our observations suggest that NPR-A could be the *bona fide* BNP receptor/guanylyl cyclase. Sequencing of the mNPR-A cDNA and comparison of rNPR-A, mNPR-A, and hNPR-A sequences indicate that divergence in the extracellular domain parallels the differences in hormone pharmacology, with the human sequence being the most highly divergent among these three orthologous receptors.

Experimental Procedures

Expression of NPR-A. For mNPR-A expression we used the cytomegalovirus-based expression vector pRK, as described previously for hNPR-A (11). Using standard cloning techniques, we eliminated a 5′ untranslated region ATG codon at position 308 (34) by substituting a polymerase chain reaction fragment for the sequence from position 307 to position 1130. The resulting construct was verified by sequencing, which identified numerous differences, compared with the published sequence. As a result we resequenced both strands of the mNPR-A cDNA clone provided to us by S. Singh (University of Texas, Dallas, Texas) (34). Sequence reactions were performed with specific primers, using both the Sequenase kit (United States Biochemicals, Cleveland, OH) and the Ladderman sequencing kit (Takara Biochemicals, Berkeley, CA). For expression of rNPR-A we used the pSVL-based vector described by Chinkers *et al.* (10), and 293 cell lines expressing mNPR-A and rNPR-A were derived as described previously for stable expression of hNPR-A (33).

Synthetic peptides. Natriuretic peptides were purchased from Bachem California (Torrance, CA) or Peninsula Laboratories (Belmont, CA) or synthesized by solid-phase methods. Dried peptides were suspended in 0.1% (v/v) acetic acid and aliquoted into siliconized plastic microcentrifuge tubes. Samples were frozen on dry ice and lyophilized before storage at −20°. Duplicate aliquots were subjected to quantitative amino acid analysis to determine peptide content.

Concentration-response curves for cGMP production. Stable 293 cell lines were maintained in glycine-free F-12 medium/Dulbecco's minimal essential medium (50:50, v/v) with 10% dialyzed fetal calf serum, 10 mM HEPES, and 400 ng/ml G418 (Geneticin; GIBCO-BRL), pH 7.2, in a humidified incubator at 37° with 7% CO_2/93% air. Cells were passaged using phosphate-buffered saline (136.8 mM NaCl, 2.6 mM KCl, 7.9 mM Na_2HPO_4, 1.4 mM KH_2PO_4, pH 7.2) with 0.5 mM EDTA. For concentration-response experiments, logarithmic phase cultures of 293 cell lines were passaged to 12-well plates at a density of 2.5×10^5 cells/well. After 48 hr, cultures were approximately 50% confluent and were used for stimulation of cGMP production. Peptide treatments were carried out essentially as described (33). Briefly, dried peptide aliquots (approximately 10 μg/tube) were resuspended in 0.1% (v/v) acetic acid to a stock concentration of 50 μM and diluted to 1 μM in F-12 medium/Dulbecco's minimal essential medium (50:50, v/v) with 25 mM HEPES, 0.1% (w/v) bovine serum albumin, and 0.1 mM isobutylmethylxanthine, pH 7.2 (stimulation medium). Five-fold serial dilutions were prepared in stimulation medium using polypropylene tubes and were then prewarmed to 37° before use. Cell culture medium was aspirated before addition of 0.5 ml of stimulation medium plus peptides to monolayer cultures for 10 min at 37°. Reactions were stopped by addition of 0.5 ml of ice-cold 12% trichloroacetic acid to give a final volume of 1 ml. Culture plates were frozen on dry ice and stored at −20° before assay. For cGMP measurements, samples were thawed at room temperature and processed as described by Koller *et al.* (15) for cGMP radioimmunoassay (Biomedical Technologies). All stimulations were performed in triplicate, and samples were analyzed in duplicate for cGMP, by radioimmumoassay. Using the KaleidaGraph program on a Macintosh IIci computer, data were fit to a four-parameter equation. Predicted peptide concentrations were corrected based on quantitative amino acid analysis of duplicate samples from the same set of peptide aliquots used for the stimulation experiments.

Results and Discussion

Sequence comparison and expression of three species of NPR-A. To directly compare the pharmacology of rNPR-A, mNPR-A, and hNPR-A, we established stable 293 cell lines expressing these receptors. We previously described the stable expression of hNPR-A (33) and used this cell line in studies of NPR-A signal transduction (35, 36). During the construction of the mNPR-A expression vector, we found numerous differences, compared with the published cDNA sequence (34). To resolve these differences we resequenced the published mNPR-A cDNA clone from nucleotide 348 to nucleotide 3548 (34) and found 34 nucleotide differences, resulting in 16 amino acid substitutions.[1] Homology alignment of mNPR-A, rNPR-A, and hNPR-A is shown in Fig. 1. For the complete precursor sequences, the overall amino acid identity is 98% between rNPR-A and mNPR-A and 91% for both the rNPR-A/hNPR-A and mNPR-A/hNPR-A comparisons. In the mature extracellular domains of 441 amino acids, there is 96.6% identity between rNPR-A and mNPR-A, 85% between rNPR-A and hNPR-A, and 85.5% between mNPR-A and hNPR-A.

Agonist potency and maximum stimulation. Using recombinant receptors, we can unequivocally evaluate both hormone structure-activity relationships and the specificity for signal transduction among different species of NPR-A. For the natriuretic peptides and NPR-A we measured the pD_2 (−log ED_{50}) for cGMP production with nine ANP variants, three species of the 32-amino acid BNP, and 22-amino acid CNP (Table 1). Monolayer cultures of 293 cells were treated with the entire set of natriuretic peptides, using cells from the same

[1] The revised sequence of mNPR-A is available under GenBank accession number L31932.

