receptor occupancy in the absence of (–)-[^{3}H]NMS). The dissociation constant of (–)-[^{3}H]NMS (0.070 nM) was determined independently by Scatchard analysis of seven-point (–)-[^{3}H]NMS binding isotherms using a centrifugation assay.[35]

Acknowledgment. We thank Drs. Catarina Ludwig and Lars-Göran Wistrand for valuable advice regarding the preparation of 1-acetyl-2-[(trimethylsilyl)ethynyl]-pyrrolidine. We also thank professor Costa Steliou for kindly providing a personal copy of PCMODEL. Support for this study was provided by grants from the Swedish Natural Science Research Council, Astra Alab AB, U.S. Public Health Service (Grants GM-37816 and MH-17691) and C.D. Carlssons Stiftelse.

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Registry No. 4, 118800-02-5; 4·$C_2H_2O_4$, 118800-20-7; 5, 118800-21-8; 5·$C_2H_2O_4$, 118800-22-9; 6, 118800-03-6; 7, 118800-04-7; 7·$C_2H_2O_4$, 118800-23-0; 8, 118800-05-8; 8·$C_2H_2O_4$, 118800-24-1; 9, 118800-06-9; 10, 118800-07-0; 10·$C_2H_2O_4$, 118800-25-2; 11, 118800-08-1; 11·$C_2H_2O_4$, 118800-26-3; 12, 118800-09-2; 13, 57790-32-6; 14, 57735-00-9; 15, 111886-97-6; 16, 111886-98-7; 17, 105457-63-4; 18, 63853-74-7; 19, 22050-10-8; 20, 88761-59-5; 21, 118800-10-5; 22, 118800-11-6; 23, 118800-12-7; 24, 118800-13-8; 25, 118800-14-9; 26, 118800-15-0; 27, 118800-16-1; 28, 118800-17-2; 29, 63050-21-5; 30, 118800-18-3; 31, 118800-19-4; methyl acrylate, 96-33-3; 4-amino-6-(trimethylsilyl)-5-hexynoic acid, 111886-96-5; bis(trimethylsilyl)acetylene, 14630-40-1; 3-(methoxycarbonyl)-propionyl chloride, 1490-25-1; pyrrolidine, 123-75-1; dimethylamine, 124-40-3.

Supplementary Material Available: ^{13}C NMR chemical data for compounds 4–12, 15–17, and 21–28 and ^{1}H NMR chemical data for compounds 5 and 7–12 (3 pages). Ordering information is given on any current masthead page.

Identification of Structural Requirements for Analogues of Atrial Natriuretic Peptide (ANP)† To Discriminate between ANP Receptor‡ Subtypes

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The structure–activity relationships for affinity and selective binding of atrial natriuretic peptide (ANP) and analogues to guanylate cyclase coupled (CC) and non-cyclase coupled (NC) receptors in rabbit lung membranes are described. We have designed a series of peptides to try to identify the minimal sequence involved in specific recognition of each receptor subtype. The affinity of the peptides was determined from competitive binding experiments. Several peptides derived from the rat ANP sequence, e.g., des-[Phe^{106},Gly^{107},Ala^{115},Gln^{116}]ANP-(103–125)NH_2 (**4**), des-[Cys^{105,121}]ANP-(104–126) (**5**), and [Acm-Cys^{105}]ANP-(105–114)NH_2 (**9**) have high affinity and selectivity for the noncoupled site. Peptide **4** was the most selective ligand with an affinity superior to that of ANP-(103–126). This compound does not displace the radiolabeled ligand from the guanylate cyclase coupled receptor at the highest concentration tested (100 nM). The structure–activity relationship for affinity and selectivity is discussed. Comparison of the peptide sequences suggests that the structural feature responsible for recognition of the NC site resides in a single sequence of seven contiguous amino acids from the cyclic core of the hormone. The corresponding heptapeptide retains affinity to the guanylate cyclase uncoupled binding site and is proposed to encompass the minimal sequence for specific recognition of the non-guanylate cyclase coupled ANP receptor.

