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Atrial Natriuretic Peptide Antagonists: Biological Evaluation and Structural Correlations

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SUMMARY

A collection of analogues of atrial natriuretic peptide (ANP) were screened for their ability to inhibit ANP-induced cGMP stimulation. The antagonists revealed through this screen are structurally related; almost all are substituted at either aspartate-13 or phenylalanine-26. This tendency is consistent throughout several families of small ANP analogues, suggesting that these two amino acid residues are involved in the process of ANP/cGMP signal transduction. One compound, A74186, was studied in some detail. A74186 is a potent inhibitor of the activation of guanylate cyclase by ANP; it acts with a pA_2 of 7.12 in rat vascular smooth muscle cells and shifts the ANP/cGMP dose-response curve by 3 orders of magnitude at a 10 μM concentration. It also inhibits cGMP release *in vivo*, and at an infusion rate of 10 μg/kg-min it completely abolishes ANP-induced natriuresis and diuresis. A74186 does not, however, antagonize the hypotensive or vasorelaxant effects of ANP; in fact it acts as an agonist in these assays. It thus appears that cGMP, although it may mediate the renal responses to ANP, is not responsible for the vascular and hemodynamic effects that result from the action of the hormone.

The ANPs are a family of peptide hormones released from atrial cardiocytes in response to increases in central venous pressure (1–3). They are intimately involved in the regulation of blood pressure and fluid volume status and, for this reason, have attracted recent attention as possible therapeutic agents (4, 5). ANPs are known to have a variety of microscopic and macroscopic actions; they stimulate sodium and water excretion by the kidneys, cause a shift of fluid from the circulation into extravascular space, enhance the production of cGMP by the membrane-bound form of guanylate cyclase while lowering intracellular cAMP levels, inhibit aldosterone biosynthesis, and oppose the actions of the renin-angiotensin system.

This complex cascade of responses is mediated through a number of different receptors (6–11). In particular, two major classes of peripheral ANP receptors have been closely studied. ANP-R1, the high molecular weight or B-type receptor, has been shown to be functionally coupled to the production of cGMP. There is strong physical evidence for this linkage; the receptor has recently been sequenced and found to consist of an extracellular binding domain and an intracellular domain that bears close homology to soluble guanylate cyclase (12). ANP-R2, the C-receptor, is also membrane bound but contains only a vestigial intracellular domain. This receptor, which predominates in most cell lines, has been suggested to serve a clearance function (13); recently it has been linked to both phosphoinositide turnover (14) and the adenylate cyclase/cAMP system (15) via guanine nucleotide-binding proteins. Despite these and other efforts, characterization of the various ANP receptor/effector systems remains incomplete.

Receptor antagonists have traditionally provided an important tool for helping to reveal the pathways linking ligand binding to function. Additionally, such compounds might aid in the understanding of the physiological role of ANPs, an area that remains the subject of some debate. A number of reports have described preliminary efforts to antagonize the effects of ANPs. Sakata and Needleman (16) studied autoimmune rats producing an anti-ANP antibody that removes the native peptide from the circulation and observed that the animals showed a decreased natriuretic-diuretic response to acute volume expansion. Richards and co-workers (17) described an ANP degradation product that exhibited some antagonist properties. More recently, Kitajima *et al.* (18) have reported that certain linear ANP analogues antagonize ANP-induced cGMP accumulation in vascular smooth muscle cells.

In this communication, we report the results of our own studies on ANP antagonism. Specifically, we have uncovered a group of ANP analogues that dramatically interfere with ANP-induced cGMP stimulation. These antagonists are structurally

ABBREVIATIONS: ANP, atrial natriuretic peptide; BTAEC, bovine transformed aortic endothelial cells; rVSMC, rat vascular smooth muscle cells.

related; on the basis of these structural relationships, we have identified two amino acid residues in the native peptide that serve to couple the ANP binding message to guanylate cyclase activation. Antagonist effects are observed *in vitro* and *in vivo* in several models. Surprisingly, the compounds act as ANP agonists with regard to vascular and hemodynamic effects. From these results, we conclude that cGMP is not the mediator of all of the actions of the ANPs, as has previously been postulated.

Experimental Procedures

Materials. All peptides are prepared using solid-phase techniques on an automated synthesizer. After cleavage from the resin with anhydrous HF, the crude linear peptides are cyclized at high dilution (~50 mg/liter at pH 7.2), using iodine in ethanol for disulfide bond formation. The products are desalted by passage through an XAD-16 adsorption column and purified by semipreparative high performance liquid chromatography.