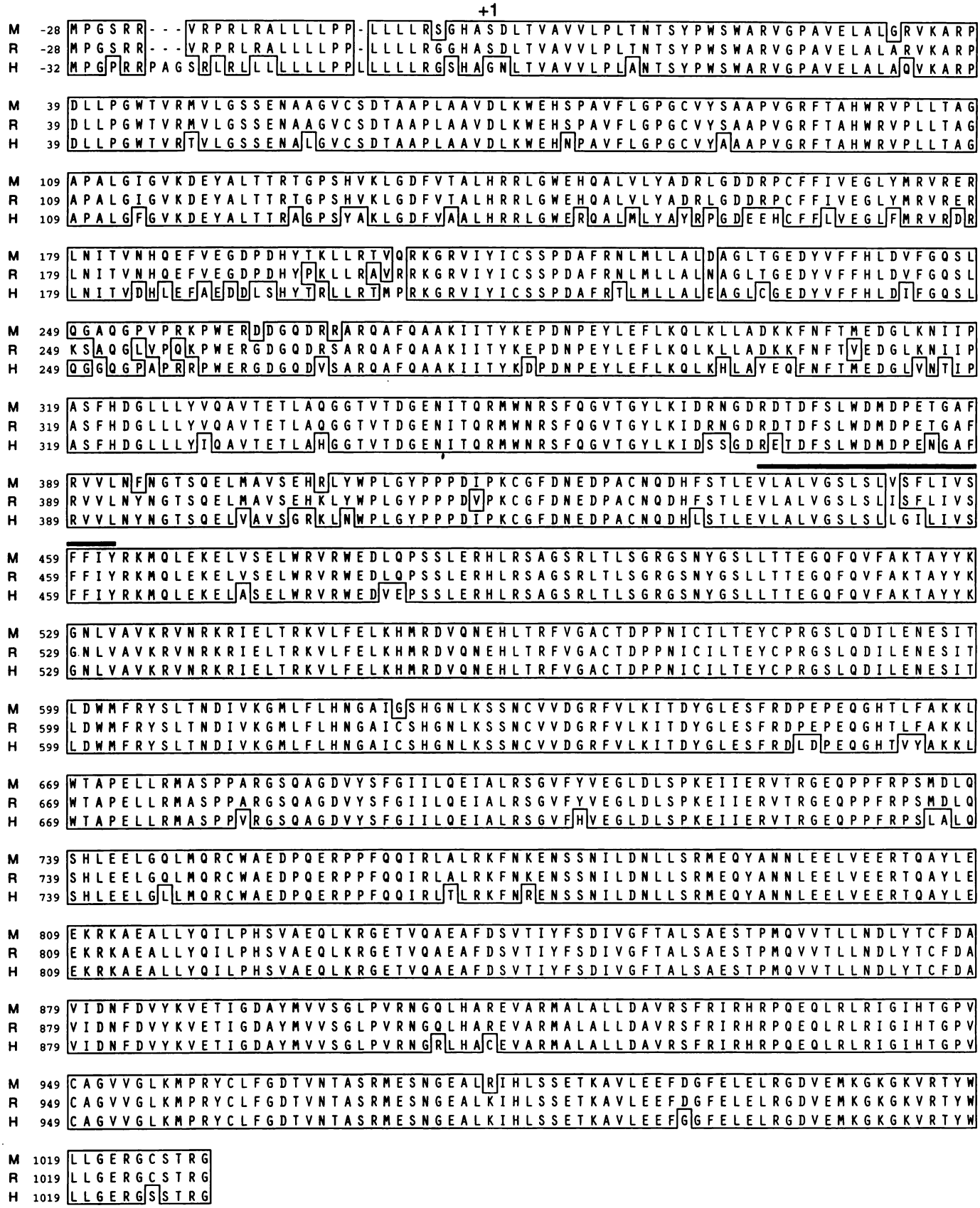

Fig. 1. Amino acid sequence alignment of rNPR-A, mNPR-A, and hNPR-A. The deduced amino acid sequence for the corrected mNPR-A cDNA sequence (*M*) is shown aligned with the rNPR-A (*R*) and hNPR-A (*H*) sequences. Position +1 was determined by amino acid sequencing of purified extracellular domain-IgG fusion protein for hNPR-A and rNPR-A. Amino acids that are identical in all three receptors are *boxed*. The GenBank accession number for the revised mNPR-A cDNA sequence is L31932. The solid overline at positions 442–462 indicates the transmembrane domain.

TABLE 1

Natriuretic peptide sequences

Amino acids different from rANP are underlined. The disulfide bond conserved in all natriuretic peptides is indicated at the top by S—S.

Peptide	Sequence
	S—S
rANP	^{1}SLRRSSCFGGRIDRIGAQSGLGCNSFRY^{28}
AP III	SSCFGGRIDRIGAQSGLGCNSFRY
AP II	SSCFGGRIDRIGAQSGLGCNSFR
AP II(F8Y)	SSCYGGRIDRIGAQSGLGCNSFR
AP I	SSCFGGRIDRIGAQSGLGCNS
hANP	SLRRSSCFGGRMDRIGAQSGLGCNSFRY
hANP(*O*-Met)[a]	SLRRSSCFGGRδDRIGAQSGLGCNSFRY
hANP(F8Y)	SLRRSSCYGGRMDRIGAQSGLGCNSFRY
hANP(R11K)	SLRRSSCFGGKMDRIGAQSGLGCNSFRY
rBNP[b]	^{1}NSKMAHSSSCFGQKIDRIGAVSRLGCDGLRLF^{32}
hBNP	SPKMVQGSGCFGRKMDRISSSSGLGCKVLRRH
pBNP	SPKTMRDSGCFGRRLDRIGSLSGLGCNVLRRY
CNP[c]	^{1}GLSKGCFGLKLDRIGSMSGLGC^{22}

[a] Oxidized methionine at position 12 is indicated by δ.
[b] Amino acids identical among all three sequences are boxed.
[c] Invariant for porcine, rat, and human sequences.

plating. In this way we could compare results for different peptides with one species of receptor, to accurately measure relative potency and maximum stimulation. Quantitative amino acid analysis of the amount of peptide used allowed us to compare results for the different species of NPR-A. A potential concern in comparing concentration-response curves between cell lines is the effect, if any, of receptor number on the ED_{50} values. For NPR-A we do not think this is a significant problem, given the agreement in ED_{50} values for hANP in cells with low expression (1.1 nM) (35), compared with overexpression (0.5–0.6 nM) (33) (Table 1).

Natriuretic peptide concentration-response curves for cGMP stimulation are shown in Fig. 2 for position 8, 11, and 12 ANP variants together with CNP, in Fig. 3 for the AP series, and in Fig. 4 for three species of BNP. The results from curve fitting of the dose-response results are presented in Table 2. For comparative purposes pD_2 values were converted to ED_{50} values and plotted as a bar graph in Fig. 5; maximum stimulation values are plotted as a bar graph in Fig. 6 for six peptides that each gave plateau responses with the three receptors.

ANP analog responses. Significant differences between species are apparent in the ED_{50} values for the analogs we tested (Fig. 2; Table 2). In comparison with rANP, hANP is 7-fold less potent with rNPR-A and 10-fold less potent with mNPR-A but shows only 20% reduced potency with hNPR-A (Fig. 5; Table 2). hNPR-A is sensitive to the methionine to isoleucine substitution in ANP, but both rNPR-A and mNPR-A are markedly more sensitive (Table 2). This result is similar to the reduced potency of hANP, compared with rANP, in the relaxation of rat and mouse aortic rings (28). hANP(*O*-Met) (Fig. 5) has relative potency similar to that of hANP with the different receptors. This analog is 12-fold less potent with hNPR-A but is >100-fold less potent with rNPR-A and mNPR-A, compared with hANP.