Recently, several reports have pointed to the existence of two distinct types of endogeneous binding proteins specific for the peptides of the atrial natriuretic peptide (ANP) family.[1,3,5,25] One subtype is coupled to guanylate cyclase and the other is not.[2,6–12] Although the circulating 28-mer, ANP (99–126), and closely related analogues bind to both types of specific binding proteins with similar subnanomolar affinities, so far, only the class of binding protein coupled to guanylate cyclase (CC, guanylate cyclase coupled) has been firmly established as a receptor correlated with the physiological responses of ANP.[6,21] The role of the non-guanylate cyclase coupled protein is still a matter for debate.[7] Affinity cross-linking studies have

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† Abbreviations are according to the IUPAC-IUB Commission on Biochemical Nomenclature (*Pure Appl. Chem.* **1974**, *40*, 317). The sequence of the human atrial natriuretic peptide (ANP) is as follows: S^{99}-L-R-R-S-S-C^{105}-F-G-G-R-M^{110}-D-R-I^{113}-G-A-Q-S-G-L-G-C^{121}-N-S-F-R-Y^{126}. The rat sequence has an Ile residue instead of the Met residue at position 110 and is abbreviated rat ANP-(99–126). An alternative nomenclature for ANP is published in *New Engl. J. Med.* **1987**, *316*, 1278.

‡ Receptor is taken in a broad sense. As described in the text, only one of the ANP binding proteins has been shown to be associated with a second messenger system producing physiological responses. The second binding protein is actually a binding site and its physiological significance is under study.

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Table I. Sequences and Characteristics of Peptides 1–10

peptides sequences[c,d]	no.	number of aa	M_r
99 105 109 113 121 126			
SSCFGGRIDRIGAQSGLGCNSFRY	1	24	2550.25
DRIGAQSGLGCNSFRY[c]	2	16	1815.01
SLRRSSCFGGRIDRIGAQC[a]	3	19	1951.28
SSC--GRIDRIG--SGLGCNSFR[a]	4	19	1982.25
S-FGGRIDRIGAQSGLG-NSFRY	5	21	2258.5
SSCFGGRIDRIGAQSGLGCNS	6	21	2083.67
SLRRSSCFGGRIDRIGAQSGLGCNSFRY	7	28	3602.27
SLRRSSCFGGR	8	11	1225.56
CFGGRIDRIG[a,c]	9	10	1163.38
GRIDRIG[b]	10	7	826.96

[a] Peptides 3, 4, and 9 have a C-terminal primary carboxamide function. [b] Peptide 10 has a C-terminal primary carboxamide function and is N-acetylated at the N-terminus. [c] The peptides synthesized have been characterized by FAB mass spectrometry and their composition analyzed by amino acid analysis. In most cases the sequence has been verified by Edman degradation sequencing. The peptides containing two cysteines are cyclic by the virtue of a disulfide bond; peptides 2 and 9 have an acetamidomethyl protecting group on the cysteine sulfhydryl side chain. Peptide 8 has a free sulfhydryl group. [d] Gaps resulting from deletions (indicated by dashes) are allowed in the sequences to facilitate the perception of alignment.

shown that the relative proportion of cyclase-coupled (CC, guanylate cyclase coupled) and non-cyclase coupled binding sites (NC, guanylate cyclase uncoupled, "C-receptor") depends on the type of cells or tissues.[7,21,25] The cyclase coupled receptor usually constitutes only 1 to 30% of the total ANP binding sites.[4,7,21,25]

The finding of analogues endowed with selectivity and high affinity for the different subtypes of binding sites is of great importance since they will contribute to further investigations of the pharmacology of ANP. Previous structure–activity relationships in bovine aortic smooth muscle (BASM) cells have indicated that, in a series of rat ANP and human ANP analogues, deletion of the N-terminal amino acids decreases cGMP production activity but preserves binding to the ANP-specific binding sites.[21] In subsequent reports, we and others have reported that various linear analogues of ANP bind specifically to the NC binding site in rabbit lung membranes,[4,25] in cultured vascular cells, in isolated perfused kidney,[14] and in rat Leydig tumor cells.[34] The smallest reported fragment of native ANP sequence that still binds to receptors in cultured vascular cells is an octapeptide, ANP-(106–113)-NH_2.[14] Other groups have reported no or very weak binding activity for various other linear peptides.[17,32,33]

Several peptidic hormones of medium size (15–50 amino acids) have been shown to contain a minimal sequence of 5–10 residues that constitutes the recognition site for binding to their receptor (for a review, see ref 13). We have designed and synthesized a series of model peptides derived from ANP primary sequence in an effort to identify the minimal sequences responsible for binding of ANP to its receptors. We report the results of this study in which we determine the ability of our model peptides to bind and,

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possibly, to discriminate between the CC and NC binding proteins present in rabbit lung membranes. By utilizing this approach, we address the following questions: (1) What are the minimal sequences for specific recognition of the ANP binding proteins? (2) Are these sequences (if identified) distinct or overlapping?

