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Functionally Active Catalytic Domain Is Essential for Guanylyl Cyclase-Linked Receptor Mediated Inhibition of Human Aldosterone Synthesis

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ABSTRACT

An aspartate-to-alanine point mutation in the catalytic domain (D853A) of guanylyl cyclase-C (GC-C), the heat-stable enterotoxin (STa) receptor, rendered the enzyme catalytically inactive. Mn^{2+}/Triton X-100-stimulated guanylyl cyclase activity was detected in membranes from COS7 cells overexpressing GC-C but not GC-CD853A. STa treatment of paired cells resulted in cGMP production in those transiently expressing GC-C but not GC-CD853A. GC-C and GC-CD853A showed similar B_{max} and K_d values for [^{125}I]STa binding in these cells, indicating that the lack of catalytic activity in the latter was not due to differing expression levels or reduced binding affinity. The involvement of the catalytic domain in aldosteronogenesis was studied in human adrenocortical H295R cells. COS7 and H295R cells infected with vaccinia virus-expressing GC-C and GC-CD853A (VVGC-CD853A) had [^{125}I]STa-binding characteristics akin to those in transfected cells. Immunoblot confirmed that both GC-C and GC-CD853A formed similar higher order oligomers in infected cells. Virus-mediated expression of GC-C in H295R cells revealed concentration-dependent STa-stimulated cGMP formation that was undetectable in VVGC-CD853A-infected cells. STa decreased angiotensin II-stimulated human aldosterone generation in a concentration-dependent manner in vaccinia virus-expressing GC-C-infected cells but not in those infected with VVGC-CD853A. These results demonstrate that a catalytically active guanylyl cyclase is required for the inhibition of aldosteronogenesis.

ANP was originally discovered in the early 1980s as a bioactive substance isolated from cardiac atria (deBold *et al*., 1981; Currie *et al*., 1983). The biological activity of ANP has long been known to be antihypertensive in nature, yet the receptors and mechanisms by which this peptide mediates its effects remain unclarified (Anand-Srivastava and Trachte, 1993; Drewett and Garbers, 1994). Some groups have suggested that ANP may mediate its action through binding to the natriuretic peptide clearance receptor (Anand-Srivastava and Trachte, 1993), a protein established to remove the cardiac peptide from the circulation (Maack, 1992). However, the majority of studies conclude that ANP results in blood pressure-lowering activities including vasodilation, natriuresis, and diuresis through the stimulation of GC (EC 4.6.1.2) activity (Chinkers *et al*., 1989; Lowe *et al*., 1989; Drewett and Garbers, 1994), including two recent studies using gene knock-out mice (Lopez *et al*., 1995, 1997).

Two forms of membrane-associated GCs are known to bind natriuretic peptides. Natriuretic peptide receptor A, or GC-A, is known to selectively bind ANP (Chinkers *et al*., 1989; Lowe *et al*., 1989) and a related type B natriuretic peptide (Schoenfeld *et al*., 1995) whereas natriuretic peptide receptor B, or GC-B (Schulz *et al*., 1989; Chang *et al*., 1989), is a receptor for type C natriuretic peptide (Koller *et al*., 1990). These receptors are related to GC-C, the STa receptor, found mainly in the gastrointestinal tract (Schulz *et al*., 1990) and other forms of particulate GC isolated from sensory tissues (Shyjan

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ABBREVIATIONS: Ang, angiotensin; ANOVA, analysis of variance; ANP, type A atrial natriuretic peptide; BSA, bovine serum albumin; HEK, human embryonic kidney; GC, guanylyl cyclase; MOI, multiplicity of infection; PBS, phosphate-buffered saline; sANP, natriuretic peptide receptor/GC-A selective agonist; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; STa, heat-stable enterotoxin; TBS, Tris-buffered saline; VVWT, wild-type vaccinia virus; VVGC-C, recombinant vaccinia virus expressing GC-C; VVGC-C D853A, recombinant vaccinia virus expressing GC-C D853A.

et al., 1992; Yu *et al*., 1997). All members of the particulate GCs have the same general topology: an extracellular ligand-binding domain, a transmembrane region, and two intracellular regions composed of a protein kinase-homology domain and a catalytic domain (Shyjan *et al*., 1992; Drewett and Garbers, 1994; Yu *et al*., 1997).

Several cell types and tissues exhibit ANP-mediated increases in intracellular cGMP levels, including adrenocortical cells (Anand-Srivastava and Trachte, 1993; Drewett and Garbers, 1994), where the peptide is also found to reduce Ang II-evoked synthesis of aldosterone, an important salt-retaining hormone involved in the maintenance of blood volume and pressure. Goodfriend *et al*. (1984) were the first to report that synthetic ANP inhibited aldosterone synthesis. Subsequent studies originating at the turn of the decade have provided the strongest evidence that this effect is mediated through a GC-linked natriuretic peptide receptor. MacFarland *et al*. (1991) obtained interesting data indicating that ANP increased cGMP production, resulting in the concomitant stimulation of a cGMP-sensitive or type II phosphodiesterase to degrade cAMP in bovine zona glomerulosa cells. This effect of ANP is consistent with an inhibition of cAMP-dependent steroidogenesis in the same cells. Furthermore, Oda *et al*. (1992) demonstrated pharmacologically that a GC-linked natriuretic peptide receptor antagonist (HS-142–1) of microbial origin blocked the ability of ANP to reduce aldosterone synthesis. The results of these studies strongly suggested that a GC-linked natriuretic peptide receptor was responsible for the ANP-mediated attenuation of steroidogenesis. At odds with these results were those previously demonstrating that sodium nitroprusside, an activator of nitric oxide-sensitive GC, markedly increased cGMP production but did not block steroidogenesis in rat and bovine zona glomerulosa cells (Matsuoka *et al*., 1987; Okamoto, 1988). Furthermore, in the same studies and in another (Ganguly *et al*., 1989), ANP inhibited steroidogenesis, whereas membrane-permeable cGMP analogs failed to mimic this effect. Taken together, these data left unclear the necessity of increases in intracellular cGMP levels to an ANP-mediated inhibition of steroidogenesis.

The ability to construct catalytically inactive point mutants of the GC family makes it possible to specifically test for the necessity of a catalytically active enzyme, and hence an increase of cGMP synthesis, in mediating intracellular responses. Thompson and Garbers (1995) have shown that a mutant of GC-A, GC-A D893A, retained the same binding characteristics associated with the wild-type receptor except for the catalytic ability to convert GTP to cGMP. In the current study, the homologous amino acid in GC-C was mutated to form GC-C D853A, which was found to be catalytically inactive while it retained the abilities to bind [^{125}I]STa and to self-associate, forming higher order oligomers.

