0026-895X/96/020430-06$3.00/0

MOLECULAR PHARMACOLOGY, 50:430–435 (1996).

Novel Natriuretic Peptide Receptor/Guanylyl Cyclase A-Selective Agonist Inhibits Angiotensin II- and Forskolin-Evoked Aldosterone Synthesis in a Human Zona Glomerulosa Cell Line

LINDA J. OLSON, DAVID G. LOWE, and JAMES G. DREWETT

Department of Pharmacology and Toxicology and Cardiovascular Research Center, Medical College of Wisconsin, Milwaukee, Wisconsin 53226 (L.J.O., J.G.D.), and Cardiovascular Research Department, Genentech, Inc., South San Francisco, California 94080 (D.G.L.)

Received February 21, 1996; Accepted May 8, 1996

SUMMARY

We report the production of a novel human natriuretic peptide receptor/guanylyl cyclase A (hNPR-A)-selective agonist ANP [G9T, R11S, G16R] (sANP). This agonist has similar affinity to ANP for hNPR-A and 1,000–10,000-fold reduced affinity for the human natriuretic peptide clearance receptor (hNPR-C). sANP was used to directly test the hypothesis that hNPR-A mediates the inhibitory effect of natriuretic peptides on aldosterone generation in a human zona glomerulosa cell line, H295R. Human type A natriuretic peptide and sANP (10^{-11} to 10^{-6} M) resulted in concentration-dependent increases in cGMP levels and decreases in forskolin (100 nM)- and angiotensin II (5 nM)-induced aldosterone and pregnenolone production. These results revealed an inhibitory effect of both peptides on the agonist-stimulated conversion of cholesterol to pregnenolone (i.e., cytochrome P-450 cholesterol monooxygenase side-chain cleaving enzyme, EC 1.14.15.6). H295R cells also exhibited angiotensin II- and forskolin-evoked conversion of [^{3}H]corticosterone to [^{3}H]aldosterone (i.e., cytochrome P-450 steroid 11β-monooxygenase/aldosterone synthase, EC 1.14.15.4). Human type A natriuretic peptide and sANP (10^{-7} M) inhibited the angiotensin II-stimulated late pathway but did not affect forskolin-facilitated conversion of corticosterone to aldosterone. Our results directly demonstrate inhibitory effects of hNPR-A-mediated signal transduction on cytochrome P-450 cholesterol monooxygenase side-chain cleaving enzyme and steroid 11β-monooxygenase/aldosterone synthase complex depending on the steroidogenic agonist used.

Aldosterone is an essential mineralocorticoid intimately involved in the regulation of sodium and fluid homeostasis (1). Increases in the levels of this hormone act to retain sodium and raise blood pressure *in vivo*. ANP is an endogenous antihypertensive agent (1, 2). It is thought to lower systemic sodium concentrations through direct effects on kidney transport as well as indirect actions to inhibit both renin release and aldosterone synthesis (1, 2). Therefore, ANP effects to attenuate steroidogenesis in the adrenal zona glomerulosa would be expected to result in decreases in sodium retention (1, 2).

ANP inhibits aldosterone production in several species (3–6). In rat zona glomerulosa cells, ANP is thought to reduce angiotensin II-evoked aldosterone production through effects on the biosynthetic pathway at both CYP11A1 (EC 1.14.15.6; early pathway, cholesterol to pregnenolone) and CYP11B2 (EC 1.14.15.4; late pathway, corticosterone to aldosterone (7). Barrett and Isales (8) reported that ANP both increased cGMP levels and decreased steroid production, which is consistent with the possibility that the latter response was the result of guanylyl cyclase activation. However, in the same study, reductions in cAMP concentrations were also necessary to completely account for the effects of ANP on steroidogenesis (8). The combination of ANP-mediated reductions in cAMP levels, the inability of membrane-permeable cGMP analogs to mimic ANP effects on aldosterone production, and

This work was supported by American Heart Association, Wisconsin Affiliate, Grant-in-Aid 94-GB-38 (J.G.D.) and by National Heart Foundation (a program of the American Health Assistance Foundation) Research Starter Grant and the 1995 Ruth Salta Junior Investigator Award (J.G.D.). Technical assistance was supported in part by the National Institutes of Health and National Center for Research Resources Minority High School Research Apprentice Program Grant (1R25-RR10113-01) to the Medical College of Wisconsin.