Vasorelaxant assays. Rabbit aortic rings (4–5 mm) are suspended in 2-ml jacketed tissue baths containing a Krebs-Henseleit buffer (118 mM NaCl, 4.7 mM KCl, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 2.5 mM $CaCl_2$, 25 mM $NaHCO_3$, 11.1 mM D-glucose). The baths are gassed with 95:5 O_2/CO_2, and pH is adjusted and maintained at 7.4. The rings are connected to an isometric force transducer for monitoring of tension changes. Baseline tension is adjusted to 2 g and maintained over a 2-hr period. To study a test compound as an ANP agonist, the rings are contracted with 3 μM methoxamine, and then cumulative additions of six half-log concentration increments of peptide to each bath are completed over a period of 90 min. To study antagonist effects, a series of separate assays are performed in which single concentrations of potential antagonist are added to the bath before the recording of a dose-response curve using ANP[5–28] as the relaxation agonist. All data points represent triplicate determinations.

Receptor binding assays. Confluent BTAEC in 100 μl of assay buffer (RPMI 1640, 0.1% bovine serum albumin, 0.2 mM 3-isobutyl-1-methylxanthine) are incubated with ~10,000 cpm I-ANP and various analogue concentrations for 4 hr at 4°. Free I-ANP is removed by washing with cold phosphate-buffered saline containing 0.1% bovine serum albumin; bound label is determined after solubilization with 200 μl of 0.5 M NaOH. All data points represent duplicate determinations.

Cyclic GMP assays. Confluent monolayers of BTAEC or rVSMC are incubated in assay medium with an antagonist dose at 37° for 15 min. ANP[5–28] is added, and the incubation is continued for 2 hr. After termination of the incubation by acidification with $HClO_4$ to 1.6%, the supernatant is collected by centrifugation. Cyclic GMP is determined after acetylation by radioimmunoassay. Data are expressed relative to the maximum response (=100%) observed for ANP[5–28]. For the determination of agonist activity, the analogue to be tested is used in place of ANP in the above experiment and there is no preincubation. All data points represent duplicate determinations.

***In vivo* studies.** All *in vivo* experiments are performed using male Sprague-Dawley rats. The animals are anesthetized with Inactin (100 mg/kg, intraperitoneally) and catheters are placed in the femoral artery and vein for measurement of arterial blood pressure and infusion of drugs, respectively. A bladder catheter is inserted for timed collections of urine. After completion of surgical procedures, the animals are allowed to equilibrate for 90 min. Test agonists are studied via a stepped-dose protocol, in which eight half-log incremental doses of peptide are infused sequentially for 15-min intervals. Mean arterial pressure, urine volume, and urinary sodium and cGMP are measured over each infusion period. For antagonism studies, antagonist infusion is initiated 60 min before the first, control period. The stepped-dose protocol is then repeated using ANP[5–28] as the reference agonist. Use of animals is in accordance with the Guide for the Care and Use of Laboratory Animals, as adopted and promulgated by the United States National Institutes of Health, and was approved by Abbott's Institutional Animal Care and Use Committee.

Results and Discussion

We have previously described (Fig. 1) our discovery of several classes of small ANP receptor agonists[1] (19, 20). These analogues are generally less potent than the parent peptides but exhibit all of the observed *in vitro* and *in vivo* effects of ANP. These prototypical small agonists have been the subject of extensive structure-activity relationship studies, with over 500 analogues produced via addition, deletion, and/or substitution of the various amino acid components. Because it is not unusual for peptide antagonists to have structures closely related to that of the native compound, we suspected that we might, in the course of these manipulations, have serendipitously modified our small agonists to produce compounds having ANP antagonist properties.

We chose to begin our studies of ANP antagonism by looking at the stimulation of guanylate cyclase in BTAEC. Cyclic GMP has often been proposed as the primary second messenger for ANP action (21, 22). Consistent with this view, exposure of BTAEC to ANP causes cGMP levels to increase up to 100-fold, in a dose-dependent fashion. We employed this ANP-induced cGMP stimulation as the basis for an antagonist screen. In order to ensure that the compounds selected could function as pure antagonists, we first screened for agonist effects. We eliminated from consideration any analogue that, at a concentration of 10 μM, elevated cGMP levels to more than one third of the maximum response resulting from ANP treatment. Dose-response experiments, employing ANP[5–28] as agonist, were then recorded in the presence of each of the remaining analogues (Fig. 2). We consider a compound to be an antagonist if it produces at least a half-log shift in the ANP dose-response curve when added at a concentration of 10 μM. Our screen revealed 21 compounds that satisfy these conditions (Table 1). A number of these are quite potent inhibitors of the action of ANP; the best antagonists in this assay (e.g., A71915, K106) shift the cGMP response by 3 orders of magnitude.

It is apparent from examination of the compounds listed in Table 1 that the process of ANP antagonism is a general one, not limited to a specific structural type. Our search revealed antagonists related to all of the agonist families that we have explored. Further examination of the differences between these antagonists and their agonist "parents" suggests that two amino acid residues in ANP are intimately involved in its cGMP response. Fourteen of the 21 antagonists contain replacements for Phe^{26} (ANP numbering); 13 carry modifications at Asp^{13}. In fact, eight of the 21 have been altered at both positions, and only two lack substitution at either Asp^{13} or Phe^{26}; in both of these latter cases (A66932, A71984), an adjacent amino acid has been altered, and this may have a secondary effect on the crucial element. Structure-activity studies in our group have previously indicated that neither Asp^{13} nor Phe^{26} is critically involved in receptor binding (20, 23). It appears that these two residues act jointly in the ANP/cGMP signal transduction process; a change at either position can disrupt normal trans-

[1] ANPs, as referred to in this paper, are naturally occurring analogues of rat ANP (shown in Fig. 1). Human ANP differs only in replacement of Ile^{12} by methionine. ANP[1–28] and ANP[5–28] have both been isolated from natural sources; these peptides have similar effects in all the assays described here and are used interchangeably for our experiments.