Chemistry: Design and Synthesis of the Model Peptides

The basic strategy to identify a minimal sequence that is responsible for a biological activity of a peptide relies on the gradual shortening of its sequence. However, one limitation of this strategy is that it does not consider the secondary structure. There is the possibility that the structural elements responsible for recognition are distributed on nonadjacent sections of the primary sequence but that the folding of the peptide brings them together in the binding conformation. Shortened fragments might then either lack one or several important residues or be unable to fold properly. These limitations are particularly crucial for ANP when truncations affect the cyclic core. Thus, progressive shortenings of the ANP sequence can be done in two ways. In one approach, the cysteine is deleted along with other amino acids, leading to a linear peptide. In the other approach, residues within the cyclic core are deleted while the cystine bridge is preserved, leading to peptides with reduced ring size. Both approaches have been followed, resulting in the selection of peptides **2**, **3**, **4**, **8**, and **9** (Table I) as models for this study. As a means to assess the effect of the cyclic structure on the binding, the linear analogue **5**, in which the cystine bridge is deleted, was included.

The peptides designed on the basis of these considerations were synthesized by established methodologies of solid-phase synthesis on 4-methylbenzhydrylamine (MBHA) or on (phenylacetamido)methyl (PAM) resin with the aid of an Applied Biosystems Inc. peptide synthesizer, Model 430A. Reverse-phase high-performance liquid chromatography (RP-HPLC) was used for the final purification. The details of the synthetic and purification steps are reported in the Experimental Section. The sequence and characteristics of the peptides studied are reported in Table I. The peptides used in this study are derived from the rat ANP sequence and our reference compound **1** is rat ANP-(103–126) (atriopeptin III[15]).

Biology

Competitive binding studies and affinity cross-linking experiments using a selective ANP analogue have revealed the presence in rabbit lung membranes of two populations of specific ANP binding sites.[4,25] This tissue has been shown to contain the CC and NC binding proteins in a ratio of 27:73. The smaller population of ANP binding proteins is associated with activation of guanylate cyclase activity while the larger population is not coupled to any known second messenger system.[25] In the present study, the affinities of the peptides for the CC and/or the NC binding sites subtypes existing in rabbit lung membranes were simultaneously assessed by the examination of the competitive displacement curves of ^{125}I-ANP-(103–126) performed as described previously[25] (see Experimental Section). This binding assay provided us with a very powerful tool to dissociate the specific binding to each site by deconvolution of the displacement curves and determination of the IC_{50} for each binding site.[27] The methods are further described in the Experimental Section and in ref 25.

Results and Discussion

From the data reported in Table II and in Figures 1–3, only two peptides, [Acm-Cys^{121}]ANP-(111–126) (**2**) and ANP-(99–109) (**8**) fail to displace any radiolabeled ligand (Figure 2) at the highest concentration tested (100 nM).

Table II. Comparison of the Inhibition Constants (IC_{50} in nM) of Peptides 1 to 10 for the ANP Guanylate Cyclase Coupled Receptor (CC) and Non-Guanylate Cyclase Coupled (NC) Binding Site in Rabbit Lung Membranes

peptide	IC_{50}^{NC}, nM[a]	rel affin[d]	IC_{50}^{CC}, nM[a]	rel affin[d]	selectivity factor[c]
1	0.29	1.0	0.29	1.000	1
2	>100	<0.003	>100	<0.003	*b*
3	0.16	2.4	52	0.008	312
4	0.1	3.2	>100	<0.003	>1000[b]
5	0.35	0.7	>100	<0.003	>285[b]
6	0.78	0.2	36	0.005	46
7	0.054	3.5	0.054	3.5	1
8	>100	<0.003	>100	<0.003	*b*
9	0.28	0.5	>100	<0.003	>357
10	65	0.005	>100	<0.003	>1.5[b]

[a] Concentration of the peptide inhibiting specific binding by 50% (IC_{50}) was derived from analysis of plots of the percentage of specific binding versus the log concentration of the peptides (at least ten concentrations ranging over 5 orders of magnitude were used). Data are derived from one experiment performed with doses in duplicate. [b] By definition of the selectivity factor, this is a minimal value for the peptide; in fact, the selectivity might be considered as total when no binding can be detected up to 100 nM peptide. [c] Selectivity factor is the ratio of the IC_{50}^{CC} to the IC_{50}^{NC} for each compound considered. [d] Relative affinities are determined by using ANP-(103–126) as a standard (given an arbitrary value of 1.0) in each individual binding experiment.