ABBREVIATIONS: ANP, type A natriuretic peptide; ACTH, adrenocorticothyrotropic hormone; ANOVA, analysis of variance; CNP, type C natriuretic peptide; CYP11A1, cytochrome P-450 cholesterol monooxygenase side-chain cleaving enzyme; CYP11B2, cytochrome P-450 steroid 11β-monooxygenase/aldosterone synthase; KHD, protein kinase-homology domain; hANP, human type A natriuretic peptide; NPR-A, natriuretic peptide receptor/guanylyl cyclase A; NPR-B, natriuretic peptide receptor/guanylyl cyclase B; NPR-C, natriuretic peptide clearance receptor; PBS, phosphate-buffered saline, pH 7.4; rANP, rat type A natriuretic peptide; sANP, natriuretic peptide receptor/guanylyl cyclase A-selective agonist type A natriuretic peptide (G9T, R11S, G16R); vANP, variant type A natriuretic peptide; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

some indications that the NPR-C had activity in zona glomerulosa cells (9) led to extensive debate surrounding the mechanism of action of ANP in adrenocortical cells (2, 6, 10).

MacFarland *et al.* (11) began to unravel a role for guanylyl cyclase (EC 4.6.1.2) in steroidogenesis by showing that ANP activated the type II, cGMP-stimulatable phosphodiesterase, presumably through NPR-A, to degrade cAMP in isolated membranes and resulted in inhibition of ACTH-stimulated aldosterone synthesis in intact bovine zona glomerulosa cells. Furthermore, they showed that the commercially available cGMP analogs were not effective allosteric modulators of the type II phosphodiesterase in zona glomerulosa cells (11). Their results are consistent with the hypothesis that guanylyl cyclase mediates the inhibitory action of ANP on aldosterone synthesis and offer plausible explanations for the ANP-mediated decreases in intracellular cAMP concentrations and the inability of cGMP analogs to reduce ACTH-stimulated aldosterone generation (2, 6, 11). Their study (11) did not address whether this mechanism could account for the effects of ANP on angiotensin II-evoked steroidogenesis.

Previous reports (6–9, 11), including that of MacFarland *et al.* (11), have been unable to provide a specific association between NPR-A and reductions in steroidogenesis because no NPR-A-selective agonists have been available (2, 6, 12–21). Therefore, pharmacological studies have not been done to more specifically define the role of NPR-A in signaling ANP-dependent inhibition of aldosterone synthesis.

An NPR-A-selective variant of ANP was developed by differential panning for mutant ANP display phage that bind NPR-A in an excess of competing NPR-C (22). The first novel analog, vANP, contained six mutations conferring NPR-A selectivity or high secretion/expression for the production of the peptide in *Escherichia coli* (Fig. 1; Ref. 22). Here, we report the pharmacological activity of a chemically synthesized analog of the *E. coli*-derived vANP (21). It is called sANP (Fig. 1) and contains only the three mutations from vANP required for NPR-A selectivity (Fig. 1; Ref. 22). The selectivity of sANP for NPR-A provides a unique pharmacological tool to study the role of specific receptor targeting in response to ANP.

In the current study, we use the newly identified human zona glomerulosa cell line, H295R (23), with which to examine the effects of hANP and sANP on aldosterone production. H295R cells provide a convenient immortalized cellular model that recapitulates responses seen in primary cultures of rat or bovine zona glomerulosa cells such as angiotensin II- and forskolin-stimulation of aldosterone production (6, 23). The use of the combination of the H295R cells and the NPR-A-selective agonist sANP allows a complete test of the hypothesis that NPR-A mediates an inhibition of angiotensin II- and/or forskolin-evoked aldosterone synthesis.

```
                1                    28
rANP    SLRRSSCFGGRIDRIGAQSGLGCNSFRY
vANP    --D-----T-SL-S-R------------
sANP    --------T-S----R------------
```

Fig. 1. Peptide sequences of NPR-A-selective agonists vANP and sANP in comparison to rANP. The mutations in vANP and sANP are shown below the sequence of rANP. Mutations in vANP that confer receptor selectivity (22) have been incorporated in sANP. The isoleucine at position 12 in rANP is a methionine in human ANP.

Materials and Methods

Natriuretic peptides, except for sANP, were obtained from Peninsula Laboratories (Belmont, CA). sANP was synthesized by solid-phase methods using standard t-BOC chemistry and purified by high performance liquid chromatography. Angiotensin II, forskolin, aldosterone, leupeptin, pepstatin A, phenylmethylsulfonyl fluoride, and pregnenolone were from Sigma Chemical (St. Louis, MO). Aldosterone, pregnenolone, and cGMP antibodies were the gracious gifts of the National Institutes of Health Pituitary Distribution Center (Lot No. 088; Bethesda, MD), Dr. Celso E. Gomez-Sanchez (Truman Veterans Affairs Medical Center, Columbia, MO), and Dr. David L. Garbers (Howard Hughes Medical Institute, University of Texas Southwestern Medical Center, Dallas, TX), respectively.