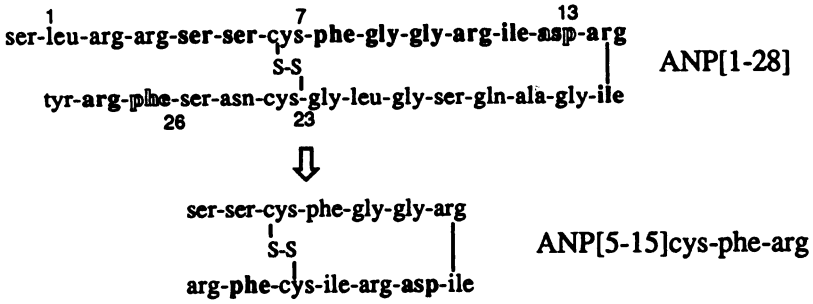


Fig. 1. Amino acid sequences of the natural hormone ANP[1–28] and a protypical small ANP analogue, ANP[5–15]Cys-Phe-Arg,[2] prepared by combining highlighted portions of the parent molecule. Critical residues Asp[13] and Phe[26] are in *open lettering* in the *upper* structure and *bold* in the *lower*.

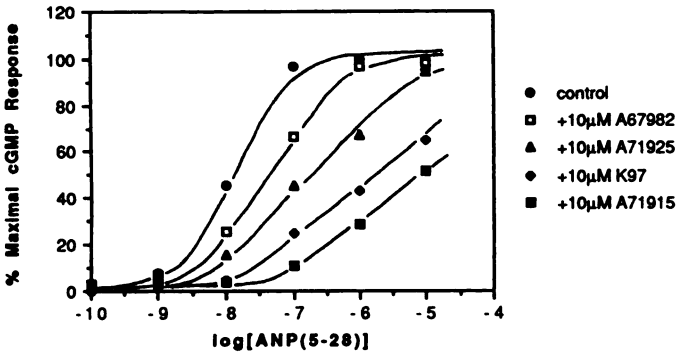


Fig. 2. Effect of antagonists on ANP-induced cGMP stimulation. Representative curves show the shift to higher concentrations resulting from preincubation of BTAEC with compounds from Table 1 before performance of an ANP/cGMP dose-response study. Values for cGMP response are scaled to the maximum stimulation (generally ~100-fold) produced by ANP[5–28].

membrane processing of the ANP binding message. Our results are consistent with and help to explain the earlier observation of Waldman *et al.* (24) that the carboxy-terminal dipeptide Phe[26]-Arg[27] is important for guanylate cyclase activation.

Our analysis of the requirements for ANP signal transduction suggests that the potency of an antagonist should reflect the degree of disruption of the critical transducing elements. As expected, there is a tendency for analogues that have replacements for both Asp[13] and Phe[26] (e.g., K106, A72747) to be some of the more potent antagonists in our study. However, an additional factor is reflected in the heirarchy of compounds in Table 1; analogues that bind tightly to ANP receptors also tend to be more effective as antagonists. This result is consistent with our view of competitive antagonism; in order to be an effective antagonist a compound must compete efficiently with the native ligand. Some combination of these two qualities

[2] Standard amino acid three-letter codes are used throughout. For unnatural amino acids, abbreviations are as follows: Cha, cyclohexylalanine; Tic, 1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid; Pen, penicillamine; Mpr, 3-mercaptopropionic acid; Aoa, 8-aminooctanoic acid. Compounds are named by analogy to ANP; thus, Arg[6],D-Ala[7a],des-gly10[ANP 6–15] begins with the ANP[6–15] sequence and then replaces Ser[6] by Arg, inserts D-Ala between positions 7 and 8, and deletes Gly[10].

TABLE 1

ANP antagonists

All compounds in this table satisfy two criteria; they cause at least a half-log shift in the ANP/cGMP dose-response curve at a concentration of 10 μM and exhibit weak intrinsic cGMP stimulation (<35% of the maximum response) at the same dose. Binding of antagonists to the cell line (BTAEC) used for the cGMP studies is also tabulated.