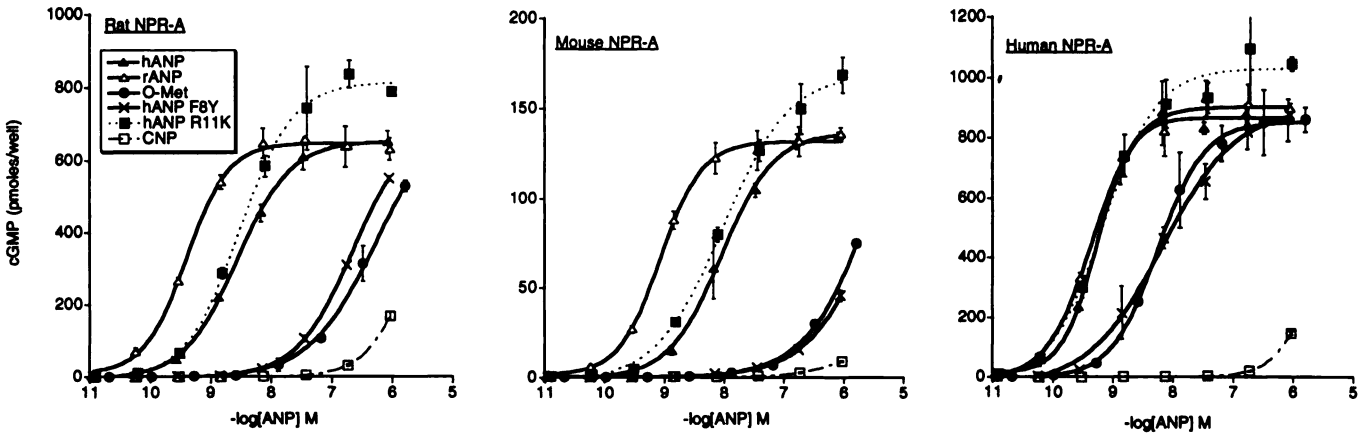

Fig. 2. NPR-A cGMP concentration-response curves for full-length ANP variants and CNP. Monolayer cultures of cell lines expressing rNPR-A, mNPR-A, or hNPR-A were treated in triplicate with serial dilutions of natriuretic peptides (see *key at left*). Samples were analyzed in duplicate for cGMP content, by radioimmunoassay. Results are plotted as the average ± standard deviation of cGMP produced in each well, using a four-parameter curve fit (see Experimental Procedures).

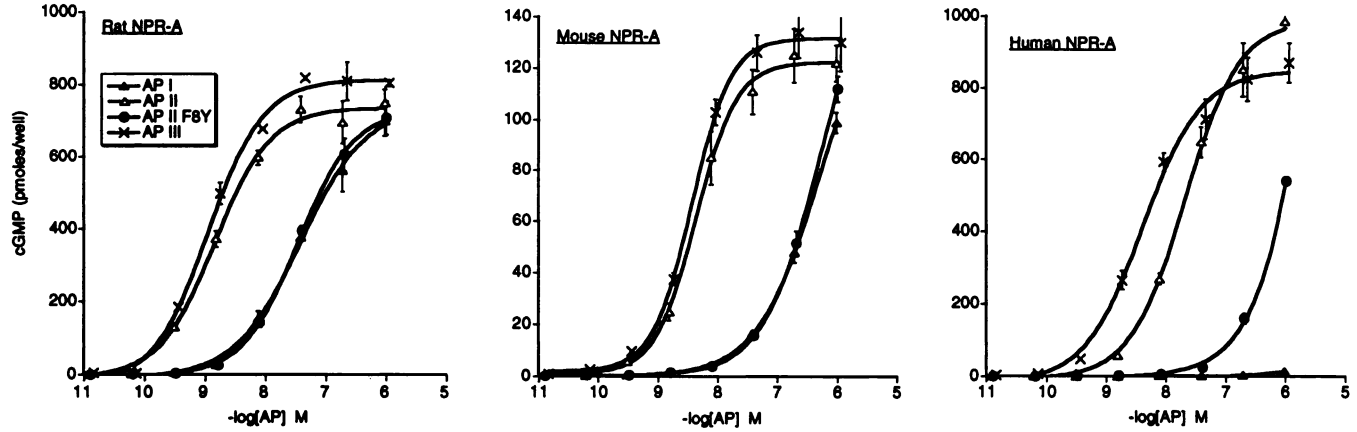

Fig. 3. AP concentration-response curves. Cell lines were treated with natriuretic peptides (see *key at left*) and results are plotted as described in the legend to Fig. 2.

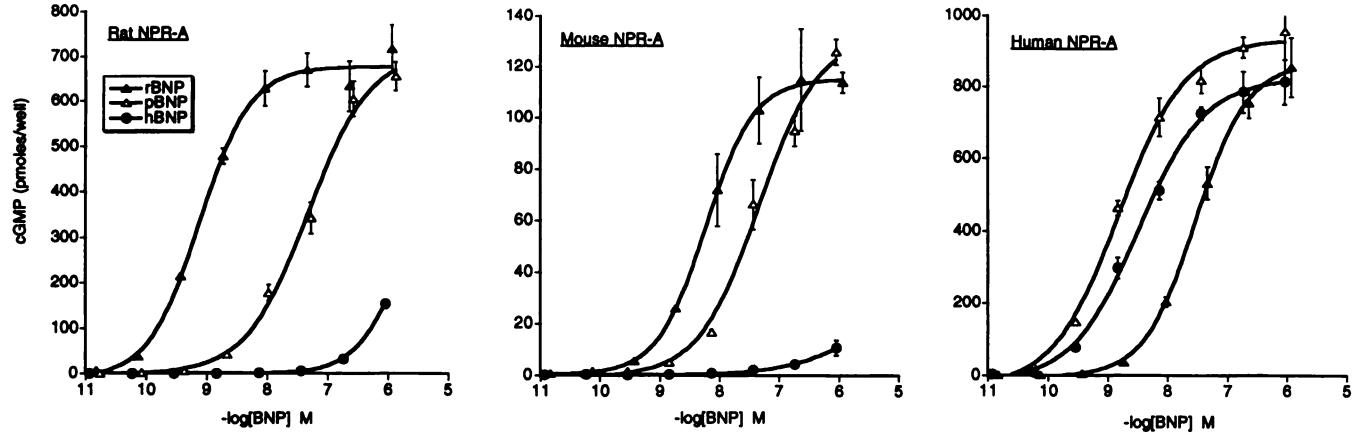

Fig. 4. BNP concentration-response curves. Cell lines were treated with natriuretic peptides (see *key at left*) and results are plotted as described in the legend to Fig. 2.