Human embryonic kidney 293 cell lines expressing stable transfectants of hNPR-A (24) or hNPR-C (22) were cultured as described. Cells were removed from culture plates with 0.5 mM EDTA in PBS, transferred to ice-cold PBS, and pelleted (228 × *g* for 10 min at 4°). Cell pellets were resuspended in ice-cold buffer containing 50 mM HEPES, 1 mM EDTA, 1 mM dithiothreitol, 0.25 M sucrose, 0.7 mg/ml pepstatin A, 0.5 mg/ml leupeptin, and 1 mM phenylmethylsulfonyl fluoride and homogenized (Brinkman polytron, 30 sec at setting 5). The homogenate was centrifuged (400 × *g* for 10 min at 4°), and the resultant supernate was centrifuged (100,000 × *g* for 30 min at 4°). The membrane pellet was resuspended in 50 mM HEPES, 0.1 mM EDTA, 5 mM $MgCl_2$, 100 mM NaCl, 0.7 mg/ml pepstatin A, 0.5 mg/ml leupeptin, and 1 mM phenylmethylsulfonyl fluoride, pH 7.4, using a dounce homogenizer. A BioRad (Hercules, CA) protein assay kit was used to determine membrane protein content, with IgG as a standard.

hANP has a single substitution of a methionine at position 12 compared with rANP (Fig. 1), but hANP and rANP have been shown to have identical potency on recombinant hNPR-A (25). Therefore, labeled rANP was used in these binding assays. Duplicate assays were performed three times in 96-well microtiter plates with membranes diluted into buffer containing 50 mM HEPES, pH 7.4, 5 mM $MgCl_2$, 0.1 M NaCl, 0.1 mM EDTA, 0.2% (w/v) bovine serum albumin, competing peptides, and [^{125}I]rANP at a final concentration of 15 pM (2 hr at 22°). Bound tracer was separated from free tracer by vacuum filtration (Packard filtermate 196 cell harvester) with polyethylenimime (1%)-pretreated Packard unifilter 96GFB filter plates. Plates were air-dried; then, scintillant was added before counting (Packard Top-Count Scintillation Counter, Meriden, CT).

H295R cells were kindly provided by Dr. William E. Rainey (University of Texas Southwestern Medical Center, Dallas, TX) from original isolated passages (23) and were grown (95% air/5% CO_2 at 37°) in 24-well plates to confluence in Dulbecco's modified Eagle's/Ham's F-12 medium (GIBCO BRL, Gaithersburg, MD) supplemented with 3% Nuserum (Collaborative Research, Bedford, MA) and 1% penicillin/streptomycin/fungizone (GIBCO). Before steroidogenesis experiments, the medium was supplemented with 20 nM angiotensin II and allowed to stand overnight (26). Pregnenolone synthesis was evoked with an EC_{50} for angiotensin II (5 nM)[1] or forskolin (100 nM)[1] for 2 hr. Experiments were performed in the presence of steroidogenic agonist vehicle, agonist, or agonist plus natriuretic peptide treatments. (The angiotensin II and natriuretic peptide vehicle was deionized, distilled water. The forskolin vehicle was 95% ethanol.) Cyanoketone (10 μM, Sterling-Winthrop Research Institute, Rensselaer, NY) was present in experiments designed to assess the effects on pregnenolone synthesis to block 3β-hydroxyΔ^5-steroid dehydrogenase (EC 1.1.1.145) (27, 28).

Aldosterone generation was stimulated in a similar manner for 6 hr, and natriuretic peptide treatments were the same as above. Steroidogenesis experiments were performed in fresh Ham's F12 supplemented with 1% bovine serum albumin, 2.6 mM $CaCl_2$, and 25 mM HEPES, pH 7.4. Cell supernates were subsequently stored at

[1] L. J. Olson, D. G. Lowe, and J. G. Drewett, unpublished observations.

−80° until assay. Aldosterone and pregnenolone production were quantified by radioimmunoassay according to the method of Campbell *et al.* (7). Conversion of [^{3}H]corticosterone to [^{3}H]aldosterone was assessed in the presence of cyanoketone to block the endogenous production of corticosterone. Steroid products were separated by thin layer chromatography and quantified as described previously (7).

Experiments designed to assess the effects of peptides on cGMP synthesis were performed in confluent 24-well plates of H295R cells at 37°. Cells were rinsed twice with PBS and preincubated with the same medium for 10 min as used in steroid production experiments, supplemented with 0.25 mM isobutylmethylxanthine. The preincubation medium was removed, and fresh buffer that included isobutylmethylxanthine and peptide vehicle, hANP, sANP, C-ANP (4–23), or CNP (vehicle or one concentration, 10^{-11} to 10^{-6} M, in each well) was added for 5 min before removal of the buffer, the addition of 1 N perchloric acid, and freezing at −80°. cGMP was purified and quantified by radioimmunoassay as described previously (29).