	Compound	BTAEC binding, pK_i[a]	cGMP stimulation at 10 μM	cGMP antagonism at 10 μM antagonist
			% of maximum	*log shift*
A61197	Asn[13][ANP 5–28]	8.40	26.8	1.40
A66865	D-Ala[7a],Ala[13][ANP 5–15]Cys-Phe-Arg	6.80	18.8	1.00
A66932	D-Ala[7a],Ala[14][ANP 5–15]Cys-Phe-Arg	7.20	19.8	0.85
A67243	D-Ala[7a][ANP 5–15]Cys-D-Tic-Arg	8.70	31.1	0.60
A67982	D-Ala[7a][ANP 5–15]Cys-L-Tic-D-Arg	8.40	<0.1	0.50
A68684	Arg[6],Cha[8][ANP 6–15]Ala-Arg-Cys	8.65	8.2	2.30
A69782	Arg[6],Leu[13][ANP 6–15]Phe-Arg-Cys	6.50	29.8	0.95
A70538	D-Val[7a][ANP 5–15]Cys-DL-Tic-Arg	8.12	4.1	0.85
A71815	Arg[6],Cha[8][ANP 6–15]L-Tic-Arg-Cys-NH_2	7.80	5.5	2.45
A71915	Arg[6],Cha[8][ANP 6–15]D-Tic-Arg-Cys-NH_2	8.30	5.3	2.80
A71925	Arg[6],Cha[8],Gly[13][ANP 6–15]Phe-Arg-Cys-NH_2	7.70	14.5	1.20
A71984	Arg[6],Cha[8][ANP 6–15]Phe-Pen-NH_2	8.68	33.8	2.90
A72747	Mpr[7],D-Ala[13],Ala[14],Des-15,16,17,Leu[26][ANP 7–27]-NH_2	7.24	0.4	2.78
A73342	Arg[6],Cha[8],Ala[13][ANP 6–15]L-Tic-Arg-Cys-NH_2	6.84	1.0	1.20
A73393	Arg[6],Cha[8],Ala[13][ANP 6–15]D-Tic-Arg-Cys-NH_2	7.18	6.8	1.50
A74186	Mpr[7],D-Ala[13,16],Ala[14],Leu[26][ANP 7–27]-NH_2	8.27	13.6	2.03
K96	Mpr[7],Leu[12],D-Ala[13]ANP 7–15]Cys-Phe-Arg-NH_2	6.25	19.9	1.35
K97	Mpr[7],D-Ala[7a,13,20],Des-Gly[10],Leu[12,26][ANP 7–27]-NH_2	6.10	0.4	2.20
K98	Mpr[7],Leu[12,26],D-Ala[13,20][ANP 7–27]-NH_2	6.85	29.0	1.25
K105	Leu[12,26],D-Ala[13,16],Aoa[20–22][ANP 7–27]-NH_2	5.15	1.6	2.75
K106	Mpr[7],Leu[12,26],D-Ala[13,16],Aoa[20–22][ANP 7–27]-NH_2	6.70	1.8	3.00

[a] We have found that BTAEC contain >90% of a single receptor, the low-molecular weight, non-guanylate cyclase-coupled receptor (8); thus, binding data reported for the antagonists reflect affinity for this receptor. Cross-linking data previously reported by some of us (27) indicate that the antagonists bind with similar affinity to both major receptor subtypes.

seems to determine the potency of compounds in this antagonist assay.

To explore further the effects of antagonists on various ANP-related functions, we chose A74186 as a typical member of the class. To confirm the generality of ANP antagonism, we first examined A74186 for inhibition of ANP-induced cGMP synthesis in a second cell line, derived without transformation from rat vascular smooth muscle (rVSMC). The compound is quite potent as an antagonist in this system; for example, a 100 nM dose of A74186 inhibits the cGMP stimulation due to a 1 μM dose of ANP[5–28] by 25%. To further examine this effect, we recorded ANP dose/cGMP response curves in the presence of four doses of antagonist (Fig. 3a). The dose dependence of inhibition may be quantified through a Schild regression analysis (Fig. 3b), which provides a pA_2 of 7.12 for the compound.

A74186 was also evaluated to see whether this antagonism manifests itself *in vivo*. It was first necessary to establish control responses (Fig. 4) to ANP and to A74186 as agonist, by measuring urine flow rate, urinary sodium and cGMP, and mean arterial pressure over 15-min intervals while sequentially infusing eight doses of peptide from 0.001 to 3.0 μg/kg-min (for A74186, six doses from 0.1 to 30 μg/kg-min). ANP produces dose-dependent increases in salt, water, and cGMP excretion in this protocol, along with a decrease in blood pressure, whereas A74186 has no renal effects and a markedly attenuated hypotensive response. To look for antagonist effects, the ANP stepped-dose protocol was then repeated, with concomitant