Human adrenocortical H295R cells do not express endogenous GC-C. These cells thus are an ideal system in which to heterologously express GC-C and the point mutant to study the potential inhibitory effect of the GC family on human steroidogenesis. To date, there are no reports of successful transfections of H295R cells with GC cDNA and resultant protein expression. Attempts to transfect H295R cells with several different plasmids containing GC-C were unsuccessful in this laboratory as well; therefore, recombinant vaccinia viruses were used as an alternative to express proteins in these cells. The expression of GC-C and GC-C D853A was monitored by both [^{125}I]STa binding and receptor-specific immunoblot. We then examined the effects of STa on cGMP production and aldosterone synthesis in H295R cells and specifically tested the hypothesis that a functionally active catalytic domain of GC is essential to mediate inhibition of human aldosteronogenesis.

Materials and Methods

Cell culture. H295R cells were kindly provided by Dr. William E. Rainey (University of Texas Southwestern Medical Center, Dallas, TX) from original isolated passages (Rainey *et al*., 1994). These cells were maintained as described previously in 3% Nuserum I (Collaborative Research, Franklin Lakes, NJ)-supplemented medium (Olson *et al*., 1996). COS7 cells were similarly cultured with other differences specified previously (Schulz *et al*., 1990). HEK 293 cells overexpressing a stable transfectant of GC-C (gracious gift of Dr. David L. Garbers, Howard Hughes Medical Institute, University of Texas Southwestern Medical Center, Dallas, TX) were grown in media of the same composition as that for COS7 cells with the addition of G418 (200 μg/ml; GIBCO, Gaithersburg, MD). T84 cells (American Type Culture Collection, Rockville, MD) were grown in the same medium as H295R cells with the exception that 5% fetal bovine serum (Atlanta Biologicals, Norcross, GA) was used instead of Nuserum I. Ltk$^-$ cells (gracious gift of Dr. Bernard Moss, National Institutes of Health, Bethesda, MD) were cultured according to the method of Mackett *et al*. (1984). All tissue culture media were purchased from GIBCO. Specific cell densities and plating configurations for each type of experiment are given below. All cells were maintained in a water-jacketed incubator (Nuaire, Plymouth, MN) at 37° in a humidified atmosphere of 95% air and 5% CO_2 (Bentley Welding Supply, Milwaukee, WI; Praxair, Grand Forks, ND).

Construction of recombinant vaccinia viral plasmid. The *Bsp*HI site at the initiation codon of rat GC-C cDNA (gracious gift of Dr. D. L. Garbers) was ligated in frame to the compatible *Nco*I site of the vaccinia viral plasmid pTM1 (gracious gift of Dr. B. Moss). The cDNA encoding rat GC-C was endonuclease-excised as two fragments from pCMV5 GC-C: a 631-bp *Bsp*HI/*Xba*I fragment including the start codon at the *Bsp*HI site and a 3.1-kb *Xba*I/*Sal*I fragment representing the remainder of GC-C. These two fragments were ligated (T4 DNA ligase) into pTM1 to yield pTM1 GC-C. All restriction endonucleases and DNA-modifying enzymes were purchased from New England Biolabs (Beverly, MA) or Promega (Madison, WI).

Development of *cyc*$^-$ GC-C mutant. Based on the development of the catalytically inactive GC-A D893A mutant (Thompson and Garbers, 1995), we constructed the homologous Asp853-to-Ala853 mutation in GC-C. Two independent amplifications were performed from pBluescript GC-C with primer sets 1 and 2: set 1, 5′-atc.g-gc.g**C**t.gcc.tac.gtg.gtg.gcc.a-3′ (sense primer) and 5′-gct.gct.cat.gt-g.aat.cc-3′ (antisense primer); and set 2, 5′-ggc.tga.cca.cct.taa.ct-3′ (sense primer) and 5′-cgt.agg.ca**G**.cgc.cga.tgg.ttt.cta.c-3′ (antisense primer) (Higuchi, 1990). The mutated base pairs are denoted in bold, uppercase text. The sense primer in set 1 was designed to be partially complementary to the antisense primer in set 2 to form two overlapping products containing the mutation. The amplifications were performed in an Ericomp Thermal Cycler (La Jolla, CA) using Vent DNA polymerase (New England Biolabs), 1 μM primer, and 1 ng of DNA under the following conditions: 1 cycle of 98° (7 min), 72° (5 min) followed by 35 cycles of 94° (1 min), 55° (1 min), and 72° (25 sec). The two amplified products then were denatured, annealed and extended with Vent DNA polymerase for 10 cycles using the same reaction conditions. The annealed product was further amplified as above with sense primer 1 and antisense primer 2 for 25 cycles.

The amplified mutated fragment was digested with *Nco*I at bp 2522 and *Sph*I at bp 2666 and substituted into pTM1 GC-C to form pTM1 GC-C D853A. The mutation and recombination were verified

by double-stranded dideoxysequencing (Amersham/USB Sequenase, Arlington Heights, IL).

Construction of recombinant vaccinia viruses. Both vaccinia viruses (WR strain and VTF7–3) and pTM1 were graciously provided by Dr. B. Moss. Recombinant vaccinia viruses containing GC-C (VVGC-C) and GC-C D853A (VVGC-C D853A) were generated by homologous recombination after transfection of human Ltk$^-$ cells previously infected with the wild-type (WR strain) vaccinia virus (Mackett *et al*., 1984). The recombinant viruses were selected by survival in 5-deoxybromouridine (Sigma Chemical, St. Louis, MO) followed by a secondary screening using slot-blot hybridization with a ~3.1-kb radiolabeled cDNA probe specific for GC-C (*Xba*I site at bp 631 through the 3′-end of the cDNA clone, ~bp 3700; Schulz *et al*., 1990). In all viral expression experiments, cells were coinfected with VVWT, VVGC-C, or VVGC-C D853A and the helper vaccinia virus, VTF7–3, which expresses the T7 RNA polymerase required for recombinant protein transcription (Elroy-Stein *et al*., 1989; see below). Henceforth, in the current report any reference to VVWT, VVGC-C, or VVGC-C D853A will include coinfection with the helper vaccinia virus as well.

Crude membrane preparation for binding studies and immunoblot. Cells were washed with PBS (138 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.4; Fisher Chemical, Fairlawn, NJ) before being scraped from the plate and resuspended in 50 mM Tris (pH 7.6), 1 mM EDTA (Fisher), and 1 mM phenylmethylsulfonyl fluoride (Sigma). Cells were lysed by sonication for 15 sec on ice and centrifuged at 30,000 × *g* for 30 min. Protein concentrations of the pellet were quantified according to the method of Bradford (1976) using BSA (Fisher) as the standard.