The ANP and sANP effects on cGMP production and agonist-evoked steroidogenesis were compared by repeated-measures ANOVA. For these experiments, each concentration of peptide was further compared with control (vehicle treatment) with the use of Dunnett's modification of a Student's paired *t* test. In regard to the conversion of [^{3}H]corticosterone to [^{3}H]aldosterone, steroidogenic agonist, agonist plus hANP or sANP (10^{-7} M), and hANP or sANP alone was also compared with control (agonist vehicle) in the same manner.

Results and Discussion

The receptor specificity of sANP was measured in equilibrium binding studies with membranes from human embryonic kidney 293 cells expressing stable transfectants of recombinant hNPR-A or hNPR-C (Fig. 2). With hNPR-A, sANP had a similar affinity (IC_{50} = 100 ± 18 pM) as rANP (IC_{50} = 105 ± 40 pM) in three experiments. Representative competition curves from one experiment are shown in Fig. 2A. For sANP competitive binding to hNPR-C, the IC_{50} was >1 μM in two experiments and 366 nM in a third (Fig. 2B). Binding of rANP to hNPR-C was much tighter in these three experiments (IC_{50} = 191 ± 62 pM). These data demonstrate that sANP has similar affinity for NPR-A as for ANP and a 1,000–10,000-fold reduced affinity for NPR-C. Therefore, sANP is an NPR-A-selective analog of ANP.

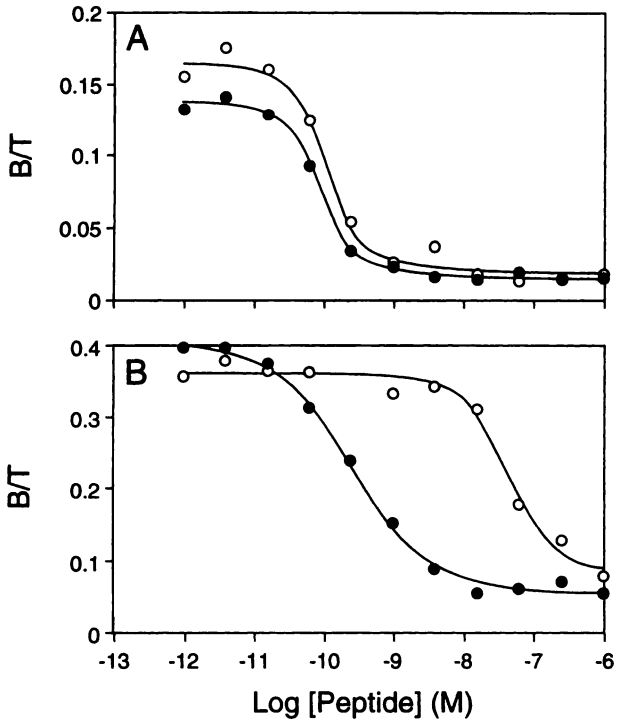

Fig. 2. Competition binding of [^{125}I]rANP with rANP (●) or sANP (○) on membranes from cells expressing recombinant NPR-A (A) and NPR-C (B). Binding is expressed as the fraction of bound [^{125}I]rANP over the total amount of [^{125}I]ANP in the reaction (*B/T*). A, IC_{50} = 50 pM for rANP and 66 pM for sANP on NPR-A. B, IC_{50} = 116 pM for rANP and 366 nM for sANP on NPR-C.

Fig. 3 demonstrates that in H295R cells, hANP and sANP have the same potency (EC_{50} = 2 nM) and maximal effect (10^{-6} M) in stimulating cGMP production. [There was no greater effect of 10^{-5} M peptides (two experiments).][1] Both ANP and sANP result in concentration-dependent increases in cGMP (repeated-measures ANOVA, $p < 0.0001$). There was no significant difference between the response curves for the peptides. The effectiveness of hANP and sANP, combined with the inability of CNP, the NPR-B-selective agonist (30), to increase cGMP production demonstrates that the only NPR in the H295R cells is NPR-A. This result is consistent with the reported absence of NPR-B in calf zona glomerulosa cells (31). The inability of C-ANF(des[Gln^{18},Ser^{19},Gly^{20},Leu^{21},Gly^{22}]ANF-4-23-NH_2) to increase cGMP production is commensurate with its selectivity for NPR-C (32).

Fig. 4A shows the effect of both peptides in attenuating forskolin- and angiotensin II-stimulated aldosterone production in H295R cells. The results in Fig. 4A represent the net inhibitory effect of hANP and sANP on the entire aldosteronogenic pathway (Fig. 5). The effects of hANP and sANP on forskolin- and angiotensin II-evoked aldosterone production are concentration dependent (repeated-measures ANOVA, $p < 0.0001$) and consistent with an effect of both natriuretic peptides through NPR-A. This result is very interesting given previous suggestions that ANP acts through differing receptors to modulate both forskolin- and angiotensin II-stimulated steroidogenesis (6). Until the current study and associated development of sANP, this hypothesis could not be directly tested. Fig. 4B shows that both peptides have similar concentration-dependent effects (repeated-measures ANOVA, $p < 0.0001$) to reduce angiotensin II- or forskolin-induced production of pregnenolone in the presence of cyanoketone, an inhibitor of 3β-hydroxyΔ^5-steroid dehydrogenase, which blocks conversion of pregnenolone to downstream products and allows a measure of effects on the conversion of cholesterol to pregnenolone (Fig. 5). These results show an effect of NPR-A signal transduction on CYP11A1 in human cells and are