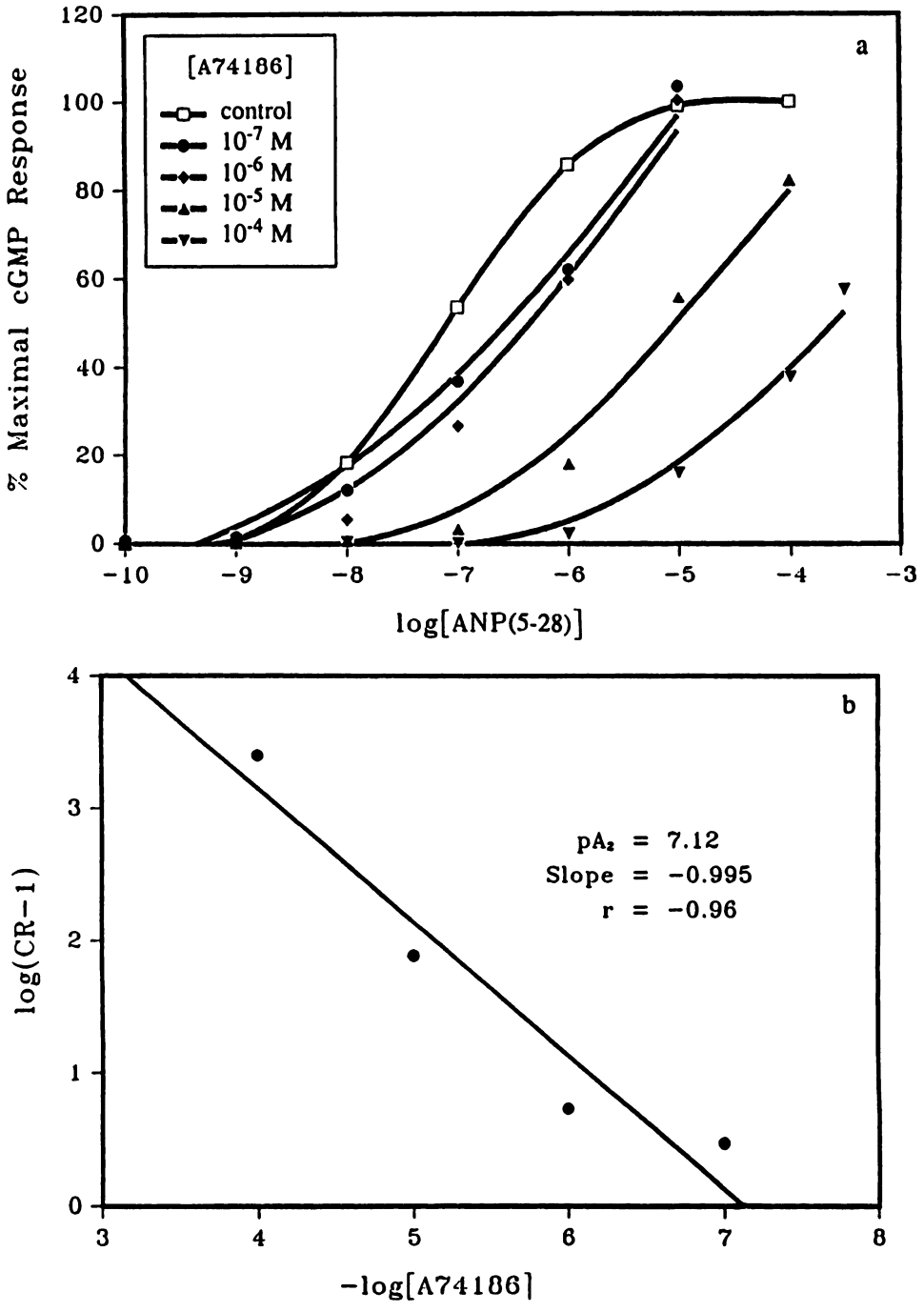


Fig. 3. Antagonist effect of A74186 on cGMP production in rVSMC. a, ANP dose/cGMP response curves in the presence of four concentrations of antagonist. A74186 clearly antagonizes the response to ANP in a dose-dependent fashion. b, Quantification of the effect through use of a Schild regression, which plots the concentration ratio (*CR*) resulting from addition of antagonist to the assay mixture versus the antagonist dose. Based on this analysis, A74186 has a pA_2 of 7.12 for cGMP inhibition.

