Molecular and Cellular Physiology of the Dissociation of Atrial Natriuretic Peptide from Guanylyl Cyclase A Receptors*

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Guanylyl cyclase subtype A (GCA) is the main receptor that mediates the effects of atrial natriuretic peptide (ANP) in the regulation of plasma volume and blood pressure. The dynamics of the dissociation of ANP from GCA were investigated in cultured Chinese hamster ovary (CHO) cells stably transfected with wild-type (WT) or mutant GCA receptors. The rate of dissociation of specifically bound ANP from intact CHOGCAWT cells at 37 °C was extremely rapid ($K_{off} = 0.49 \pm 0.02 \text{ min}^{-1}$), whereas in isolated membranes prepared from these cells, the dissociation at 37 °C was 10-fold slower ($K_{off} = 0.035 \pm 0.006 \text{ min}^{-1}$). The dissociation of ANP from CHOGCAWT cells showed remarkable temperature dependence. Between 22 and 37 °C, $K_{off}$ increased 8 times, whereas between 4 and 22 °C, it increased only 1.5 times. Total deletion of the cytoplasmic domain or of the catalytic guanylyl cyclase sequence within this domain abolished ANP-induced increases in cGMP, dramatically slowed receptor-ligand dissociation by at least 10-fold, and abolished the temperature dependence of the dissociation of ANP. Deletion of the kinase-like domain led to maximal constitutive activation of guanylyl cyclase, markedly decreased $K_{off}$ to $0.064 \pm 0.006 \text{ min}^{-1}$, and also abolished the temperature dependence of dissociation. Substitution of Ser506 by Ala and particularly the double substitution of Gly505 and Ser506 by Ala within the kinase-like domain markedly reduced ANP-induced increases in cGMP, whereas $K_{off}$ decreased modestly (albeit significantly) to $0.36 \pm 0.03$ and $0.24 \pm 0.02 \text{ min}^{-1}$, respectively. As a whole, the results demonstrate for the first time that temperature perception by ATP alone cannot account for rapid GCA receptor-ligand dissociation under physiological conditions and suggest that ligand dissociation is modulated in part by the interaction of still unidentified cytosolic factors with the cytoplasmic domain of GCA.

Atrial natriuretic peptide (ANP), 1 a member of the natriuretic peptide family that includes brain natriuretic peptide and C-type natriuretic peptide, plays a fundamental role in the regulation of blood pressure, plasma volume, and renal function (1, 2). Two distinct classes of ANP receptors, named clearance and guanylyl cyclase (GC) receptors, have been biochemically and functionally well characterized (1, 3).

Clearance receptors of ANP, the most abundant class of the natriuretic peptide receptors, have a single transmembrane domain, a short cytoplasmic tail of 37 amino acids, and an extracellular binding domain that has a significant homology to the extracellular domain of GC receptors (1, 4–6). An extensive series of physiological, pharmacological, cellular, and genetic studies have shown that clearance receptors are importantly involved in the systemic and local clearance of ANP (7–11). This clearance function is accomplished by an efficient mechanism of receptor-mediated endocytosis. Endocytosed ANP is delivered to lysosomes, where it is hydrolyzed to its constituent amino acids, and the internalized receptors are recycled to the cell membrane (10, 12). The efficiency of this receptor-mediated endocytic mechanism is enhanced by a relatively low rate of dissociation of ANP from cell-surface receptors (12).

Guanylyl cyclase subtype A (GCA) receptors mediate all of the known cardiovascular and renal effects of ANP (2, 13). GCA receptors have a single transmembrane domain, an extracellular ligand-binding domain, and a cytoplasmic domain constituted by a catalytic GC sequence and a tyrosine kinase-like (TK) sequence interposed between the transmembrane and the catalytic domains (14). Between the TK and GC sequences there is an amphiphilic α-helical region that is involved in higher order oligomerization of GCA receptors (15, 16). Under basal conditions, the TK domain has an inhibitory effect on GC activity. It has been postulated that upon ANP (or brain natriuretic peptide) binding to the extracellular domain, ATP binds to the TK domain and allosterically activates the catalytic GC domain (14, 17).