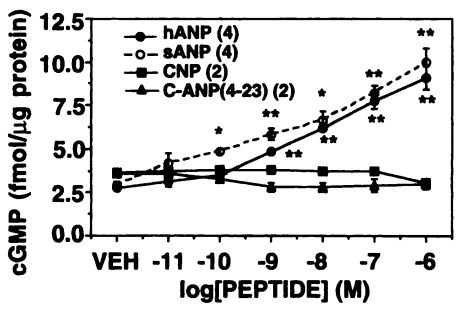

Fig. 3. Natriuretic peptide effects on cGMP generation in H295R cells. The number of experiments for each treatment are given in parentheses. Data are mean ± standard error. *VEH*, peptide vehicle. *, $p < 0.05$. **, $p < 0.01$.

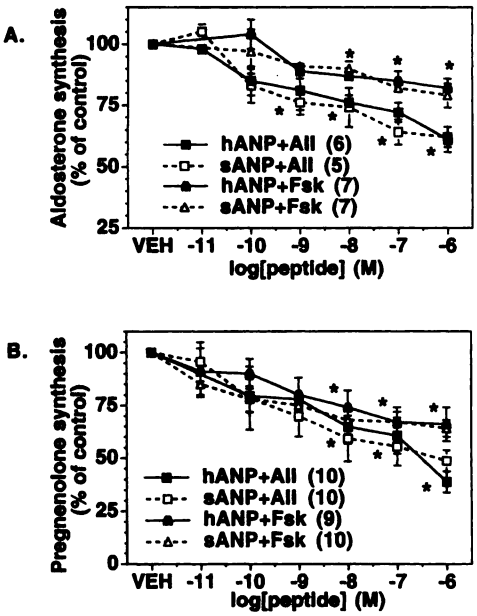

Fig. 4. Effects of ANP and NPR-A-selective agonist sANP on forskolin (*Fsk*)- and angiotensin II (*AII*)-evoked aldosterone (A) or pregnenolone (B) production in H295R cells. A, Basal levels of aldosterone produced were 544 ± 83 pg/hr/mg protein. After forskolin (100 nM) and angiotensin II (5 nM) treatment, the levels were 3667 ± 294 and 1367 ± 150 pg/hr/mg protein, respectively. B, Basal levels of pregnenolone were 2444 ± 56 pg/hr/mg protein. After forskolin and angiotensin II treatment, the levels were 10,888 ± 333 and 3,389 ± 611 pg/hr/mg protein (four experiments), respectively. Data are presented as a percentage of production in the presence of the peptide vehicle (*VEH*). The number of experiments for each treatment are given in parentheses. Average protein content was 178 ± 10 μg of protein/well. Data are mean ± standard error. A and B, Comparisons between the effectiveness of ANP and sANP were not statistically different by ANOVA. *, $p < 0.05$.

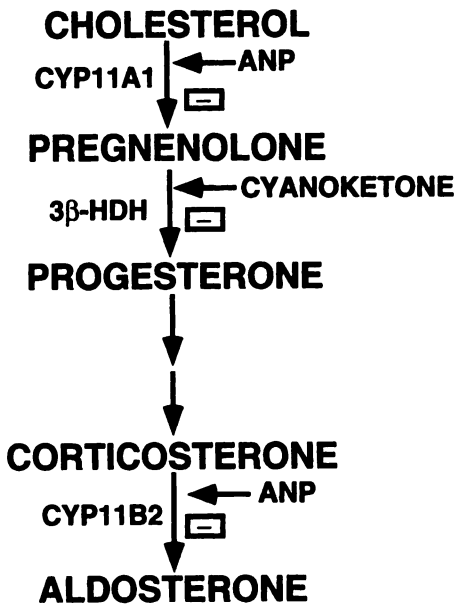

Fig. 5. Biosynthetic pathway of aldosterone synthesis. Relevant enzyme complexes noted are CYP11A1, 3β-hydroxyΔ^5-steroid dehydrogenase (3β-*HDH*), and CYP11B2. Data relevant to hANP and sANP effects on the agonist-evoked entire pathway, early pathway (CYP11A1), and late pathway (CYP-11B2) are presented in Fig. 4, A and B, and Table 1, respectively. Cyanoketone is shown as an inhibitor of 3β-HDH. The points at which ANP attenuates the pathway are also shown.