TABLE 2

Potency of natriuretic peptides for cGMP production with cloned NPR-A from three species

All values are mean ± standard error from three determinations.

Peptide	rNPR-A		mNPR-A		hNPR-A	
	pD_2[a]	Maximum cGMP	pD_2	Maximum cGMP	pD_2	Maximum cGMP
		pmol/well		*pmol/well*		*pmol/well*
rANP	9.4 ± 0.04	648 ± 8	9.1 ± 0.04	132 ± 2	9.4 ± 0.06	903 ± 18
AP-I	7.4 ± 0.07	746 ± 30	6.3 ± 0.04	ND[b]	<6	ND
AP II	8.8 ± 0.06	738 ± 17	8.4 ± 0.04	122 ± 2	7.7 ± 0.05	991 ± 27
AP III	8.9 ± 0.07	814 ± 19	8.4 ± 0.03	132 ± 1	8.4 ± 0.08	850 ± 27
AP II(F8Y)	7.5 ± 0.02	734 ± 10	6.3 ± 0.01	ND	<6	ND
hANP	8.6 ± 0.02	653 ± 6	8.1 ± 0.04	137 ± 3	9.3 ± 0.04	86 ± 14
hANP(*O*-Met)	6.3 ± 0.15	ND	<6	ND	8.3 ± 0.03	854 ± 9
hANP(F8Y)	6.7 ± 0.02	ND	<6	ND	8.2 ± 0.08	898 ± 34
hANP(R11K)	8.5 ± 0.05	816 ± 17	8.0 ± 0.06	170 ± 5	9.2 ± 0.1	1032 ± 37
rBNP	9.1 ± 0.08	677 ± 18	8.2 ± 0.02	115 ± 1	7.5 ± 0.03	869 ± 13
hBNP	<6	ND	<6	ND	8.5 ± 0.05	825 ± 18
pBNP	7.3 ± 0.1	ND	7.4 ± 0.1	ND	8.8 ± 0.08	935 ± 26

[a] pD_2, $-\log ED_{50}$.
[b] ND, not determined.

rANP (Ile-12) is more active than hANP (Met-12) in muscle relaxation and natriuresis assays, and oxidation of Met-12 in hANP severely impairs activity (27, 28). Koyama *et al.* (37) have suggested that hANP(*O*-Met) is relatively selective for the non-guanylyl cyclase NPR-C, compared with NPR-A. In examining the effects of hANP and hANP(*O*-Met) on kidney function in rats, Willenbrock *et al.* (38) found that oxidized hANP could stimulate diuresis but not natriuresis or cGMP secretion. Our results are consistent with the reduced vessel relaxation and kidney natriuresis being due to weak potency of hANP(*O*-Met) with NPR-A.

In comparison with hANP, nearly identical relative potency

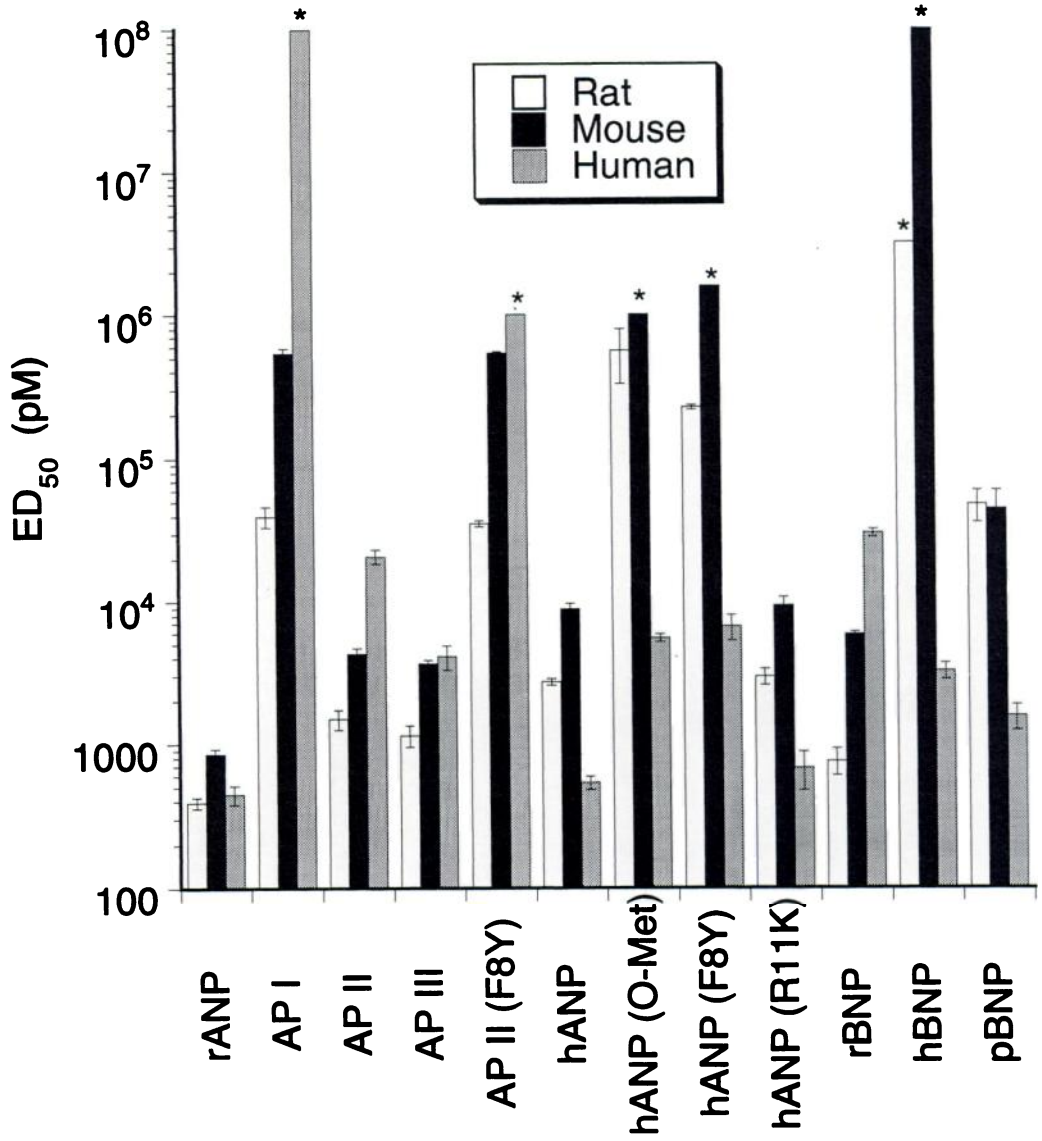

Fig. 5. Bar graph of ED_{50} values for cGMP stimulation. To compare the relative potencies, the pD_2 (−log ED_{50}) values in Table 2 were converted to picomolar values to highlight differences between the peptides. Values for each peptide with the three species of NPR-A are plotted together. *, Values greater than 10^6 pM (1 μM), which are estimates based on extrapolation of the dose-response curves in Figs. 2–4.