Previous studies in our laboratory have demonstrated that the native GCA in cultured glomerular mesangial and renomedullary interstitial cells is a constitutive membrane resident protein that does not undergo endocytosis and does not mediate lysosomal hydrolysis of ligand (18). Moreover, the dissociation of ANP from native GCA is very slow at subphysiological temperatures and increases exponentially at near physiological temperatures (18). We postulated that the rapid dissociation of ANP from surface GCA receptors at physiological temperatures (18) is mediated by intracellular factors that are not present in cell-free systems. This work was supported in part by National Institutes of Health Grant R01 DK-53526. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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1 The abbreviations used are: ANP, atrial natriuretic peptide; GC, guanylyl cyclase; GCA, guanylyl cyclase subtype A; TK, tyrosine kinase-like; CHO, Chinese hamster ovary; GCACYT, GCA receptor with the cytoplasmic domain deleted; GCAWT, GCA receptor with the TK domain deleted; GCAGCT, GCA receptor with the GCA domain deleted; CHOGCAWT, CHO cells transfected with GCAWT; CHOCCGACYT, CHO cells transfected with GCAWT; CHOCCGACTK, CHO cells transfected with GCAWT; BSA, bovine serum albumin; ATP5′, adenosine 5′-O-(3-thiotriphosphate).
cal temperatures was due to an interaction of a cytoplasmic domain of GCA receptor. This mechanism would allow for rapid onset of ANP effects upon increasing plasma levels of the hormone and a rapid termination of effects when plasma levels of ANP fall (2, 18).

In this study, we examined the dynamics and some of the molecular mechanisms of dissociation of ANP from transfected wild-type and mutant GCA receptors stably transfected into Chinese hamster ovary (CHO) cells. It will be shown that there is a remarkable temperature dependence of receptor-ligand dissociation that is observed only in intact cells and not in isolated membrane preparations. The very fast dissociation of ANP depends on cell integrity, the intactness of the cytoplasmic domain of GCA, and near physiological temperatures.

**EXPERIMENTAL PROCEDURES**

**Materials—CHO-K1 cells were obtained from American Type Culture Collection (Manassas, VA). 125I-ANP-(1–28) (rat) and the cGMP assay system were purchased from Amersham Pharmacia Biotech. Cell culture media and all supplements were from Life Technologies, Inc. Blood of Dulbecco’s modified Eagle’s medium (Gibco-BRL, Grand Island, NY) was obtained from HyClone Laboratories (Salt Lake City, UT). The Mutagene M13 mutagenesis kit was purchased from Bio-Rad. Oligonucleotides were from Genosys Biotechnologies, Inc. (The Woodlands, TX). The MC1061 bacterial strain and Sequenase DNA sequencing kit were from U. S. Biochemical Corp. The Wizard DNA purification kits, JM109 bacterial strain, and competent cells were from Promega (Madison, WI). M13 bacteriophage vectors, DNA restriction enzymes, and other enzymes were from New England Biolabs Inc. (Beverly, MA). The protein assay reagent kit was from Pierce. The pAXNEO mammalian expression vector and full-length clones of the human kidney GCA receptor (GCAWT) and the cytoplasmic domain-deleted GCA receptor (GCAYCT) were a kind gift from Dr. John Lewicki (Scios Inc., Mountain View, CA). Rat ANP-(1–28) was from Peninsula Laboratories, Inc. (Belmont, CA), and GFC filter membranes were from Whatman (Kent, United Kingdom). Trypsin, leupeptin, aprotinin, BSA, 3-isobutyl-1-methylxanthine, and all other chemicals were from Sigma.

**Site-directed and Deletion Mutagenesis—**For mutagenesis constructs, full-length transfected GCAWT (19) was first cloned into SalI-XbaI cloning sites of the M13 bacteriophage vector. The mutagenesis procedures were performed following the method of Kunkel et al. (20) using the Mutagen-M13 M13 in vitro mutagenesis kit. Point mutations within the TK domain of GCA were introduced in Human CK1α, which is well conserved in the GCA receptor (16, 17, 21). GCAA505 was constructed by substituting the GGC codon (codon 1653 in full-length GCAWT), coding for Gly505, for GCC, coding for alanine. GCAA506 was obtained by substituting the serine codon TCC (codon 1656 in full-length GCAWT) for GCC, coding for alanine. The double mutant GCAA506/505 was obtained by the single-mutation in the same codon using GCAA505 as a template. Deletion of the GC domain was accomplished by introducing the stop codon TGA in place of TCC (codon 1656 in full-length GCAWT) to Thr768 (codon 2442). All mutations were confirmed by sequencing. GCAWT and mutant GCA receptors were subcloned into the SalI-XbaI cloning sites of the mammalian expression vector pAXNEO using standard techniques (22).