Western blot analysis. Crude membranes (50 μg) from transiently transfected COS7 cells and stably transfected HEK 293 cells overexpressing rat GC-C (gift of Dr. D. L. Garbers) were separated by SDS-PAGE (8% polyacrylamide). Crude membranes (5 μg) from virus-infected COS7 and H295R cells were separated by SDS-PAGE (5–10% gradient polyacrylamide) (Owl Scientific, Woburn, MA). High range molecular weight markers (Boehringer-Mannheim, Indianapolis, IN) were included for determination of molecular weights. Proteins in the gel were semidry transferred (Owl Scientific) onto 0.45-μm nitrocellulose membrane (Micro Separations, Westboro, MA) and blocked overnight in 5% nonfat dry milk (Sam's Choice, Bentonville, AR) in TBS (20 mM Tris-base, 137 mM NaCl, pH 7.6; Fisher) supplemented with 0.1% Tween-20 (Fisher). After three consecutive washes with TBS/0.3% Tween-20, blots were incubated with 1:3000 diluted antisera (gift of Dr. D. L. Garbers) (TBS/0.1% Tween-20 with 1% BSA) for 1 hr at room temperature. The crude membranes were washed again before incubation with horseradish peroxidase-conjugated goat anti-rabbit antiserum (Amersham) at a 1:10,000 dilution. After washing in TBS/0.3% Tween-20, the signal was detected by enhanced chemiluminescence (Pierce Chemical, Rockford, IL).

Transient transfections of COS7 cells. GC-C and GC-C D853A were subcloned into the eukaryotic expression vector pCDNA3 (InVitrogen, La Jolla, CA) to make pCDNA3 GC-C and pCDNA3 GC-C D853A, respectively. COS7 cells were transfected with either pCDNA3 (mock), pCDNA3 GC-C, or pCDNA3 GC-C D853A by the diethylaminoethyl-dextran (Sigma) method using 10 μg of DNA/10^6 cells on a 100-mm culture dish (Sarstedt, Newton, NC). Cells were grown for 48 hr to allow for expression before experimentation (Schulz *et al*., 1990) (i.e., radioligand binding, cyclase activity, immunoblot, and so on).

Vaccinia virus-mediated expression. For studies using virus-mediated expression, the rates of protein expression and incorporation into the membrane were studied in COS7 cells (2 × 10^6 cells/100-mm dish). Crude membranes were isolated 4.5, 6, 8, 14, or 24 hr after infection of cells with VVWT or VVGC-C at an MOI of 6 and with helper virus at an MOI of 1. GC activity in membranes from VVGC-C-infected cells increased from 2.5 ± 0.1 to a maximum of 36 ± 8 mmol of cGMP formed/min/μg of protein after 14 hr (six experiments) (Olson LJ and Drewett JG, unpublished observations). Therefore, assays were performed 14 hr after infection throughout this study in all cells infected with VVWT, VVGC-C, or VVGC-C D853A.

Steroidogenesis. H295R cells in Sarstedt Cell$^+$ 24-well plates (1.2 × 10^5 cells/well) were infected with the appropriate vaccinia virus (VVWT, VVGC-C, or VVGC-C D853A) as described above. Cells were subsequently rinsed twice with PBS and incubated with Ang II (5 nM) in the presence of either ANP (10^{-10} to 10^{-6} M), STa (10^{-12} to 10^{-8} M), or distilled water (vehicle) for 2 hr in serum-free medium (Ham's F-12, 0.2% BSA, 2.6 mM $CaCl_2$, 25 mM HEPES, pH 7.4) at 37° in 95% air and 5% CO_2. Aldosterone was quantified by specific radioimmunoassay as described previously (Olson *et al*., 1996). STa, aldosterone, human ANP, and human Ang II were purchased from Sigma. $CaCl_2$ and HEPES were from Fisher. Aldosterone antibody (lot 088) was obtained from the National Pituitary Distribution Center (National Institutes of Health, Bethesda, MD). [^{3}H]Aldosterone was purchased from New England Nuclear (Boston, MA).

STa-binding assays. Binding assays were conducted on crude membranes (500 μg/ml) from transiently transfected COS7 cells and virus (VVWT, VVGC-C, and VVGC-C D853A as described above)-infected COS7 and H295R cells at 37° in buffer (pH 7.6, 50 mM Tris·HCl, 500 mM NaCl, 0.67 mM cystamine, 0.1% bacitracin, and 1 mM EDTA) (Deshmane *et al*., 1995). Bacitracin and cystamine were from Sigma. STa was radioiodinated (ICN, Costa Mesa, CA), and the resulting [^{125}I-Tyr$_4$]STa was high performance liquid chromatography purified as described previously (Schulz *et al*., 1990; Deshmane *et al*., 1995). The specific activity (~1000 Ci/mmol) of the radiolabeled STa was determined according to the method of Deshmane *et al*. (1995). Reactions were terminated after 2 hr by rapid vacuum filtration through Millipore multiscreen filtration plates (1.2-μm hydrophilic low protein-binding membranes; Bedford, MA) followed by three rinses with wash buffer (20 mM sodium phosphate, pH 7.2, 1 mM EDTA, 150 mM NaCl; Fisher). Radioactivity bound to filters was quantified using a Packard 2100TR scintillation counter (Meriden, CT). K_D and B_{max} values were calculated from linear Scatchard plots of the binding data.

GC assays. Crude membranes (25 μg) from transiently transfected COS7 cells were incubated for 5 min at 37° in a total volume of 0.15 ml of buffer containing 20 mM HEPES (pH 7.4), 1 mM $MnCl_2$, 1% Triton X-100 (Fisher), 0.2 mM isobutyl-methylxanthine, 0.1 mM GTP, 0.1 mM ATP (Sigma), and 1 μCi of [α-^{32}P]GTP (ICN, Costa Mesa, CA). Reactions were stopped with 0.45 ml each of 110 mM zinc acetate and 110 mM sodium carbonate (Fisher). The samples were centrifuged (3000 × *g*, 10 min), the supernates were column purified over alumina (Hansborough and Garbers, 1981), and cyclase activity was quantified by scintillation counting (Packard 2100TR).