is observed for hANP(R11K) (Fig. 5; Table 2). However, the R11K substitution is distinguished by an up to 20% increase in the maximum level of cGMP produced (hormone efficacy), compared with hANP and rANP (Figs. 2 and 6; Table 2). Budzick *et al.* (39) reported that rANP(5–28,R11K) was more potent and more efficacious than rANP in stimulation of cGMP in rabbit vascular smooth muscle. Although the increased potency in the rabbit system is not reflected in our data, enhanced efficacy in cGMP production with the R11K mutation is, suggesting that the published reports measured responses from NPR-A.

For hANP(F8Y), the responses are very similar to those to ANP(*O*-Met) (Figs. 2 and 5). The F8Y substitution has been reported to severely impair cGMP stimulation in vascular smooth muscle cells but produces a <10-fold reduction in potency for relaxation of precontracted aortic rings (39–41). This difference may be accounted for by alternative explanations, other than potency at NPR-A (as discussed below).

Agonist potency in the AP series. In one group of ANP variants, we examined the effect of amino and carboxyl tail truncations with the AP series (Table 1) (42). Deletion of the first four amino acids of rANP to give AP III (Table 1) results in only 3- and 4-fold reductions in potency with rNPR-A and mNPR-A, respectively, but a 10-fold reduction with hNPR-A, compared with rANP. In addition, AP III shows a 25% increase in the maximum rANP-stimulated cGMP level with rNPR-A, but not the other receptors (Fig. 6). When the carboxyl-terminal tyrosine is deleted in the variant AP II (Table 1), only hNPR-A registers a major change in potency, compared with AP III (Fig. 5), with a 5-fold decrease. In addition to potency effects, there are efficacy changes between AP II and AP III, with an increase in the maximal response with hNPR-A and a decrease with rNPR-A (Fig. 6). Further deletion at the carboxyl terminus in AP I (Table 1) results in a >1000-fold loss of potency with hNPR-A, compared with AP II. In comparison, CNP (Fig. 2), which lacks a carboxyl-terminal tail (Table 1), is more potent than AP I (Fig. 3) with hNPR-A. Both rNPR-A and mNPR-A are less sensitive than hNPR-A to the carboxyl-terminal deletion in AP I, with 26- and

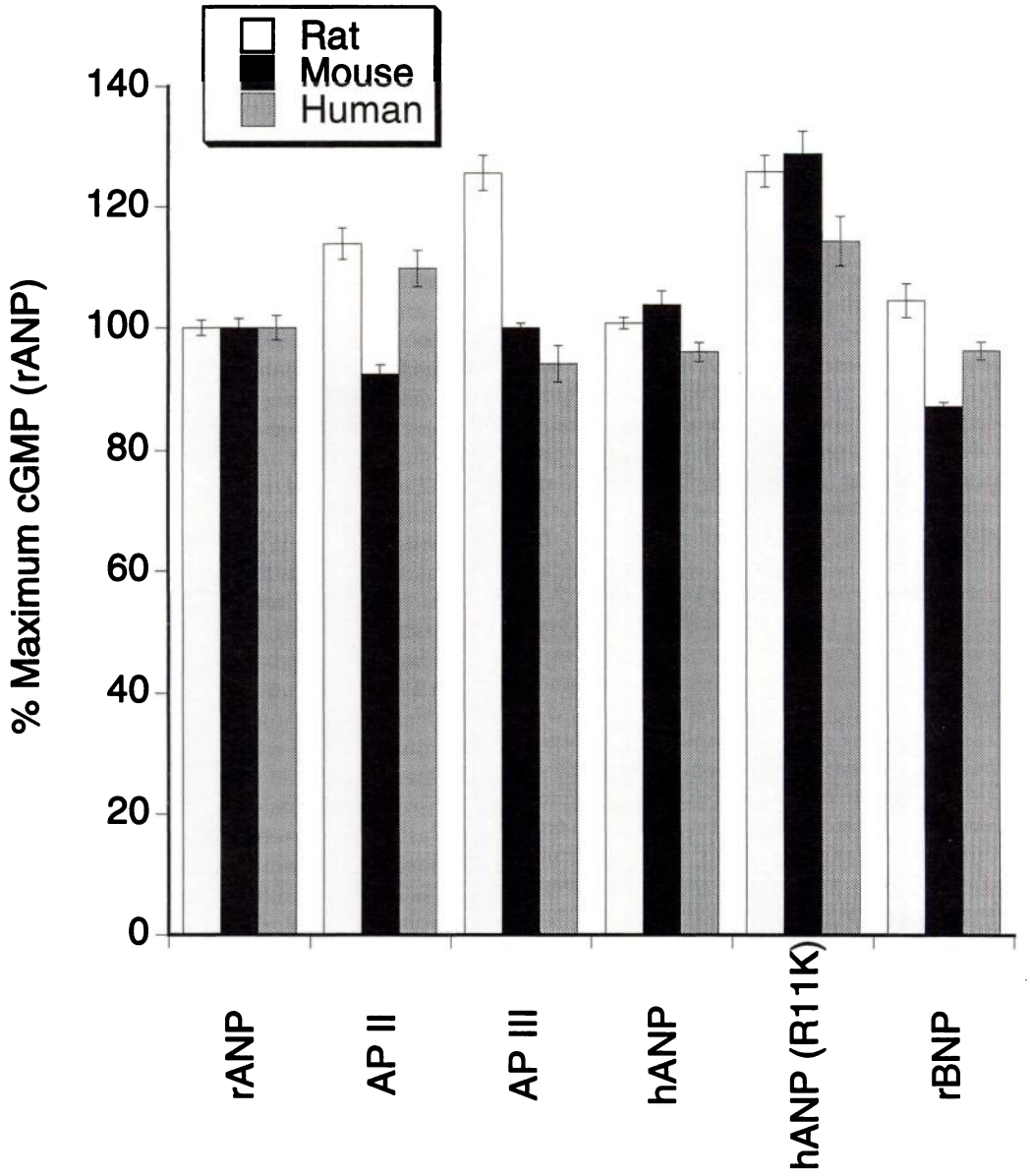

Fig. 6. Bar graph of maximum stimulation values. Results are graphed as the percentage of maximum stimulation observed with rANP (Table 2) for six peptides that showed maximal responses with all three receptors.