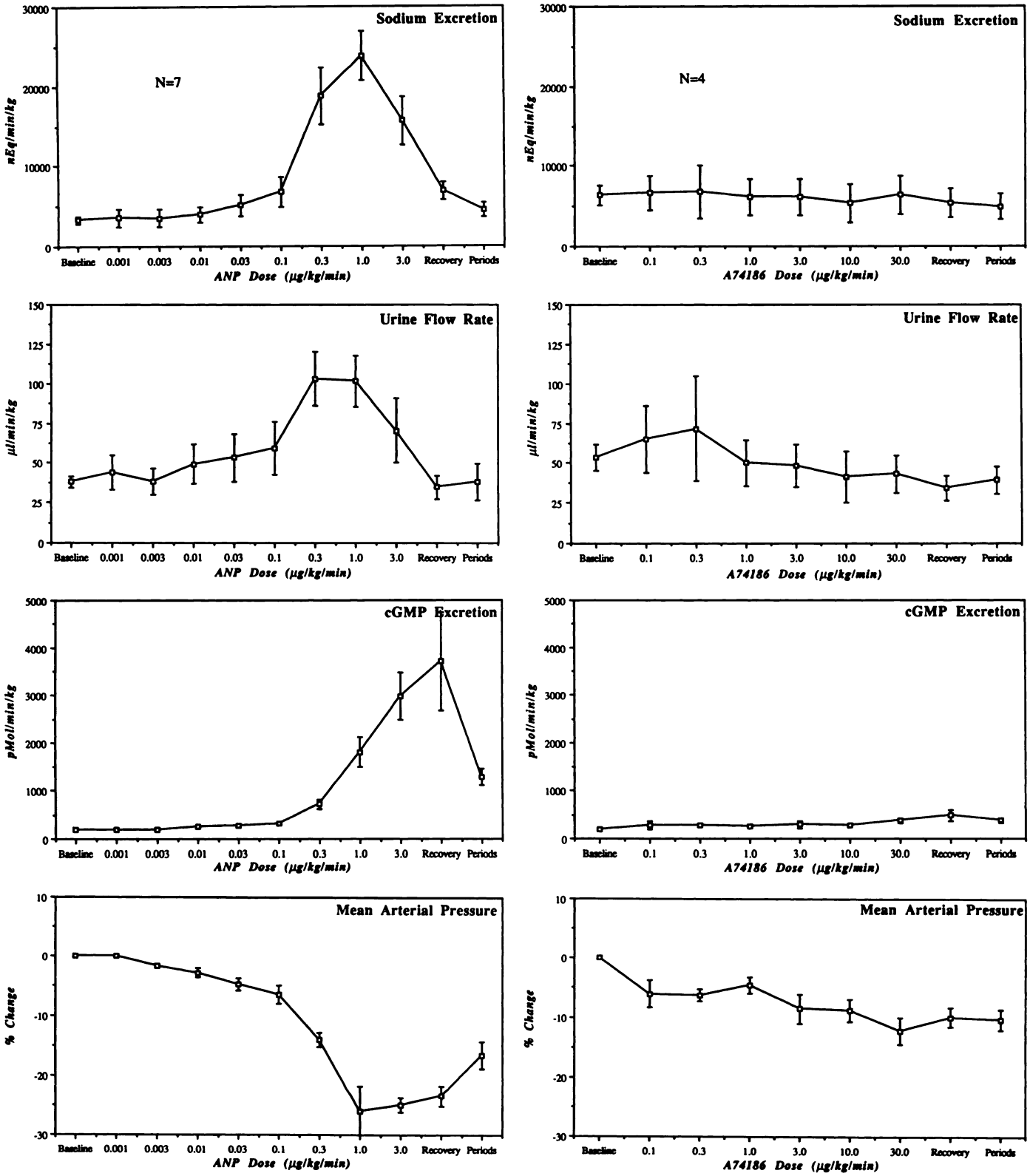
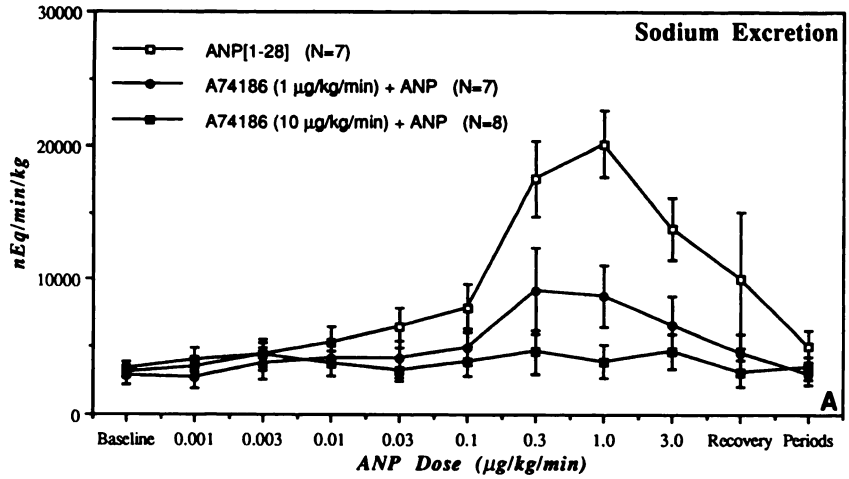
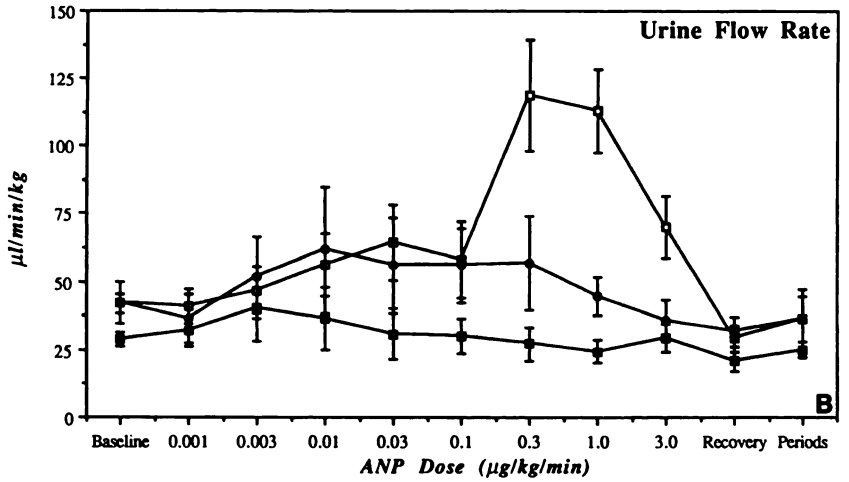
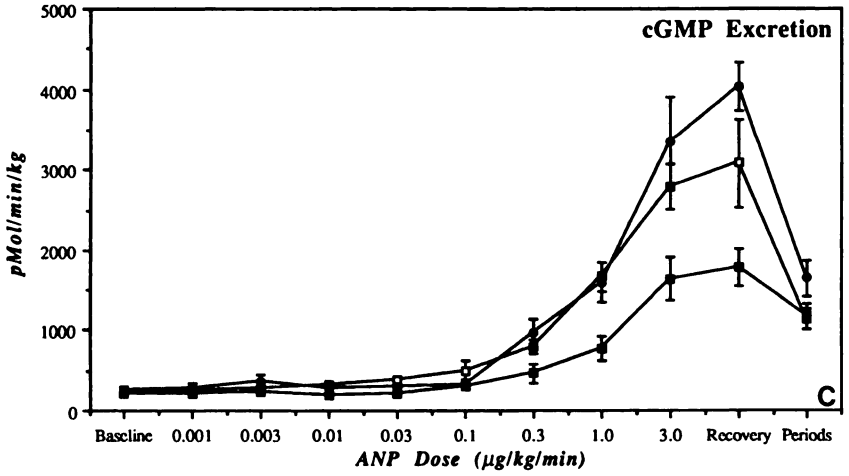