Whole-cell cGMP assays. Cells in plates were rinsed twice with PBS and preincubated 10 min at 37° in serum-free medium supplemented with 0.25 mM isobutyl-methylxanthine before treatment. Cells in individual plates were mock transfected, GC-C transfected, or GC-C D853A transfected as described above. One plate each was incubated in fresh treatment buffer containing either vehicle or 10^{-7} M STa. Uninfected and virus-infected H295R cells (24-well Sarstedt Cell$^+$ plate; 1.2 × 10^5 cells/well; MOI as described above for virus infection) were incubated in fresh treatment buffer containing vehicle, STa (10^{-11} to 10^{-7} M), or ANP (10^{-10} to 10^{-6} M). All reactions were terminated after 5 min by rapid aspiration and addition of 0.5 ml of 1 N $HClO_4$ (Fisher) before freezing at −80°. Cell supernates were subjected to anion-exchange column chromatography and radioimmunoassay as described previously (Hansborough and Garbers, 1981). cGMP antibody was graciously provided by Dr. D. L. Garbers. [^{125}I]cGMP and unlabeled cyclic nucleotide were purchased from ICN.

Cell viability assay. The Eukolight Cytotoxicity Assay (Molecular Probes, Eugene, OR) was used to determine the effect of vaccinia virus infection, STa and ANP treatment, or both on H295R cellular viability. This kit simultaneously determines the number of live and

dead cells by measuring two parameters of cell viability, esterase activity associated with the conversion of calcein AM to a fluorescent product and the uptake of ethidium homodimer by cells with weakened membrane permeability associated with toxicity. The procedures were identical to those outlined in the manufacturer's specifications with the following changes. H295R cells (1.2×10^5 cells/35-mm Sarstedt dish) were uninfected or infected with the appropriate titer of each virus (VVWT, VVGC-C, or VVGC-C D853A) for 14 hr as described above and in the results for a given experiment. In experiments involving treatment with STa or ANP (10^{-6} M), the cells were incubated with these peptides and Ang II (5 nM) for 2 hr as described to emulate the same treatment used during steroidogenesis studies. After the treatment period, the cells were rinsed twice with PBS and treated with 60 μl of the calcein AM/ethidium homodimer mixture (2 μM each). After 40 min at 25°, the cells were visualized using fluorescent microscopy as a monolayer in each dish. The average of cell counts (green, live; red, dead) from three fields was taken as the value for each experiment. Each experiment was repeated three times. The average number of cells per field for a typical experiment was 195 ± 11 cells. Results were expressed as percentage of live cells (mean ± standard error).

Statistics. The concentration dependence for a given reagent (STa, ANP, or Ang II) in all concentration-response curves was determined by repeated measures ANOVA. Dunnett's modification of Student's paired *t* test also was used to compare base-line cGMP and aldosterone levels in VVGC-C-infected H295R cells with those uninfected, VVGC-C D853A infected, or VVWT infected. Statistical significance was declared as ***, $p < 0.001$, **, $p < 0.01$, and *, $p < 0.05$.

Results

GC activity of GC-C and GC-C D853A. COS7 cells were transiently transfected with either pCDNA3 (mock), pCDNA3 GC-C, or pCDNA3 GC-C D853A. Membranes isolated from pCDNA3 GC-C-transfected cells exhibited Mn^{2+}/Triton X-100-stimulated GC activity (i.e., 416 ± 70 fmol of cGMP formed/min/mg of protein; four experiments) as assessed by the conversion of [α-^{32}P]GTP to [^{32}P]cGMP. Such an increase in GC activity was not observed in the membranes from mock- or pCDNA3 GC-C D853A-transfected cells (i.e., 49 ± 15 and 56 ± 16 fmol of cGMP formed/min/mg of protein, respectively; four experiments). STa (10^{-7} M) markedly increased cGMP synthesis ~50-fold in both COS7 cells transiently transfected wth GC-C and H295R cells infected with VVGC-C (Fig. 1). No effect of STa on cGMP generation was observed in paired mock-infected, GC-C D853A-transfected/infected cells (Fig. 1), untransfected/uninfected cells (Olson LJ and Drewett JG, unpublished observations), or VVWT-infected cells (Olson LJ and Drewett JG, unpublished observations).

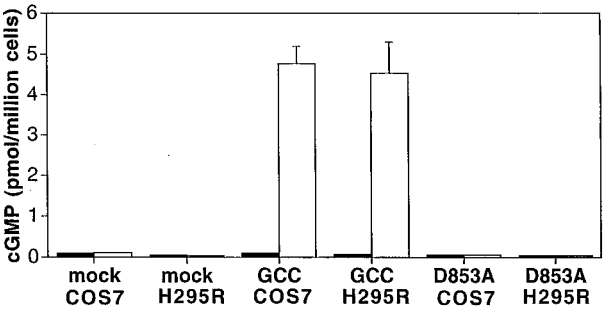

Fig. 1. STa stimulation of cGMP production in COS7 and H295R cells expressing GC-C and GC-C D853A. COS7 cells that were transfected with pCDNA3 (*mock*), pCDNA3 GC-C (*GCC*), or pCDNA3 GC-C D853A (*D853A*). H295R cells were VVWT (mock), VVGC-C (GC-C), or VVGC-C D853A-(D853A) infected. cGMP levels were measured in the absence of STa (■) and the presence of 10^{-7} M STa (□). Columns are mean ± standard error for four experiments.

Radiolabeled STa binding to GC-C and GC-C D853A. Expression of GC-C and GC-C D853A in the above experiments was confirmed by radiolabeled STa binding. [^{125}I]STa bound to crude cell membranes transiently transfected with GC-C or GC-C D853A with similar K_D and B_{max} values (Table 1) with no statistical difference. Untransfected cells demonstrated only nonspecific [^{125}I]STa binding (Olson LJ and Drewett JG, unpublished observations).

Crude cell membranes from virus-infected COS7 and H295R cells also were used for radioligand binding studies similar to those for transiently transfected COS7 cells. GC-C and GC-C D853A exhibited K_D values that were not statistically different from each another in virus-infected H295R cells and COS7 cells (Table 1) and were similar to those in transiently transfected cells (Table 1). Experiments with membranes from uninfected cells or VVWT-infected H295R cells showed only nonspecific binding under the same conditions (Olson LJ and Drewett JG, unpublished observations). The expression level of the receptor as assessed by B_{max} values also was not significantly different between GC-C and GC-C D853A in virus-infected H295R and COS7 cells (Table 1).