123-fold reductions in potency, respectively, compared with AP II. With the AP series of analogs, hNPR-A is distinguished by its sensitivity to truncations in both the amino and carboxyl tails.

One ring substitution mutant, AP II(F8Y), was studied in the rANP-derived AP series. Compared with the parental molecule AP II, there is a loss of potency with all three receptors (Fig. 5; Table 2). The most sensitive is mNPR-A, with a 125-fold reduction, followed by hNPR-A with an approximately 50-fold reduction and rNPR-A with a 23-fold reduction. These results are to be compared with the activity of hANP(F8Y), which shows that hNPR-A is least sensitive to the F8Y substitution. Overall, the combination of several differences between the two F8Y variants makes it difficult to evaluate the relative contribution of this change; however, in both cases the murine receptor is the most sensitive to the position 8 substitution.

The published data on AP show decreased potency with both amino and carboxyl truncations for relaxation of rabbit aorta (43). As with other natriuretic peptide variants, the APs show differences in relative potency in chicken, rat, rabbit, mouse, and guinea pig spasmolytic assays (26–28). Weis (28) reported that AP I and hANP were equipotent in relaxing precontracted rat aorta, but we observe a 10-fold lower potency for AP I with rNPR-A. With mouse aorta AP I is 50% as potent as hANP (28), compared with 50-fold less potent with the cloned receptor.

BNP specificity. The BNPs exhibit species-specific reactivity in a variety of systems (17, 32). Concentration-response data for rBNP, hBNP, and pBNP (Fig. 4) demonstrate marked preferences of the different species of NPR-A for these ligands (Fig. 5; Table 2). hNPR-A is the least sensitive to the sequence variation in this series, with pD_2 values in the range of 8.8–7.5 (Table 2). rNPR-A and mNPR-A are very unresponsive to hBNP, but each has a pD_2 of 7.4–7.3 for pBNP, the most potent BNP with hNPR-A. In comparisons of the cognate ligands for

rNPR-A and hNPR-A, hBNP is less potent with hNPR-A than rBNP is with rNPR-A (Table 2). With rNPR-A, hBNP shows a >100-fold decrease in potency, compared with rBNP, but with hNPR-A rBNP is only 10-fold less potent than hBNP (Fig. 5). In rat thoracic aorta relaxation, the rank order of BNP potency is the same as we observed for stimulation of cGMP with rNPR-A, i.e., rBNP > pBNP > hBNP (32).

Implications of species-specific pharmacology. The data we have presented establish that NPR-A, at least in part, is responsible for the differential activity of natriuretic peptide variants in different test systems. Where direct comparisons can be made with published data, there is concordance between the rank orders of potency in muscle relaxation (28, 32) and stimulation of cGMP production by NPR-A. Differences in the absolute potencies in aortic ring relaxation versus the cGMP assay with cloned receptors are possibly due to the design of the two assay systems. cGMP responses are measurements of cyclic nucleotide produced in 10 min, whereas spasmolytic assays measure relaxation as an indirect effect of receptor occupancy and hence are subject to additional rate-limiting steps beyond the production of cGMP. Alternatively, we cannot formally rule out the possibility of an as yet unidentified receptor guanylyl cyclase subtype that is partly responsible for differences in pharmacology between assay systems.

Several other aspects of the natriuretic peptide system could also, in theory, contribute to different responses *in vivo* or *ex vivo* between species. Differences in the affinity of peptides for the more abundant NPR-C (17, 44), together with different ratios of NPR-A and NPR-C coexpression among species, could be contributing factors. hBNP is more resistant to proteolysis by neutral endopeptidase (EC 3.4.24.11) than is ANP (45), but the sensitivity of other species of BNP is not known. This degradative pathway could have less relative importance for BNPs, compared with ANP. Differences in neutral endopeptidase abundance in various tissues should also be considered as a potential factor in differential responses.

With respect to natriuretic peptide structure-activity relationships, the data we present demonstrate that results obtained using non-human assay systems are of limited predictive value for extrapolating to the human system. To develop natriuretic peptide analogs for therapeutic applications in humans, structure-activity assays most likely need to be based on the human receptors. Recent work on the development of a hNPR-A-specific ANP variant that does not bind to hNPR-C or hNPR-B (46) supports this conclusion. This variant has only three amino acid substitutions in the ANP sequence that impart receptor specificity, but it is unable to bind to or stimulate rNPR-A, despite having full agonist potency with hNPR-A. Hence, the ligand binding site of hNPR-A is different from that of mNPR-A and rNPR-A. This is not surprising, given the degree of divergence (15%) in the extracellular domain of hNPR-A, compared with the other receptors. These sequence differences could be responsible for changes in ligand affinity, agonist potency, or both. The very close identity between rNPR-A and mNPR-A, with only 14 substitutions among 441 amino acids in the extracellular domain, still results in potency differences for AP II(F8Y) and rBNP (Table 2). It should therefore be possible to map residues in rNPR-A and mNPR-A important for signal transduction or ligand binding, using receptor chimeras and point mutagenesis.

Concerted evolution of ligand and receptor. Our data support the conclusion that the structural determinants of the ligand for agonist potency, and presumably binding affinity,[2] have changed since the time of divergence for the three receptors we studied. This is illustrated in particular for hNPR-A, which is much less sensitive to the ligand sequence variation in the BNPs and several ring substitutions in ANP. Conversely, hNPR-A is more sensitive to deletions in the carboxyl-terminal region of ANP, compared with rNPR-A and mNPR-A. The markedly different selectivity of orthologous NPR-A forms suggests that each receptor has been evolving in parallel with its BNP ligand, while under the constraint of maintaining full agonist potency for a ligand (ANP) that is relatively invariant across species. As a result, the elements of the ANP polypeptide sequence that are important for receptor activation have apparently changed. Although we cannot rule out a BNP-specific receptor/guanylyl cyclase, the current knowledge of BNP pharmacology suggests that NPR-A may be the *bona fide* BNP receptor. Additional factors, such as NPR-C binding, may not play a role in selective pressure on the hormones, because this receptor has less stringent sequence requirements for ligand binding. Concerted evolution of ligand-receptor pairs has been described for a variety of systems (47, 48), and so the natriuretic peptides are not unique in this regard. A more complete knowledge of the normal and pathological roles these hormones play might help us to understand the selective pressures that have influenced the evolution of ligand and receptor (47).