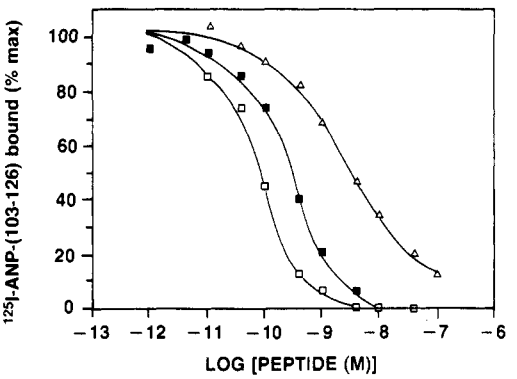

Figure 1. Competition for ^{125}I-ANP-(103–126) binding to rabbit lung membranes by various ANP analogues. The unlabeled peptides were incubated with 130 pM ^{125}I-ANP-(103–126) and 100 μg of rabbit lung membranes as described in the Experimental Section: [■] ANP-(103–126) (**1**); [□] ANP-(99–126) (**7**); [△] ANP-(103–123) (**6**).

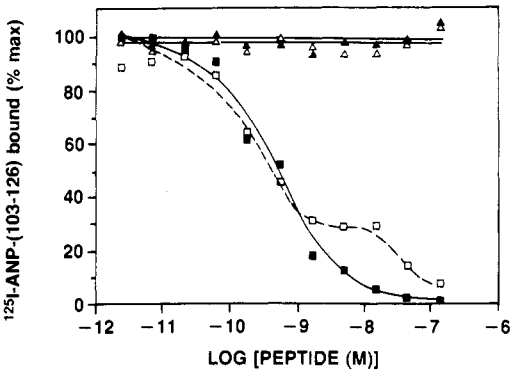

Figure 2. Competition for ^{125}I-ANP-(103–126) binding to rabbit lung membranes by various ANP analogues. The unlabeled peptides were incubated with 130 pM ^{125}I-ANP-(103–126) and 100 μg of rabbit lung membranes as described in the Experimental Section: [■] ANP-(103–126) (**1**); [△] [Acm-Cys^{121}]ANP-(111–126) (**2**); [□] [Cys^{116}]ANP-(99–116)NH_2 (**3**); [▲] ANP-(99–109) (**8**).

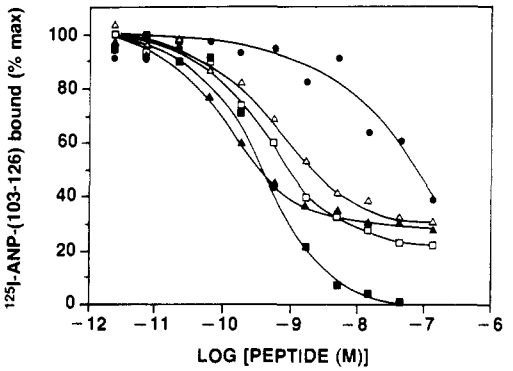

Figure 3. Competition for ^{125}I-ANP-(103–126) binding to rabbit lung membranes by various ANP analogues. The unlabeled peptides were incubated with 130 pM ^{125}I-ANP-(103–126) and 100 μg of rabbit lung membranes as described in the Experimental Section: [■] ANP-(103–126) (**1**); [▲] des-[Phe^{106},Gly^{107},Ala^{115},Gln^{116}]ANP-(103–125)NH_2 (**4**); [□] des-[Cys^{105,121}]ANP-(104–126) (**5**); [△] [Acm-Cys^{105}]ANP-(105–114)-NH_2 (**9**); [●] [N-Ac-Gly^{108}]ANP-(108–114)NH_2 (**10**).

The other analogues have similar affinities, characterized by subnanomolar IC_{50}, for the NC binding site (with the exception of **10** whose case will be discussed later). On the other hand, the affinities of analogues **1**–**9** for the CC receptor extend over a wide range of values. On the basis of this property, the analogues can be separated in three classes.

The first class is constituted by the 28-mer hormone **7** and its N-terminal deletion analogue **1**, ANP-(103–126). Both have equal affinity for both binding sites as previously described[16] and display a displacement curve that fits a one-site model (Figure 1).

The second class is represented by two peptides, ANP-(103–123)NH_2 (**6**) and [Cys^{116}]ANP-(99–116)NH_2 (**3**). Both exhibit considerably reduced affinities for the CC receptor site. This situation leads to displacement curves that fit a two-site model. This is particularly striking for **3** (Figure 2).