Formation of similar higher order oligomers on expression of GC-C or GC-C D853A. Crude membranes from virus-infected COS7 and H295R cells were separated by SDS-PAGE (5–10% polyacrylamide gradient) on paired gels under reducing and nonreducing conditions. For each cell type, one gel was probed with the antibody to the carboxyl terminus of GC-C and the antigenic peptide (1 μM), and the other was probed with only antibody. Under nonreducing conditions, GC-C and GC-C D853A form higher order oligomers in COS7 cells with calculated molecular masses of 320 and 285 kDa and monomer molecular mass bands of 130 and 110 kDa (Fig. 2A). The same electrophoretic separation and immunoblotting conditions yielded similar results for crude membranes isolated from VVGC-C- and VVGC-C D853A-infected H295R cells (Olson LJ and Drewett JG, unpublished observations). The ability of both the mutant and wild-type receptors to form similar higher order structures under nonreducing conditions (Fig. 2A) indicates that the single point mutation does not block self-association. The lower two bands also are observed under reducing conditions (Fig. 2B) and may represent different glycosylation states of GC-C as reported by Vaandrager *et al.* (1994). The presence of higher molecular mass bands is consistent with data suggesting that GC-C may form trimers in stable HEK 293 cells expressing the receptor (Vaandrager *et al.*, 1994). Results similar to those presented in Fig. 2B were obtained after Western blot analysis of crude membranes from HEK 293 cells overexpressing GC-C and transiently transfected COS7 cells expressing GC-C or GC-C D853A (Olson LJ and Drewett JG, unpublished observations). Crude membrane protein from uninfected and VVWT-infected COS7 and H295R cells showed no chemiluminescent signal after Western blotting (Olson LJ and Drewett JG, unpublished observations) as described above.

Base-line cGMP production in H295R cells expressing GC-C and GC-C D853A. Fig. 3 presents basal cGMP

levels in H295R cells that were uninfected (no VV) and infected with VVWT, VVGC-C, and VVGC-C D853A. Uninfected cells and those infected with either VVWT or VVGC-C D853A had similar basal cGMP levels in contrast to cells infected with VVGC-C. The latter cells had slightly but significantly elevated base-line cGMP concentrations in comparison to those infected with VVGC-C D853A ($p < 0.05$).

Concentration-response curves for STa-stimulated cGMP production in VVGC-C- and VVGC-C D853A-infected H295R cells. The stimulatory effect of STa on cGMP synthesis was examined further in VVGC-C-infected H295R cells. STa (10^{-11} to 10^{-6} M) stimulated cGMP generation in a concentration-dependent (Fig. 4) manner in H295R cells similar to that in the colonic, epithelial T84 cell line known to express GC-C endogenously (Currie *et al*., 1992). The same STa treatment did not increase cGMP synthesis in paired VVWT- or VVGC-C D853A-infected cells (Fig. 4). In T84 cells or VVWT-, VVGC-C-, and VVGC-C D853A-infected H295R cells, no greater stimulation of STa-mediated cGMP synthesis was observed after treatment with 10^{-5} M STa (Olson LJ and Drewett JG, unpublished observations). The concentration response for ANP (10^{-10} to 10^{-6} M) on cGMP production in H295R cells also is shown in Fig. 4 and is similar to the lower portion of the curve for STa in cells overexpressing GC-C. Similar results to those presented in Fig. 4 also were observed after ANP treatment of VVWT-, VVGC-C-, and VVGC-C D853A-infected H295R cells (Olson LJ and Drewett JG, unpublished observations). Base-line cGMP concentrations for H295R cells were presented in Fig. 3. Those for T84 cells were 11.1 ± 2.0 fmol/μg of protein (mean ± standard error).

Base-line aldosterone synthesis in H295R cells expressing GC-C and GC-C D853A. Uninfected cells and those infected with VVWT or VVGC-C D853A were found to have nearly identical base-line aldosterone levels (Fig. 3). However, cells infected with VVGC-C exhibited significantly lower base-line aldosterone concentrations ($p < 0.001$) in addition to significantly elevated base-line cGMP concentrations ($p < 0.05$) compared with VVGC-C D853A-infected cells expressing the catalytically inactive point mutant (Fig. 3). There was no difference in H295R cellular viability (three experiments) between uninfected cells (94.7 ± 1.2%) relative to those infected with VVWT (95.0 ± 0.6%), VVGC-C (94.7 ± 0.7%), or VVGC-C D853A (94.7 ± 0.9%).

Ang II-stimulated aldosterone production in H295R cells. As shown in Fig. 5, Ang II (10^{-10} to 10^{-7} M) increased aldosterone production in uninfected and VVGC-C-infected H295R cells in a similar concentration-dependent manner (ANOVA, $p < 0.001$). No greater effect was observed after treatment with 10^{-6} M Ang II. The EC_{60} value for Ang II was ~5 nM, and maximal stimulation (~4-fold) was attained at 0.1 μM Ang II. Similar concentration-dependent effects of Ang II (10^{-10} to 10^{-6} M) on aldosterone production also were observed in VVGC-C D853A- and VVWT-infected cells (Olson LJ and Drewett JG, unpublished observations).

STa effect on Ang II-stimulated aldosterone production in VVGC-C- or VVGC-C D853A-infected H295R cells. From the results of Fig. 5 and a previous study (Olson *et al*., 1996), an approximate EC_{60} concentration (5 nM) that produces a 2-fold stimulation of steroidogenesis (Fig. 5) was chosen to examine the effect of STa (10^{-12} to 10^{-8} M) on aldosterone production in paired H295R cells expressing either GC-C or GC-C D853A. Ang II (5 nM)-induced aldosterone synthesis was attenuated by STa only in VVGC-C-infected cells but not in paired VVGC-C D853A-infected cells (Fig. 6A), VVWT-infected cells (Olson LJ and Drewett JG, unpublished observations), or uninfected cells (Olson LJ and Drewett JG, unpublished observations). A maximal suppression of ~20–25% was attained at 10^{-9} M STa (Fig. 6A) in VVGC-C-infected cells. STa (10^{-6} M) treatment did not affect H295R cell viability (three experiments) in uninfected cells (94.3 ± 0.3%) or those infected with VVGC-C (95.0 ± 0.6%) or VVGC-C D853A (94.7 ± 0.9%) in comparison to infected cells not receiving STa (see above).

ANP effect on Ang II-stimulated aldosterone synthesis in H295R cells. ANP treatment produced a concentration-dependent inhibition of Ang II (5 nM)-stimulated aldosterone synthesis in uninfected H295R cells (Fig. 6B). These results are consistent with a previous study from this laboratory (Olson *et al*., 1996) using both ANP and a human GC-A-selective agonist (sANP). It is noteworthy that the maximal effect of STa to diminish Ang II-stimulated aldosterone production by ~20–25% (Fig. 6A) is similar to that observed at a maximally effective ANP concentration (Fig. 6B) and also is consistent with the effectiveness of the GC-A-selective agonist sANP in H295R cells (Olson *et al*., 1996). ANP (10^{-6} M) did not affect H295R cell viability (i.e., for three experiments, ANP vehicle treated, 94.7 ± 1.2%; ANP treated, 95.0 ± 1.2%). Similar concentration-dependent effects of ANP on Ang II (5 nM)-evoked aldosteronogenesis were observed in VVGC-C- and VVGC-C D853A-infected cells (Olson LJ and Drewett JG, unpublished observations).