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References

1. Brenner, B. M., B. J. Ballermann, M. E. Gunning, and M. L. Zeidel. Diverse biological actions of atrial natriuretic peptide. *Physiol. Rev.* **70**:665–699 (1990).
2. Sudoh, T., K. Kangawa, N. Minamino, and H. Matsuo. A new natriuretic peptide in porcine brain. *Nature (Lond.)* **332**:78–81 (1988).
3. Sudoh, T., N. Minamino, K. Kangawa, and H. Matsuo. A new member of natriuretic peptide family identified in porcine brain. *Biochem. Biophys. Res. Commun.* **168**:863–870 (1990).
4. Aburaya, M., N. Minamino, J. Hino, K. Kangawa, and H. Matsuo. Distribution and molecular forms of brain natriuretic peptide in the central nervous system, heart and peripheral tissue of rat. *Biochem. Biophys. Res. Commun.* **165**:880–887 (1989).
5. Ruskoaho, H. Atrial natriuretic peptide: synthesis, release, and metabolism. *Pharmacol. Rev.* **44**:479–602 (1993).
6. Mukoyama, M., K. Nakao, K. Hosoda, S. Suga, Y. Saito, Y. Ogawa, G. Shirakami, M. Jougasaki, K. Obata, H. Yasue, Y. Kambayashi, K. Inouye, and H. Imura. Brain natriuretic peptide as a novel cardiac hormone in humans. *J. Clin. Invest.* **87**:1402–1412 (1991).
7. Suga, S., K. Nakao, H. Itoh, Y. Komatsu, Y. Ogawa, N. Hama, and H. Imura. Endothelial production of C-type natriuretic peptide and its marked augmentation by transforming growth factor-β. *J. Clin. Invest.* **90**:1145–1190 (1992).
8. Stingo, A. J., A. L. Clavell, L. L. Aarhus, and J. C. Burnett. Cardiovascular and renal actions of C-type natriuretic peptide. *Am. J. Physiol.* **262**:H308–H312 (1992).
9. Clavell, A. L., A. J. Stingo, C. M. Wei, D. M. Heublein, and J. C. Burnett. C-type natriuretic peptide: a selective cardiovascular peptide. *Am. J. Physiol.* **264**:R290–R295 (1993).
10. Chinkers, M., D. L. Garbers, M.-S. Chang, D. G. Lowe, H. Chin, D. V. Goeddel, and S. Schulz. A membrane form of guanylate cyclase is an atrial natriuretic peptide receptor. *Nature (Lond.)* **338**:78–83 (1989).
11. Lowe, D. G., M. S. Chang, R. Hellmiss, E. Chen, S. Singh, D. L. Garbers, and D. V. Goeddel. Human atrial natriuretic peptide receptor defines a new paradigm for second messenger signal transduction. *EMBO J.* **8**:1377–1384 (1989).
12. Fethiere, J., and A. De Lean. Pharmacological evidence for the heterogeneity of atrial natriuretic factor-R_1 receptor subtype. *Mol. Pharmacol.* **40**:913–922 (1991).
13. Chang, M. S., D. G. Lowe, M. Lewis, R. Hellmiss, E. Chen, and D. V. Goeddel.

[2] P. Sehl, J. R. Schoenfeld, D. G. Lowe, unpublished observations.