Fig. 4. *In vivo* agonist effects of ANP[1–28] and A74186. Plots on the *left* show the effects of ANP[1–28] recorded during stepped-dose infusion experiments, as described in Experimental Procedures; on the *right* are the effects of A74186 in a similar protocol. ANP causes a dose-dependent tripling of urine volume, a 7-fold increase in urinary sodium excretion, and a 15-fold increase in urinary cGMP, along with a 25% decrease in blood pressure. A74186 is devoid of renal effects and causes only a small decrease in mean arterial pressure.







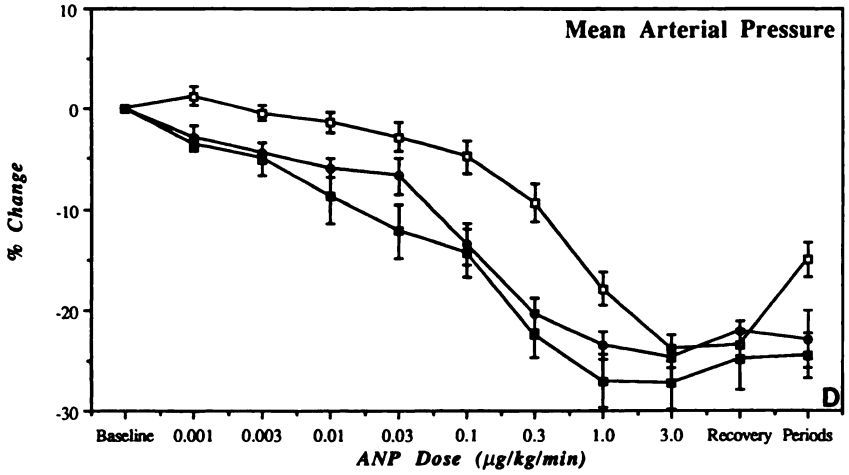


Fig. 5. *In vivo* antagonist effects of A74186. The antagonist is infused at a constant rate, beginning 1 hr before the first measurement; a standard stepped-dose experiment is then performed using ANP[1–28] as the agonist. A74186 at an infusion rate of 1 µg/kg/min decreases the ANP-induced stimulation of urine flow rate and urinary sodium; at 10 µg/kg/min the ANP effects are completely abolished. The higher dose of antagonist also attenuates ANP-induced cGMP release into the urine; however, A74186 coinfusion decreases blood pressure beyond the normal levels resulting from ANP administration.