Representative of the third class are the peptides des-[Phe^{106},Gly^{107},Ala^{115},Gln^{116}]ANP-(103–125)NH_2 (**4**), des-[Cys^{105,121}]ANP-(104–126) (**5**), and [Acm-Cys^{105}]-ANP-(105–114)NH_2 (**9**), which could only displace approximately 75% of the radiolabeled ^{125}I-ANP-(103–126) at the highest concentration (100 nM) tested (Figure 3). In the case of **5**, this has been shown to correlate with selective binding to the NC binding protein by cross-linking experiments and absence of stimulation of guanylate cyclase activity upon binding.[25]

The set of analogues that we have reported here allows us to make the following statements about the structure–activity relationships. The structural elements responsible for binding to the cyclase coupled receptor span the entire sequence of the hormone. None of the model peptides showed preference for this receptor and the cystine bridge was required for binding. Amino acids at the C-terminal end influence the selectivity more than the N-terminal residues which influence overall affinity for both sites.

Structural requirements for binding to the NC site are better defined. First, neither the cyclic structure nor the presence of cysteines is required for binding to the NC site in sharp contrast with what is observed for the CC receptor. Second, a sequence of seven adjacent amino acids from the cyclic core (Gly^{108} to Gly^{114}) is the only element common to all the peptides that bind to the NC site. Therefore this sequence is proposed to have a unique role in the recognition of the NC site. In support of this, it can be pointed out that the two peptides lacking affinity in the binding assay (**2** and **8**) were also lacking part of that sequence.

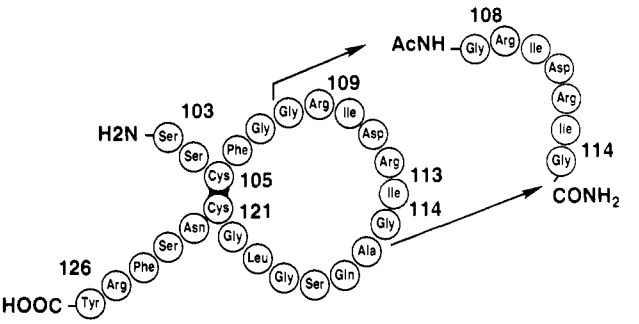

Figure 4. Primary sequence of rat ANP-(103–126) and its N-terminal-acetylated ANP-(108–114)NH_2 fragment in which reside the structural elements responsible for specific recognition of the non-guanylate cyclase coupled receptor. The heptapeptide must have the charged groups at both termini masked in order to bind.

Stimulated by the observation that a short sequence was a common fragment of all active peptides, we synthesized the heptapeptide comprising the sequence Gly^{108}–Gly^{114} of rat ANP. The peptide was prepared as a C-terminal amide and had to be acetylated on the N-terminal position. These modifications were introduced to mask the terminal charged groups in the model peptide. Indeed, we have observed that the same analogue, without the acetyl group at the N-terminus, does not displace any radiolabeled ligand at 100 nM (data not shown). This can be rationalized by the consideration that coulombic interactions resulting from the presence of charged groups on both termini of the analogue **10** are not tolerated by the binding site. In accord with our hypothesis, the protected peptide **10** was found to bind very tightly to the guanylate cyclase uncoupled binding site with an IC_{50} of the order of 60 nM. As expected, no displacement of the radiolabeled ligand from the guanylate cyclase coupled receptor was observed. As calculated from the experimental data[35,36] the free energy of binding ($\Delta G° = -RT \ln K$) of **10** to the NC receptor is approximately 80% of that determined for **1**. Thus, most of the specific binding of the hormone ANP to the NC receptor could be accounted for by interaction with no more than seven amino acid residues.[36] When compared to the decapeptide **9**, our observations with **10** suggest that, in rabbit lung membranes, the sequence Cys^{105}-Phe^{106}-Gly^{107} is not required for specific binding but provides additional affinity. These observations correlate well with the results reported by Scarborough et al. in that the shortest fragment of ANP which binds the "C-receptor" in cultured vascular cells is rat ANP-(106–113)NH_2.[14] The high affinity of our analogue **4** in which Phe^{106}-Gly^{107} is deleted indicate that this additional affinity can be provided by other residues. On the other hand, we have observed the absolute necessity for the Ile^{113} carboxylic group to be masked: we have tested ANP-(106–113) in rabbit lung membranes and found it devoid of affinity to either binding sites (data not shown). Thus, the NC binding site has well-defined specific structural requirements for its ligand (Figure 4).