Differential activation by atrial and brain natriuretic peptides of two different receptor guanylate cyclases. *Nature (Lond.)* **341**:68–72 (1989).
14. Schulz, S., S. Singh, R. A. Bellet, G. Singh, D. J. Tubb, H. Chin, and D. L. Garbers. The primary structure of a plasma membrane guanylate cyclase demonstrates diversity within this new receptor family. *Cell* **59**:1155–1162 (1989).
15. Koller, K. J., D. G. Lowe, G. L. Bennett, N. Minamino, K. Kangawa, H. Matsuo, and D. V. Goeddel. Selective activation of the B natriuretic peptide receptor by C-type natriuretic peptide (CNP). *Science (Washington D. C.)* **252**:120–123 (1991).
16. Bennett, B. D., G. L. Bennett, R. V. Vitangcol, J. R. S. Jewett, J. Burnier, W. Henzel, and D. G. Lowe. Extracellular domain-IgG fusion proteins for three human natriuretic peptide receptors. *J. Biol. Chem.* **266**:23060–23067 (1991).
17. Suga, S., K. Nakao, K. Hosoda, M. Mukoyama, Y. Ogawa, G. Shirakami, H. Arai, Y. Saito, Y. Kambayashi, K. Inouye, and H. Imura. Receptor selectivity of natriuretic peptide family, atrial natriuretic peptide, brain natriuretic peptide, and C-type natriuretic peptide. *Endocrinology* **130**:229–239 (1992).
18. Fuller, F., J. G. Porter, A. E. Arfsten, J. Miller, J. W. Schilling, R. M. Scarborough, J. A. Lewicki, and D. B. Schenck. Atrial natriuretic peptide clearance receptor: complete sequence and functional expression of cDNA clones. *J. Biol. Chem.* **263**:9395–9401 (1988).
19. Anand-Srivastava, M. B., and G. J. Trachte. Atrial natriuretic factor receptors and signal transduction mechanisms. *Pharmacol. Rev.* 455–497 (1993).
20. Nussenzveig, D. R., J. A. Lewicki, and T. Maack. Cellular mechanisms of the clearance function of type C receptors of atrial natriuretic factor. *J. Biol. Chem.* **265**:20952–20958 (1990).
21. Maack, T., J. Okolicany, G. Y. Koh, and D. A. Price. Functional properties of atrial natriuretic factor receptors. *Semin. Nephrol.* **13**:50–60 (1993).
22. Anand-Srivastava, M., M. R. Sairam, and M. Cantin. Ring deleted analogs of atrial natriuretic factor inhibit adenylate cyclase/cAMP system. *J. Biol. Chem.* **265**:8566–8572 (1990).
23. Hirata, M., C.-H. Chang, and F. Murad. Stimulatory effects of atrial natriuretic factor on phosphoinositude hydrolysis in cultured bovine aortic smooth muscle cells. *Biochim. Biophys. Acta* **1010**:346–351 (1989).
24. Levin, E. R. Natriuretic peptide C-receptor: more than a clearance receptor. *Endocrinol. Metab.* **27**:E483–E489 (1993).
25. Bovy, P. R. Structure activity in the atrial natriuretic peptide (ANP) family. *Med. Res. Rev.* **10**:115–142 (1990).
26. Fok, K. F., F. S. Tjoeng, J. A. Houbion, K. L. Spear, S. T. Nugent, S. R. Eubanks, M. E. Zupec, G. M. Olins, D. J. Blehm, and S. P. Adams. Structure-activity studies of atriopeptins, in *Peptides: Structure and Function* (C. M. Deber, V. J. Hruby, and K. D. Kopple, eds.). Pierce Chemical Co., Rockford, IL, 953–956 (1985).
27. Watanabe, T. X., Y. Noda, N. Chino, Y. Nishiuchi, T. Kimura, S. Sakakibara, and M. Imai. Structure-activity relationships of α-human atrial natriuretic peptide. *Eur. J. Pharmacol.* **147**:49–57 (1988).
28. Weis, J. Different inhibitory potencies of various ANF-analogues in the isolated aorta from four rodent species. *Pharmacol. Toxicol.* **68**:282–283 (1991).
29. Kojima, M., N. Minamino, K. Kangawa, and M. Matsuo. Cloning and sequence analysis of cDNA encoding a precursor for rat brain natriuretic peptide. *Biochem. Biophys. Res. Commun.* **159**:1420–1426 (1989).
30. Sudoh, T., K. Maekawa, M. Kojima, N. Minamino, K. Kangawa, and H. Matsuo. Cloning and sequence analysis of cDNA encoding a precursor for human brain natriuretic peptide. *Biochem. Biophys. Res. Commun.* **159**:1427–1434 (1989).
31. Seilhamer, J. J., A. Arfsten, J. A. Miller, P. Lundquist, R. M. Scarborough, J. A. Lewicki, and J. G. Porter. Human and canine gene homologs of porcine brain natriuretic peptide. *Biochem. Biophys. Res. Commun.* **165**:650–658 (1989).
32. Kambayashi, Y., K. Naka, H. Kimura, T. Kawabata, M. Nakamura, K. Inouye, N. Yoshida, and H. Imura. Biological characterization of human brain natriuretic peptide (BNP) and rat BNP: species specific actions of BNP. *Biochem. Biophys. Res. Commun.* **173**:599–605 (1990).
33. Lowe, D. G., and B. M. Fendly. Human natriuretic peptide receptor-A guanylyl cyclase: hormone cross-linking and antibody reactivity distinguish receptor glycoforms. *J. Biol. Chem.* **267**:21691–21697 (1992).
34. Pandey, K., and S. Singh. Molecular cloning and expression of murine guanylate cyclase/atrial natriuretic factor receptor cDNA. *J. Biol. Chem.* **265**:12342–12348 (1990).
35. Lowe, D. G. Human natriuretic peptide receptor-A guanylyl cyclase is self-associated prior to hormone binding. *Biochemistry* **31**:10421–10425 (1992).
36. Jewett, J. R. S., K. J. Koller, D. V. Goeddel, and D. G. Lowe. Hormonal induction of low affinity receptor guanylyl cyclase. *EMBO J.* **12**:769–777 (1993).
37. Koyama, S., T. Terai, T. Inoue, K. Inomata, K. Tamura, Y. Kobayashi, Y. Kyogoku, and M. Kobayashi. An oxidized analog of α-human atrial natriuretic polypeptide is a selective agonist for the atrial-natriuretic-polypeptide clearance receptor which lacks a guanylate cyclase. *Eur. J. Biochem.* **203**:425–432 (1992).
38. Willenbrock, R. C., J. Tremblay, R. Garcia, and P. Hamet. Dissociation of natriuresis and diuresis and heterogeneity of the effector system of atrial natriuretic factor in rats. *J. Clin. Invest.* **83**:482–489 (1989).
39. Budzik, G. P., S. L. Firestone, E. N. Bush, P. J. Connolly, T. W. Rockway, V. K. Sarin, and W. H. Holleman. Divergence of ANF analogs in smooth muscle cell cGMP response and aorta vasorelaxation: evidence for receptor subtypes. *Biochem. Biophys. Res. Commun.* **144**:422–431 (1987).
40. Holleman, W. H., E. E. Bush, E. M. Devine, S. L. Firestone, T. W. Rockway, V. K. Sarin, and G. P. Budzik. Atrial natriuretic factor: structural requirements of the peptide for receptor binding, biological activity, and cGMP stimulation. *Drug Dev. Res.* **12**:109–117 (1988).
41. Minamitake, Y., Y. Kitajima, M. Furuya, M. Yoshida, and S. Tanaka. Importance of hydrophobic residues in *alpha*-human atrial natriuretic peptide (*alpha*-hANP) for vasorelaxant activity. *Biochem. Biophys. Res. Commun.* **172**:971–978 (1990).
42. Currie, M. G., D. M. Geller, B. R. Cole, N. R. Siegel, K. F. Fox, S. P. Adams, S. R. Eubanks, G. R. Galluppi, and P. Needleman. Purification and sequence analysis of bioactive atrial peptides (atriopeptins). *Science (Washington D. C.)* **23**:67–69 (1984).
43. Wakitani, K., T. Oshima, A. D. Loewy, S. W. Holmberg, B. R. Cole, S. P. Adams, K. F. Fok, M. G. Currie, and P. Needleman. Comparative vascular pharmacology of the atriopeptins. *Circ. Res.* **56**:621–627 (1985).
44. Engel, A., J. R. Schoenfeld, and D. G. Lowe. A single residue determines the distinct pharmacology of rat and human natriuretic peptide receptor-C. *J. Biol. Chem.* **269**:17005–17008 (1994).
45. Kenny, A. J., A. Bourne, and J. Ingram. Hydrolysis of human and pig brain natriuretic peptides, urodilatin, C-type natriuretic peptide and some C-receptor ligands by endopeptidase-24.11. *Biochem. J.* **291**:83–88 (1993).
46. Cunningham, B. C., D. G. Lowe, B. Li, B. D. Bennett, and J. A. Wells. Production of an atrial natriuretic peptide variant that is specific for type A receptors. *EMBO J.* **13**:2508–2515 (1994).
47. Murphy, P. M. Molecular mimicry and the generation of host defense protein diversity. *Cell* **72**:823–826 (1993).
48. Moyle, W. R., R. K. Campbell, R. V. Myers, M. P. Bernard, X. Wang, and Y. Wang. Co-evolution of ligand-receptor pairs. *Nature (Lond.)* **368**:251–255 (1994).

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