Discussion

Goodfriend *et al*. (1984) were the first to report that synthetic ANP reduced Ang II-, K^+-, and membrane-permeable cAMP analog-facilitated aldosterone synthesis in bovine zona glomerulosa cells. The ANP-mediated reduction of aldosterone synthesis is commensurate with the natriuretic and diuretic activities of this cardiac peptide, which is known to bind to a membrane-associated GC to mediate most of its

TABLE 1

K_D and B_{max} values for [^{125}I]STa-binding to GC-C and GC-C D853A in COS7 and H295R cells

For each expression method, the K_D and B_{max} values (mean ± standard error) between GC-C and GC-C D853A were not significantly different by Student's paired *t* test. The number of times each experiment was repeated in duplicate is shown in the column labeled *n*.

Cell type	*n*	Expression method	K_D		B_{max}	
			GC-C	GC-C D853A	GC-C	GC-C D853A
			nM		*pmol/mg of protein*	
COS7	4	DEAE/Dextran	0.31 ± 0.05	0.18 ± 0.04	0.37 ± 0.04	0.33 ± 0.02
COS7	4	Vaccinia virus	0.23 ± 0.04	0.12 ± 0.06	0.16 ± 0.01	0.11 ± 0.01
H295R	3	Vaccinia virus	0.15 ± 0.01	0.17 ± 0.02	0.14 ± 0.01	0.19 ± 0.01

biological activity (Chinkers *et al.*, 1989; Lowe *et al.*, 1989; Drewett and Garbers, 1994). Studies in more recent years have focused on defining the exact relationship between the ANP-mediated inhibition of aldosterone synthesis and stimulation of cGMP production in zona glomerulosa cells (MacFarland *et al.*, 1991; Oda *et al.*, 1992; Ganguly *et al.*, 1989) and H295R cells (Bodart *et al.*, 1996; Olson *et al.*, 1996). Taken together, the results of these studies are somewhat confusing because neither membrane-permeable cGMP analogs (Matsuoka *et al.*, 1987; Okamoto, 1988; Ganguly *et al.*, 1989) nor treatment with sodium nitroprusside, an activator of soluble GC (Matsuoka *et al.*, 1987; Okamoto, 1988), has mimicked the effects of ANP. In 1991, MacFarland *et al.* obtained compelling evidence that supported the hypothesis that cGMP was responsible for ANP-mediated inhibition of adrenocorticothyrotropic hormone-evoked aldosterone synthesis. They proposed that ANP resulted in increased synthesis of cGMP, which activated cGMP-sensitive, type II phosphodiesterase to decrease intracellular cAMP concentrations and aldosterone synthesis. Moreover, type II phosphodiesterase purified from bovine zona glomerulosa cells was stimulated by cGMP but not by membrane-permeable cGMP analogs, consistent with a previous study (Erneux *et al.*, 1985). MacFarland *et al.* (1991) suggested that this phenomenon (Erneux *et al.*, 1985) may account for the previous disparity between the ability of ANP, but not cGMP analogs, to inhibit steroidogenesis (Matsuoka *et al.*, 1987; Okamoto, 1988; Ganguly *et al.*, 1989). The data of MacFarland *et al.* (1991), although potentially better characterizing the action of ANP, left unclear at least two issues. First, the results of Goodfriend *et al.* (1984) demonstrated that ANP diminished aldosterone synthesis evoked by N^6-2′-*O*-dibutyryl cAMP, an analog that is not degraded by type II phosphodiesterase in the same cell type (MacFarland *et al.*, 1991). Second, the inability of sodium nitroprusside-mediated production of native cGMP to result in concomitant attenuation of aldostero-

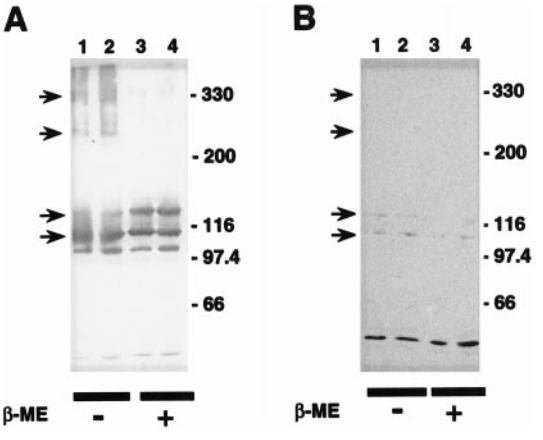

Fig. 2. Oligomerization of GC-C and GC-C D853A. A, Crude membrane protein (5 μg) from COS7 cells infected with either VVGC-C (*lanes 1* and *3*) or VVGC-C D853A (*lanes 2* and *4*) were separated by SDS-PAGE (5–10% polyacrylamide gradient) before immunoblotting with an anti-peptide antibody specific to GC-C. Proteins in *lanes 1* and *2* were not reduced before loading, whereas those in *lanes 3* and *4* were reduced with 70 mM β-mercaptoethanol (β-*ME*) before loading. The positions of protein molecular mass markers are denoted on the right in kDa. B, An identically prepared SDS-PAGE as shown in A with exception that it was probed with antibody in the presence of the antigenic carboxyl-terminal peptide (1 μM, peptide block). *Arrows*, bands eliminated by peptide block.

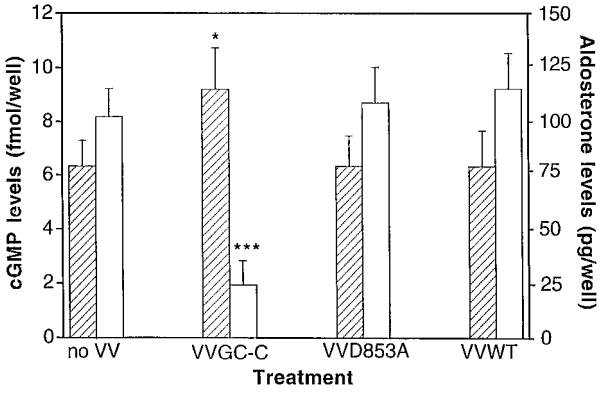

Fig. 3. Effects of expression of GC-C and GC-C D853A on base-line cGMP and aldosterone levels in H295R cells. Basal cGMP (▨) and aldosterone (□) levels were measured in paired uninfected cells (*no VV*), VVGC-C-infected cells, VVGC-C D853A-infected cells (*VVD853A*), and VVWT-infected cells (mean ± standard error for four experiments; *, $p < 0.05$; ***, $p < 0.001$ by Dunnett's paired t test for VVGC-C versus VVD853A).