TABLE 1

hANP and sANP effects on the agonist-evoked conversion of corticosterone to aldosterone

Percent conversion = [aldosterone cpm × 100] ÷ [corticosterone added (i.e., 58,000 cpm)]. Values are mean ± standard error for six experiments.

Treatment	Conversion
	%
Vehicle	16 ± 1
Forskolin	23 ± 3*
Forskolin + hANP	24 ± 3*
Forskolin + sANP	25 ± 3*
Angiotensin II	21 ± 3*
Angiotensin II + hANP	17 ± 2
Angiotensin II + sANP	17 ± 3
hANP	16 ± 1
sANP	14 ± 1

*$p < 0.05$.

consistent with a previous study examining the effect of ANP in rat zona glomerulosa cells (7).

CNP and C-ANP (4–23) (10^{-10} to 10^{-6} M) had no effect on angiotensin II- or forskolin-stimulated aldosterone or pregnenolone synthesis (four experiments; mean range, 100–115%). These results demonstrate that NPR-B and NPR-C do not modulate aldosteronogenesis or that these receptors are not present in H295R cells. In regard to NPR-B, the latter possibility is consistent with the inability of CNP to increase cGMP concentrations in this cell line (Fig. 3).

It is noteworthy that hANP and sANP are equipotent at blocking steroid production in response to either forskolin or angiotensin II. The degree of ANP- or sANP-mediated maximal suppression on each stimulus is different, with a less efficacious reduction of forskolin-facilitated aldosteronogenesis as assessed by repeated-measures ANOVA ($p < 0.05$) and shown in Fig. 4A. Furthermore, hANP and sANP completely block angiotensin II stimulation of the late pathway of aldosterone production without an effect on forskolin-facilitated conversion of [^{3}H]corticosterone to [^{3}H]aldosterone (Table 1 and Fig. 5). The ability of the peptides to reduce angiotensin II stimulation of both CYP11A1 and CYP11B2 (7) and diminish only forskolin-mediated activation of the former enzyme agrees with earlier findings in rat zona glomerulosa cells (33). In the latter study, ANP was found to inhibit the ACTH-induced stimulation of only the early pathway, not the late pathway (33). ACTH is thought to act through increases in cAMP via a G_s protein-coupled receptor, and therefore its effect on steroid production should be comparable with that of forskolin (6, 23). We were unable to use ACTH because it does not increase steroid production in H295R cells[1] (23) as a result of the absence or very low abundance of its receptor (23).

In regard to the comparison of ANP effects between both aldosteronogenic stimuli (Fig. 4 and Table 1), one possible explanation is that the activation of NPR-A differentially modulates the signaling pathways of angiotensin II (i.e., mobilization of intracellular calcium and opening of T-type calcium channels) compared with forskolin (i.e., cAMP production) in zona glomerulosa cells (6). Future studies with sANP in H295R cells may allow the relationship of NPR-A and effects on calcium-associated pathways (i.e., phospholipase C and voltage-sensitive calcium channels) to be defined in modulation of the enzymatic steps of aldosteronogenesis.

The observation that the NPR-A-selective peptide sANP attenuates the activity of both CYP11A1 and CYP11B2 complexes is a significant, novel observation (6) because the inability of ANP to inhibit angiotensin II- and forskolin-induced steroidogenesis to the same extent has been suggested to be due to differing natriuretic peptide receptors (2, 6). Our data define a central role for NPR-A in modulating the effects of both agonists on aldosterone synthesis.

This study shows that ANP and a newly identified NPR-A-selective agonist, sANP, reduce aldosterone generation. It is important to note that NPR-A, like the other membrane-associated guanylyl cyclase-linked receptors, contains an extracellular ligand (ANP)-binding domain and two intracellular domains: a KHD and a cyclase catalytic region (2). Our data pharmacologically demonstrate that the NPR-A mediates an inhibitory effect on steroidogenesis. This result is consistent with the proposed involvement of the guanylyl cyclase/cGMP system in this effect (11). However, the cytoplasmic KHD may transduce ANP-mediated cellular effects independent of the catalytic region. We cannot differentiate between these two possibilities because sANP binds selectively to the extracellular region of NPR-A to potentially activate both the KHD and catalytic domains, resulting in the signaling of downstream cellular events. The discovery or development of a membrane-permeable catalytic inhibitor of NPR-A is essential before this dichotomy can be clarified for adrenocortical steroidogenesis or any other effect of ANP.

The results reported here show that the H295R cell line represents an *in vitro* model for screening novel natriuretic peptide agonists and antagonists for their effects on hNPR-A and steroid biosynthesis. The ability of ANP to reduce aldosterone production in the human adrenal cortex is likely a significant contributor to its natriuretic and diuretic effects and associated hypotensive activity (1, 2, 6, 34). The ability to selectively target NPR-A with the novel agonist sANP will likely be of significant therapeutic relevance in that clearance by NPR-C can be avoided, maximizing desirable pharmacological effects. NPR-A-selective agonists such as sANP may be important in treating conditions associated with volume overload, including renal failure, congestive heart failure, hyperadrenalism, and primary hyperaldosteronism.