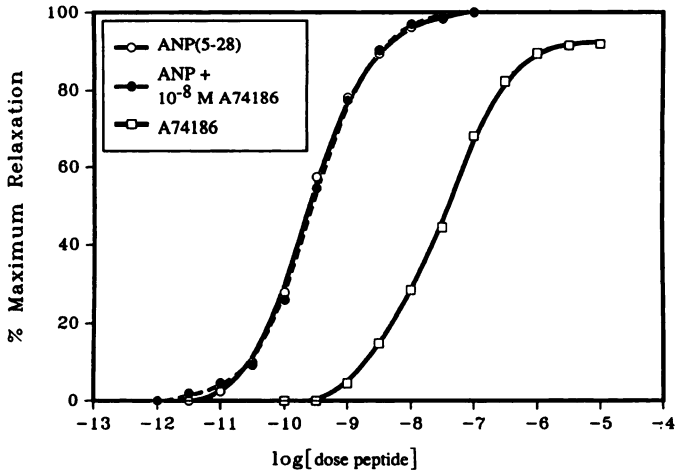


Fig. 6. Antagonist and agonist effects of A74186 on vasorelaxation. ANP relaxes methoxamine-constricted rabbit aortic rings in a dose-dependent fashion, with an EC_{50} of 0.2 nM. Addition of A74186 to the bath at a concentration of 10 nM has no antagonist effect on this relaxation; in fact, a separate experiment shows that the compound acts as a full agonist, with an EC_{50} of 40 nM.

infusion of the antagonist at a constant rate of 1 or 10 μg/kg-min (Fig. 5). Antagonist infusion was initiated 60 min before the first test period, which served as a baseline.

The effects of A74186 on kidney function are dramatic. The lower dose of antagonist produces significant reductions in urine volume and urinary sodium levels (Fig. 5, a and b); at the higher dose, the effects of ANP administration are completely eliminated. Again, the compound appears to be quite potent; for example, a 1 μg/kg-min infusion of A74186 neutralizes 70% of the increase in sodium excretion due to a similar infusion of ANP[1–28]. A similar, although less marked, antagonism of ANP-induced cGMP excretion is also observed. Although the lower dose of A74186 had no significant effect on cGMP levels in the urine (Fig. 5c), at the higher dose we see a 50% suppression of the maximum stimulatory response, with similar levels of inhibition observed throughout the dosing range.

The observations of the *in vitro* and *in vivo* actions of A74186 described above are all consistent, and support the prevalent view that cGMP is the primary mediator of the effects of ANP. Thus, we were initially confused to note that the antagonist causes no attenuation of the hypotensive effect of ANP (Fig. 5d). In fact, there is a tendency for blood pressure to drop *more* during A74186 coinfusion. Although this result is surprising in light of the absence of hemodynamic effects recorded for A74186 during the *in vivo* agonist protocol, it is clearly significant and reproducible. It also forces us to include a caveat regarding the interpretation of our previous *in vivo* results. Increases in intrarenal blood pressure often lead to increased sodium and water excretion, through a mechanism known as "pressure diuresis." Conversely, the decreases in blood pressure caused by A74186 may contribute to the observed attenuation of these responses.

This absence of antagonism of the blood pressure effects of ANP surprised us and led us to examine A74186 as an *in vitro* antagonist in a vasorelaxant assay (Fig. 6). The presence of a 10 nM concentration of analogue in the tissue bath produced no attenuation of ANP-induced relaxation. Instead, the "antagonist" functions as a potent full agonist in this assay, having an ED_{50} of 40 nM. We have tested a number of the compounds from Table 1 in this assay; each acts as an agonist in a similar fashion.

The mechanism of the observed agonist effects in the vasculature is unclear. In light of the clear antagonism of the guanylate cyclase system observed in rVSMC, a vascular cell line, this does not appear to be a question of tissue specificity. It is tempting to speculate that these responses are mediated by the C-receptor, either by displacement of bound ANP or through the agency of cAMP or phosphoinositide. Although we have no direct evidence on this point, it should be noted that all of the antagonists described above contain the ANP[11–15] sequence exploited by several groups (25, 26) to prepare analogues that specifically bind this receptor. Asp^{13}, one of the critical residues for cGMP signal transduction, falls within this C-receptor binding "domain" and so has been modified in a number of our antagonists. This modification does not appear to affect C-receptor binding, though, as is shown by two sets of binding studies in BTAEC. A72747, a typical antagonist, competes with I-ANP for two classes of binding sites, with K_i values of 1.9 and 10 nM. Identical results are observed when chemical cross-linking of I-ANP is blocked with increasing amounts of A72747 (27). SDS-gel autoradiographic data show that the 10 nM site corresponds to the high molecular weight, guanylate cyclase-coupled receptor subtype, whereas the 1.9 nM site represents the low molecular weight, C-type receptor. It is, thus, quite possible that these B receptor antagonists are at the same time potent C receptor agonists.

The availability of a group of selective antagonists has allowed us to divide the actions of ANP into two classes, those that are antagonized and those that are not. In the first class we place the renal effects of ANP and the stimulation of cGMP synthesis *in vitro* and *in vivo*; the latter includes the vasorelaxant and hemodynamic effects of ANP. Thus, we conclude that cGMP stimulation and the guanylate cyclase-coupled receptor do not appear to be responsible for the vascular actions of ANP, although they may be mediating its renal response. Although disparity between these renal and vascular effects has been debated in the past (7, 28–30), our experiments provide the first direct evidence on this point.

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