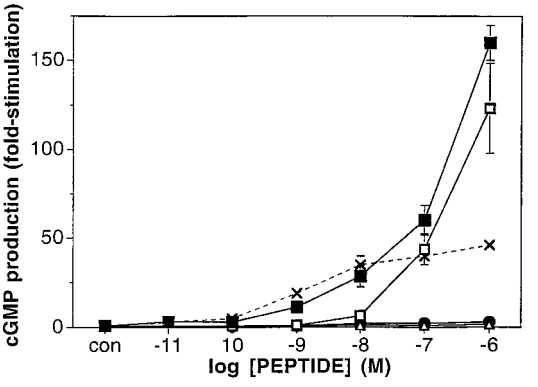

Fig. 4. Effects of STa on cGMP levels in H295R cells. STa-stimulated cGMP generation was measured in intact H295R cells that were VVGC-C infected (■), VVGC-C D853A infected (●), or VVWT infected (△). STa had no effect on cGMP production in uninfected cells similar to that observed in VVWT-infected cells (Olson LJ and Drewett JG, unpublished observations). Paired uninfected cells were treated with ANP (- -×- -) to stimulate native GC-A for comparison to STa treatment of VVGC-C-infected H295R cells. T84 cells known to express native GC-C also were treated with STa (□) as an additional comparison. For each concentration-response, *curve points* are mean ± standard error for H295R cells (four experiments) and for T84 cells (six experiments). Base-line concentrations of cGMP are given in Fig. 3 or the text, respectively. In all experiments, there was a concentration-dependent stimulation of cGMP production on STa or ANP addition as shown for the appropriate cell type and experiment (ANOVA, ***$p < 0.001$).

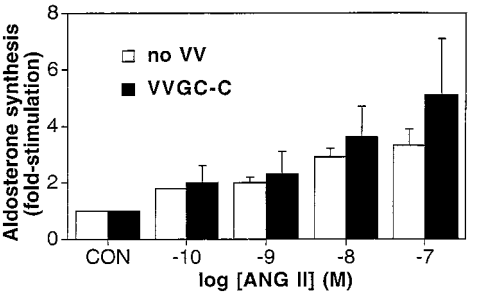

Fig. 5. Ang II-induced aldosterone production in uninfected or virus-infected H295R cells. Ang II-stimulated aldosterone production in uninfected (□) and VVGC-C-infected (■) H295R cells. Columns represent mean ± standard error for four experiments. Base-line levels of aldosterone (ng/10^6 cells) are presented in Fig. 3. Ang II induced a concentration-dependent stimulation of aldosterone synthesis (ANOVA, $p < 0.01$). *CON*, control.

nogenesis (Matsuoka *et al*., 1987; Okamoto, 1988). The latter point is of key importance to the current study because like ANP, sodium nitroprusside-mediated increases in cGMP activate the type II phosphodiesterase in a similar manner (Whalin *et al*., 1991) but fail to alter steroidogenesis (Matsuoka *et al*., 1987; Okamoto, 1988). Both points taken together suggest that ANP may act by a mechanism independent of increases in cGMP levels and/or concomitant stimulation of the type II phosphodiesterase. This possibility is highly relevant in that MacFarland *et al*. (1991) were unable to dissociate the inhibitory effect of ANP on steroidogenesis from its binding to GC-A and associated enzyme activation.

Two subsequent studies used natriuretic peptide receptor-selective reagents in an attempt to better define the involvement of GC-A. The first reported that a GC-linked natriuretic peptide receptor antagonist, HS-142–1, blocked the ability of ANP to reduce aldosterone synthesis (Oda *et al*., 1992), and the second reported that a GC-A-selective agonist (selective ANP or sANP) inhibited both forskolin- and Ang II-evoked human aldosterone synthesis in H295R cells (Olson *et al*., 1996). Based on the selective pharmacology, these studies concluded that ANP attenuated steroid synthesis through binding to a GC-linked receptor, likely GC-A. Even though the natriuretic peptide receptor-selective reagents indicated that GC-A mediated the ANP effect on steroidogenesis, they contributed limited information toward identifying the importance of catalytic activity (i.e., cGMP synthesis) to relaying the cellular signal. Ideally, a membrane-permeable enzymatic inhibitor of GC-A would be required to more specifically study this phenomenon. Although a recent report suggests that a selective inhibitor is available for nitric oxide-sensitive or cytosolic GC (Garthwaite *et al*., 1995), none is known for GC-A or other membrane-associated members of this enzyme family.

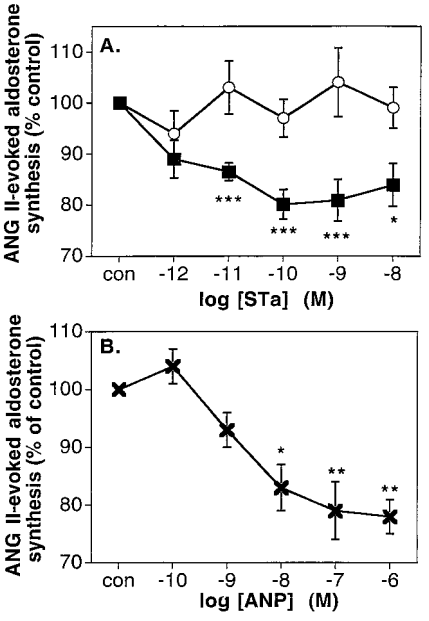

Fig. 6. Effects of STa or ANP on Ang II-induced aldosterone synthesis. A, Effects of increasing concentrations of STa on Ang II (5 nM)-stimulated aldosterone production were measured in H295R cells infected with VVGC-C (■, 10 experiments) or VVGC-C D853A (○, eight experiments). B, The effect of ANP on Ang II-stimulated (5 nM) aldosterone synthesis was measured in uninfected H295R cells (×, eight experiments) as a comparison. All values are mean ± standard error. Base-line and Ang II-stimulated levels of aldosterone (ng/10^6 cells) are presented in Figs. 3 and 5, respectively. Both STa and ANP exhibited concentration-dependent effects (ANOVA, $p < 0.001$). ***, $p < 0.001$; **, $p < 0.01$; and *, $p < 0.05$.