Interestingly, former studies have indicated that the sequence Arg^{109} to Ile^{113} plays a crucial role in the recognition of the CC receptor and in the activation of the biological response.[17–20,24] It had also been suggested that this sequence forms an a-helix in the native hormone,[24]

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although spectroscopic data (NMR[28,29] and IR[30,31]) on ANP analogues do not indicate the presence of an α-helix in solution.

With respect to the questions that this study has sought to address (see introduction), we conclude the following. The recognition sequences for the two ANP-binding proteins present in the rabbit lung membranes share a common structural element. The sequence Gly^{108} to Gly^{114} (and most probably its core Arg^{109} to Ile^{113}) plays an important role in the recognition by both binding sites. However, while this is so far the shortest sequence for binding to the NC site, additional structural elements are necessary for interaction with the CC receptor. It appears that this latter receptor site tolerates few changes with respect to the basic sequence of the circulating hormone. This is in accordance with the observations that biological activity is dramatically decreased with analogues that contain small modifications with respect to the structure of the circulating hormone.[17-20]

We have reported the design and synthesis of several peptides that discriminate between two specific binding sites for ANP. While we have found no fragment of ANP endowed with measurable affinity solely for the CC receptor, we have reported selective ligands for the NC site. One compound recognizes exclusively the guanylate cyclase uncoupled site with affinities superior to that of ANP-(103-126). Furthermore, we have identified crucial structural elements for specific recognition of the atrial natriuretic peptide NC binding site in rabbit lung membranes and have shown that a N- and C-termini protected heptapeptide binds selectively to this site.

Experimental Section

Peptide Synthesis and Purification. Synthesis and Materials. ANP-(103-126) (1) was obtained from the Searle Development group, St. Louis, MO. ANP-(99-126) (7) and ANP-(99-109) (8) were purchased from Peninsula Laboratories.

Novel peptides were synthesized by solid-phase synthesis using an Applied Biosystems Inc. Model 430A peptide synthesizer. The synthesis was performed with 4-methylbenzhydrylamine functionalized, 1% cross-linked polystyrene resin (USBC, 800 mg of 0.65 mequiv/g) or with a Boc-L-(BrZ)tyrosyl-Pam resin[23] (Applied Biosystems Inc., 700 mg of 0.7 mmol/g) serving as an insoluble support that generated respectively a carboxamide or a carboxylic acid C-terminus in the final product. Most protected amino acids were purchased from Peninsula Laboratories or Peptide International Inc. The *tert*-butyloxycarbonyl group (Boc) was used for amino protection during the coupling procedure. Reactive side-chain protections were Arg, N^g-tosyl; Asp, *O*-benzyl; Cys, *S*-acetamidomethyl; Ser, *O*-benzyl; Tyr, *O*-[2-(bromobenzyloxy)carbonyl].

The peptides were assembled by using the standard protocols of the Applied Biosystems Inc. System Software version 1.30 except for the coupling of cysteine that required a customized activation cycle using dimethylformamide (DMF) as the solvent.

All reagents and solvents were ACS grade or better and used without further purification.

Cleavage and Deprotection. The removal of the peptides from the resin and simultaneous deprotection of side chains (with the exception of the acetamidomethyl group)[26] were achieved by treatment with anhydrous hydrogen fluoride (HF, 10 mL/g of resin bound peptide) containing anisole (0.1 mL/mL of HF) and 2-mercaptopyridine (20 mg/mL of HF) for 60 to 90 min at 0 °C in a Peptide Institute type I HF reaction vessel. The HF was removed under vacuum and the crude residue was triturated and washed with a 1:1 ethyl acetate/diethyl ether mixture (3 × 60 mL). The crude peptide was extracted with 80% aqueous acetic acid, concentrated in vacuo, and lyophilized. Acm-cysteine protecting group can subsequently be removed by treatment with $Hg(OAc)_2$ and H_2S.[26]

Cyclization.[22] To a magnetically stirred solution of 400 mg of iodine in 200 mL of a 1:4 water/acetic acid mixture was added a 200-mg aliquot of the crude peptide to be cyclized. The progression of the cyclization reaction was monitored by analytical HPLC. After 1-6 h of stirring at 25 °C, 200 mL of deionized water was added and the resulting mixture was extracted twice with 500 mL of chloroform and once with 300 mL of diethyl ether. The aqueous phase was concentrated and lyophilized.