Acknowledgments

The authors thank Mr. Johnny Myers (Science Department, Hamilton High School, Milwaukee, WI; Minority High School Research Apprentice Program) and Ms. Patricia Sehl for their excellent technical assistance; Mr. Jeff Tom, Dr. David Oare, and the Genentech Peptide Synthesis Group for sANP; and Drs. William B. Campbell and Begonia Y. Ho for their helpful suggestions and encouragement.

References

1. Brenner, B. M., B. J. Ballerman, M. E. Gunning, and M. L. Zeidel. Diverse biological actions of atrial natriuretic peptide. *Physiol. Rev.* **70**:665–669 (1990).
2. Drewett, J. G., and D. L. Garbers. The family of guanylyl cyclase receptors and their ligands. *Endocr. Rev.* **15**:135–162 (1994).
3. Atarashi, K., P. J. Mulrow, R. Franco-Saez, R. Snajdar, and J. Rapp. Inhibition of aldosterone production by an atrial extract. *Science (Washington D. C.)* **224**:992–994 (1984).
4. Goodfriend, T. L., M. E. Elliot, and S. A. Atlas. Actions of synthetic atrial natriuretic factor on bovine adrenal glomerulosa. *Life Sci.* **35**:1675–1682 (1984).
5. Maack, T., D. N. Marion, M. J. F. Camargo, H. D. Kleinhert, J. H. Laragh, E. D. Vaughan, Jr., and S. A. Atlas. Effect of auriculin (atrial natriuretic factor) on blood pressure, renal function and the renin-aldosterone system in dogs. *Am. J. Med.* **77**:1069–1075 (1984).
6. Ganguly, A. Atrial natriuretic peptide-induced inhibition of aldosterone secretion: a quest for mediator(s). *Am. J. Physiol.* **263**:E181–E194 (1992).
7. Campbell, W. B., M. G. Currie, and P. Needleman. Inhibition of aldosterone biosynthesis by atriopeptins in rat adrenal cells. *Circ. Res.* **57**:113–118 (1985).
8. Barrett, P. Q., and C. M. Isales. The role of cyclic nucleotides in atrial natriuretic peptide-mediated inhibition of aldosterone secretion. *Endocrinology* **122**:799–808 (1988).
9. Isales, C. M., J. A. Lewicki, J. J. Nee, and P. Q. Barrett. ANP-(7–23) stimulates a DHP-sensitive Ca^{2+} conductance and reduces cAMP *via* a cGMP-independent mechanism. *Am. J. Physiol.* **263**:C334–C342 (1992).
10. Anand-Srivastava, M. B., and G. J. Trachte. Atrial natriuretic factor receptors and signal transduction mechanisms. *Pharmacol. Rev.* **45**:455–497 (1993).
11. MacFarland, R. T., B. D. Zelus, and J. A. Beavo. High concentrations of cGMP-stimulated phosphodiesterase mediated ANP-induced decreases in cAMP and steroidogenesis in adrenal glomerulosa cells. *J. Biol. Chem.* **266**:136–142 (1991)
12. 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 cGMP response and aorta vasorelaxation: evidence for receptor subtypes. *Biochem. Biophys. Res. Commun.* **144**:422–431 (1987).
13. Minamitake, Y., Y. Kitajima, M. Furuya, M. Yoshida, and S. Tanaka. Structure-activity relationships of *alpha*-human atrial natriuretic peptide (*alpha*-hANP) analogs: role of charged groups in alpha-ANP. *Chem. Pharm. Bull.* **39**:2005–2008 (1991).
14. Oda, S., T. Sano, Y. Morishita, and Y. Matsuda. Pharmacological profile of HS-142–1, a novel nonpeptide atrial natriuretic peptide (ANP) antagonist of microbial origin. II. Restoration by HS-142–1 of ANP-induced inhibition of aldosterone production in adrenal glomerulosa cells. *J. Pharmacol. Exp. Ther.* **263**:241–245 (1992).
15. Ohyama, Y., K. Miyamoto, Y. Morishita, Y. Matsuda, Y. Saito, K. Kangawa, and H. Matsuo. Stable expression of natriuretic peptide receptors: effects of HS-142–1, a non-peptide ANP antagonist. *Biochem. Biophys. Res. Commun.* **189**:336–342 (1992).
16. Toki, S., Y. Morishita, T. Sano, and Y. Matsuda. HS-142–1, a novel non-peptide ANP antagonist, blocks the cyclic GMP production elicited by natriuretic peptides in PC12 and NG108–15 cells. *Neurosci. Lett.* **135**:117–120 (1992).
17. von Geldern, T. W., G. P. Budzik, T. P. Dillon, W. H. Holleman, M. A. Holst, Y. Kiso, E. I. Novosad, T. J. Opgenorth, T. W. Rockway, A. M. Thomas, and S. Yeh. Atrial natriuretic peptide antagonists: biological evaluation and structural correlations. *Mol. Pharmacol.* **38**:771–778 (1990).
18. von Geldern, T. W., T. W. Rockway, S. K. Davidsen, G. P. Budzik, E. N. Bush, M. Y. Chu-Moyer, E. M. Devine, Jr., W. H. Holleman, M. C. Johnson, and S. D. Lucas. Small atrial natriuretic peptide analogues: design, synthesis, and structural requirements for guanylate cyclase activation. *J. Med. Chem.* **35**:808–816 (1992).
19. von Geldern, T. W., G. P. Budzik, and T. P. Dillon. A ring-reversed analog of atrial natriuretic peptide retains receptor binding, guanylate cyclase stimulation. *Biochem. Biophys. Res. Commun.* **185**:734–739 (1992).
20. Weber, W., W. Fischli, E. Hochuli, E. Kupfer, and E. K. Weibel. Anantin: a peptide antagonist of the atrial natriuretic factor (ANF). *J. Antibiot. (Tokyo)* **44**:164–180 (1991).
21. Kashiwagi, M., T. Katafuchi, A. Kato, H. Inuyama, T. Ito, H. Hagiwara, Y. Takei, and S. Hirose. Cloning and properties of a novel natriuretic peptide receptor, NPR-D. *Eur. J. Biochem.* **233**:102–109 (1995).
22. 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 receptor. *EMBO J.* **13**:2508–2515 (1994).
23. Rainey, W. E., I. M. Bird, and J. I. Mason. The NCI-H295 cell line: a pluripotent model for adrenocortical studies. *Mol. Cell. Endocrinol.* **100**: 45–50 (1994)
24. 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).
25. Schoenfeld, J. R., P. Sehl, C. Quan, J. P. Burnier, and D. G. Lowe. Agonist selectivity for three species of natriuretic peptide receptor-A. *Mol. Pharmacol.* **47**:172–180 (1995).
26. Roskelley, C. D., K. G. Baimbridge, P. C. K. Leung, and N. Auersberg. Divergent differentiation of rat adrenocortical cells is associated with an interruption of angiotensin II-mediated signal transduction. *Mol. Cell. Endocrinol.* **89**:79–89 (1992).
27. Lupo di Prisco, C., G. Materazzi, and B. Scattolini. Inhibition of steroidogenesis by cyanoketone. *Gynecol. Invest.* **2**:309–315 (1971/72).
28. Aguilera, G., and K. J. Catt. Loci of action of regulators of aldosterone biosynthesis in isolated glomerulosa cells. *Endocrinology* **104**:1046–1052 (1979).