Based on the precedence of Thompson and Garbers (1995) in creating the catalytically inactive GC-A D893A, we constructed an inactive cyc$^-$ analog called GC-C D853A. Assessment of Mn^{2+}/Triton X-100 stimulated GC activity in membranes from transiently transfected COS7 cells revealed that GC-C was active, whereas GC-C D853A was not. Moreover, STa (10^{-7} M) increased cGMP production to the same level in transiently transfected COS7 cells and virus-infected H295R cells expressing GC-C but not in those mock-transfected (pCDNA3)/infected (VVWT) or those expressing the mutant. This result demonstrates that the toxin-responsive enzyme is functionally active to a similar extent in these cells independent of the expression method used. GC-C and GC-C D853A also were expressed to nearly equal levels independent of the expression method used or cell type chosen based on similar B_{max} values for radiolabeled STa (Table 1). Binding studies with [^{125}I]STa also revealed similarities between K_D values for both GC-C and GC-C D853A in transiently transfected and virus-infected cells expressing either form. The latter result confirms that the intracellular point mutation does not reduce the affinity of the receptor for ligand. Gradient PAGE followed by immunoblot of COS7 and H295R cell membrane protein samples yielded similar migration patterns and molecular masses for GC-C and GC-C D853A. This similarity indicates that the mutation in the catalytic domain does not prevent the receptor from self-associating to form higher order oligomers. This observation is in agreement with a report that a truncated form of GC-C composed of only the ligand-binding and transmembrane domains formed dimers (Hiryama *et al*., 1993). Because our nonreducing gels were run in the absence of STa, GC-C and GC-C D853A oligomerize before ligand binding akin to GC-A (Chinkers and Wilson, 1992; Lowe, 1992). The results of the current study also support those of Vaandrager *et al*. (1994), who reported the existence of higher order oligomers of GC-C in HEK 293 cells overexpressing a stable transfectant of the protein.

Expression of GC-C resulted in a slight but significant increase in cGMP production that was accompanied by a decrease in basal aldosterone synthesis of similar magnitude in H295R cells. These effects were not due to a nonspecific effect of the viral vector used because basal aldosterone or cGMP levels were unchanged in either VVGC-C D853A-infected or VVWT-infected cells relative to uninfected cells. Furthermore, these results were not the consequence of virus-mediated cell death because there was no effect of VVWT, VVGC-C, or VVGC-C D853A infection on cell viability compared with uninfected cells. The effect of GC-C on basal aldosterone and cGMP production were completely prevented by the single point mutation rendering the catalytic domain inactive, demonstrating that GCs attenuate steroidogenesis. The increase in cGMP levels observed in these cells under basal conditions (i.e., in the absence of STa treatment) may be explained by the high levels of expression obtained using vaccinia virus. Alternatively, H295R cells may produce low levels of a natural ligand for GC-C, perhaps of the guanylin family (Currie *et al*., 1992). The latter possibility will be the focus of future studies.

Ang II was found to stimulate aldosterone synthesis in a similar concentration-dependent manner in uninfected cells and virus-infected cells. The maximal effect of Ang II was attained at 10^{-7} M, which represented ~4-fold stimulation above base-line, with an EC_{60} of ~5 nM. These results are nearly identical to those reported previously (Olson *et al.*, 1996). Therefore, 5 nM Ang II was used to examine the effect of STa on evoked aldosterone synthesis in studies comparing paired groups of cells infected with either VVGC-C or VVGC-C D853A. Those heterologously expressing GC-C demonstrated STa-mediated, concentration-dependent stimulation of cGMP production and inhibition of Ang II-evoked human aldosterone synthesis. The maximal inhibition of steroidogenesis (nearly 25%) was similar to that previously reported by this laboratory (Olson *et al.*, 1996) using ANP and sANP in H295R cells. The maximal effect of ANP on Ang II-induced human aldosterone production in the current study is similar to the 30% suppression for the same response originally reported by Goodfriend *et al.* (1984) in bovine zona glomerulosa cells. Furthermore, the potency of ANP in the H295R cells (IC_{50} ~ 1 nM) resembled nearly identically that in zona glomerulosa cells for 1 and 100 nM Ang II-stimulated aldosteronogenesis (IC_{50} ~ 3.5 nM; Goodfriend *et al.*, 1984). Relative to the effect of ANP on agonist-evoked aldosterone synthesis, STa was more potent, which was not surprising given the effect of GC-C expression on both base-line cGMP and aldosterone levels in the absence of toxin. In paired H295R cells infected with VVWT or VVGC-C D853A, there was no effect on base-line cGMP or aldosterone levels, and STa failed to increase cGMP synthesis or decrease aldosteronogenesis relative to base-line. STa treatment did not decrease cell viability in virus-infected or uninfected cells. Therefore, the STa-mediated elevation of cGMP levels and concomitant diminution of steroid synthesis in H295R cells were directly associated with the presence of an active catalytic domain in the receptor and were not the result of a nonspecific effect associated with virus infection.

The observation that heterologous expression of GC-C leads to an STa-mediated inhibition of aldosterone synthesis is consistent with previous pharmacological studies indicating that ANP, a known activator of GC-A, is capable of modulating the same cellular response (Goodfriend *et al.*, 1984; MacFarland *et al.*, 1991; Oda *et al.*, 1992; Bodart *et al.*, 1996; Olson *et al.*, 1996). In the current study, STa binds only to GC-C in VVGC-C-infected H295R cells. In paired experiments, the catalytically inactive GC-C D853A retains the same higher order structure and STa-binding characteristics in comparison to the wild-type receptor. However, GC-C D853A is unable to synthesize cGMP or mediate any effect of STa on steroidogenesis. These results demonstrate the necessity of a functionally active catalytic domain (i.e., cGMP synthesis) for GC-linked receptors to mediate an inhibition of aldosterone production.

Because the endogenous expression of GC-C is localized almost exclusively to the gastrointestinal tract in several species (Schulz *et al.*, 1990; Currie *et al.*, 1992; Drewett and Garbers, 1994), heterologous expression of the receptor/enzyme complex in various cell types can be effectively used to assess the importance of membrane-associated cGMP production to mediating cellular responses. This approach allows native cGMP to be synthesized by STa treatment in a receptor-mediated, concentration-dependent manner and is preferable to that using membrane-permeable cGMP analogs. The latter approach is limited by the fact that high concentrations of the analog are used in an attempt to reach adequate intracellular levels (Drewett and Garbers, 1994). Moreover, in studies using heterologous expression of GC-C, the specific involvement of a functional catalytic region in generating cGMP can be examined by comparison to paired cells expressing the inactive point mutant GC-C D853A.

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