Purification and Characterization. The peptide solution in water containing 0.05% trifluoroacetic (TFA) was initially filtered through a glass fiber filter (Whatman 934 AH). The peptide was purified by preparative, reverse-phase, high-performance liquid chromatography on C_{18} bonded silica gel. The crude peptide was eluted on a Vydac C_{18} column (218TP510, i.d. 10 mm, length 25 cm, 5-μm particle size or 218TP1520-22, i.d. 22 mm, length 25 cm, 15-20 μm particle size) fitted to a Waters Dual 510 pumps system with a linear gradient (4 or 9 mL/min flow rate) of aqueous acetonitrile containing a constant concentration of TFA (0.05%). The linear gradient (15 to 35% of acetonitrile in water over 25 min) was generated by a Waters automated gradient controler and the eluent was monitored at 210 nm with a Waters Model 481 detector. Fractions were collected manually and their homogeneity was established by analytical reverse-phase HPLC using a Vydac C_{18} support (218TP54, 4.5 mm × 25 cm, 5-m particle size and 30-nm pore size) with two different gradients. The fractions of homogeneity greater than 99% (estimates based on the integrated area under the peptide peak versus the total integrated area recorded at 210 nm) were pooled and lyophilized. The homogeneous peptides were further characterized by fast atom bombardment mass spectroscopy (VG ZAB E/SE), by amino acid analysis of the hydrolyzates (110 °C, 24h) on a Beckman 6300 analyzer, and by sequencing on an Applied Biosystems protein sequencer Model 470A using the Edman degradation method.

[Acm-Cys^{121}]ANP-(111-126) (2). Amino acid analysis (theoretical, found): Asp, Asn (2, 2.10); Ser (2, 1.78); Gln (1, 1.07); Gly (3, 3.07); Ala (1, 1.00); Ile (1, 0.88); Leu (1, 1.09); Tyr (1, 0.92), Phe (1, 1.03); Arg (2, 1.65). FAB mass spectrum (base peak of isotope cluster, $[M + H]^+$): 1815.

[Cys^{116}]ANP-(99-116)NH_2 (3). Amino acid analysis (theoretical, found): Asp (1, 1.21); Ser (3, 2.84); Gly (3, 3.53); Ala (1, 1.25); Ile (2, 2.10); Leu (1, 1.15); Phe (1, 1.04); Arg (4, 4.88). FAB mass spectrum (base peak of isotope cluster, $[M + H]^+$): 1952.

des-[Phe^{106},Gly^{107},Ala^{115},Gln^{116}]ANP-(103-125)NH_2 (4). Amino acid analysis (theoretical, found): Asp, Asn (2, 2.09); Ser (4, 3.61); Gly (4, 4.39); Ile (2, 1.82); Leu (1, 1.00); Phe (1, 0.94); Arg (3, 3.14). FAB mass spectrum (base peak of isotope cluster, $[M + H]^+$): 1983.

des[$Cys^{105,121}$]ANP-(104-126) (5). Amino acid analysis (theoretical, found): Asp, Asn (2, 2.08); Ser (3, 2.42); Gln (1, 1.12); Gly (5, 4.76); Ala (1, 1.00); Ile (2, 1.82); Leu (1, 1.04); Phe (2, 2.05); Arg (3, 2.84). FAB mass spectrum (base peak of isotope cluster, $[M + H]^+$): 2259.

ANP-(103-123) (6). Amino acid analysis (theoretical, found): Asp, Asn (2, 2.12): Ser (4, 3.56); Gln (1, 1.07); Gly (5, 4.78); Ala (1, 1.03); Ile (2, 1.88); Leu (1, 1.03); Phe (1, 1.00); Arg (2, 1.89). FAB mass spectrum (base peak of isotope cluster, $[M + H]^+$): 2084.

[Acm-Cys^{105}]ANP-(105-114)NH_2 (9). Amino acid analysis (theoretical, found): Asp (1, 0.97); Gly (3, 2.83); Ile (2, 1.78); Phe (1, 1.00); Arg (2, 1.84). FAB mass spectrum (base peak of isotope cluster $[M + H]^+$): 1164.

[*N*-Ac-Gly^{108}]ANP-(108-114)NH_2 (10). Amino acid analysis (theoretical, found): Asp (1, 1.05); Gly (2, 2.00); Ile (2, 1.94); Arg (2, 1.96). FAB mass spectrum (base peak of isotope cluster, $[M + H]^+$): 827.