29. Drewett, J. G., B. M. Fendly, D. L. Garbers, and D. G. Lowe. Natriuretic peptide receptor-B (guanylyl cyclase-B) mediates C-type natriuretic peptide relaxation of precontracted rat aorta. *J. Biol. Chem.* **270**:4668–4674 (1995).
30. Koller, K. J., D. G. Lowe, G. L. Bennett, N. Minamino, K. Kangawa, H. Matsuo, and D. V. Goeddel. C-type natriuretic peptide (CNP) selectively activates the B natriuretic peptide receptor (ANPR-B). *Science (Washington D. C.)* **252**:120–123 (1991).
31. Cozza E. N., M. F. Koecking, M. del Carmen Vila, and C. E. Gomez-Sanchez. Adrenal receptors for natriuretic peptides and inhibition of aldosterone secretion in calf zona glomerulosa cells in culture. *Acta Endocrinol.* **129**:59–64 (1993).
32. Maack, T., M. Suzuki, F. A. Almeida, D. Nussenzveig, R. M. Scarborough, G. A. McEnroe, and J. A. Lewicki. Physiological role for silent receptors of atrial natriuretic factor. *Science (Washington D. C.)* **238**:675–678 (1987).
33. Schiebinger, R. J., D. C. Kem, and R. D. Brown. Effect of atrial natriuretic peptide on ACTH, dibutyryl cAMP, angiotensin II and potassium-stimulated aldosterone secretion by rat adrenal glomerulosa cells. *Life Sci.* **42**:919–926 (1988).
34. Barrett, P. Q., W. B. Bollag, C. M. Isales, R. T. McCarthy, and H. Rasmussen. Role of calcium in angiotensin II-mediated aldosterone secretion. *Endocr. Rev.* **10**:496–518 (1989).

Send reprint requests to: James G. Drewett, Ph.D., Department of Pharmacology and Toxicology, Medical College of Wisconsin, 8701 Watertown Plank Road, Milwaukee, WI 53226-4801. E-mail: drewett@post.its.mcw.edu