Binding Assay. Rabbit lung membranes were used to study the competitive binding of atrial natriuretic peptides and were prepared as previously described.[16] The binding assay consisted of 0.25 mL of a solution containing 50 mM Tris pH 7.5, 0.1% bovine serum albumin, 100 μg of membrane protein, and [^{125}I]-ANP-(103-126) (2-3 × 10^4 cpm) in the absence or presence of unlabeled peptide. The competition experiment was initiated by the addition of membranes and the mixture was incubated at 25 °C for 30 min. The incubation was terminated with ice-cold 50 mM Tris pH 7.5 and the mixture was filtered to separate membrane-bound labeled peptide from free ligand by using a PHD cell harvester (Cambridge Technology, Inc., Cambridge, MS). The incubation tube and filter were washed with ice-cold buffer. Filters

were assayed for radioactivity in a Micromedic gamma counter. Nonspecific binding was defined as binding in the presence of 10^{-6} M ANP-(103-126).[25] Specific binding was calculated as total binding minus nonspecific binding. Binding data was analyzed by using a nonlinear curve fitting program.[27]

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Synthesis and D_2 Dopaminergic Activity of Pyrrolidinium, Tetrahydrothiophenium, and Tetrahydrothiophene Analogues of Sulpiride

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All of the existing dopamine receptor models recognize the amine nitrogen of agonist and antagonist drugs as playing a crucial role in receptor interactions. However, there has been some controversy as to which molecular form of the amine, charged or uncharged, is most important in these interactions. We have synthesized and examined the biological activity of permanently charged and permanently uncharged analogues of the dopaminergic antagonist, sulpiride. Sulpiride and the permanently charged pyrrolidinium (**6**, **7**) and tetrahydrothiophenium (**9**) analogues were able to antagonize the inhibitory effect of apomorphine on the K^+-induced release of [^{3}H]acetylcholine from striatal slices. In contrast, the permanently uncharged tetrahydrothiophene analogue **8** was inactive at concentrations up to 100 μM. Additionally, both sulpiride and the tetrahydrothiophenium analogue were able to displace [^{3}H]spiperone from D_2 binding sites, while the tetrahydrothiophene analogue was unable to produce any significant displacement. These results are consistent with our previous observations on permanently charged chlorpromazine analogues and provide further evidence that dopaminergic antagonists bind in their charged molecular forms to anionic sites on the D_2 receptor.

Dopamine antagonists are an important class of therapeutic agents and are useful in a variety of pathological conditions. The design of new dopamine antagonists with improved safety and efficacy has been aided in the past few years by the development of several very useful dopamine receptor models.[1-7] To a certain extent, these models complement one another; however, several discrepancies do exist. One of the major discrepancies that needs to be resolved concerns the protonation state of the amine nitrogen during its interaction with the dopamine receptor.

A common feature of all dopamine antagonists is a basic nitrogen atom. At physiological pH, an equilibrium between a charged ammonium ion and an uncharged amine exists for these compounds. The amount of each species that is present depends upon the pK_a of the amine. Since the majority of dopamine antagonists have pK_a values between 8 and 9 and are thus substantially protonated at physiological pH, several investigators have suggested that the active form of dopamine antagonists is the charged, protonated form.[1,2,8] Based upon a reported pK_a of 5.9 for butaclamol, Philipp et al.[6] proposed that the uncharged amine is the important species and binds to the dopamine receptor via hydrogen bond formation. Recently, Chrzanowski et al.[8] independently determined the pK_a of butaclamol and found it to be in the range of 7.0-7.5. They concluded that since this pK_a allows for approximately 50% protonation at physiological pH, both charged and uncharged species need to be considered when describing possible ligand-receptor interactions. Obviously, since both charged and uncharged amine species can exist in solution at physiological pH, it is difficult to establish which of these two molecular species is actually responsible for dopamine antagonist activity.[9]

The question of which molecular species, charged or uncharged, is most important for the binding of dopamine agonists to the dopamine receptor has recently been addressed.[10,11] Studies with permanently charged and permanently uncharged sulfur and selenium analogues of dopamine suggested that the charged, protonated form of dopamine is the molecular species required for maximal activation of the dopamine D_2 receptor. In order to determine if dopamine antagonists also require a charged species for maximal activity, our group originally investigated a series of permanently charged and uncharged analogues of chlorpromazine (**1**).[12] Permanently charged ammonium (**2**) and sulfonium (**3**) analogues of chlorpromazine retained the ability to both bind and act as

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