**Culture Medium and Binding Solution—**The culture medium consisted of Dulbecco’s modified Eagle’s medium/nutrient mixture F-12 supplemented with 2 g/liter NaHCO3, 10% bovine calf serum, 100 units/ml penicillin, 0.1 mg/ml streptomycin, and 0.25 μg/ml amphotericin B (pH 7.15). The binding solution consisted of Dulbecco’s modified Eagle’s medium/nutrient mixture F-12 supplemented with 3.6 g/liter HEPES, 3.7 g/liter NaCl, and 2 mg/ml BSA (pH 7). The washing solution was the same as the binding solution, except that it did not contain BSA.

**Dissociation of ANP from GCA Receptors**—Wild-type and transfected CHO-K1 cells were propagated and maintained in the culture medium described above and placed at 37 °C in a humidified atmosphere of 95% O2 and 5% CO2. Stable transfections of GCAYCT and mutant GCA receptors in CHO cells were accomplished by calcium phosphate precipitation method, followed by selection with Geneticin according to standard techniques (22). 10–20 clones were harvested by cloning cylinders and grown in 75-cm2 flasks until confluence.

**Competition Binding Experiments in Cell Monolayers—**Equilibrium competition binding experiments were performed in intact cells at 4 °C to determine the apparent dissociation constant (Kd) and apparent equilibrium dissociation constant (K) of ANP-specific binding sites as previously described (10, 12). Cells plated in 24-well plates to near confluence were incubated for 3–4 h at 4 °C with binding solution containing trace amounts of 125I-ANP-(1–28) in the absence or presence of 0.01 nM to 0.5 μM unlabeled ANP-(1–28). At the end of the incubation period, cells were washed twice with ice-cold washing solution, and membrane-bound 125I-ANP-(1–28) was removed by incubation with a hypertonic acid solution (0.2 M acetic acid and 0.5 M NaCl) for 20 min at room temperature. The radioactivity released into the acid solution was counted in a γ-counter. Specific binding was determined by the difference between total binding and binding of 125I-ANP-(1–28) in the presence of excess ANP-(1–28) (0.5 μM). Two additional wells in each 24-well plate were reserved for control experiments performed as a Coulter cell counter or manually using a hemocytometer. At 4 °C, the apparent dissociation constants (K) of ANP in transfected cells were below 1 nM, except in CHOOGCAA506 and CHOOGCAA505/AS06, in which the values for the K were 3 and 9 nM, respectively. The Bmax values were used to estimate the density of surface membrane receptors and are reported in the legends of Figs. 4 and 5.

**Membrane Preparation—**CHO-GCAYCT cells were grown to confluence in 850-cm2 roller bottles. Cell monolayers were washed twice with ice-cold Hanks’ balanced salt solution containing 5 mM HEPES and 3.7 g/liter NaHCO3 (pH 7.4) and scraped with a rubber policeman into 40 ml of buffer containing 50 mM Tris-HCl (pH 7.5), 1 mM EDTA, 1 mM dithiothreitol, 250 mM sucrose, 10 μg/ml leupeptin, 10 μg/ml aprotinin, and 1 μg/ml pepstatin A. The cells were pelleted at 250 < g for 5 min, resuspended in buffer, and sonicated with a Polytron homogenizer. After centrifugation at 250 × g for 3 min to remove unbroken cells and nuclei, the supernatant was recentrifuged at 100,000 × g for 1 h. The resulting supernatant was discarded, and the final membrane pellet was resuspended in 2 ml of buffer and briefly homogenized with a Dounce homogenizer. The aliquots were frozen in liquid nitrogen and stored at −80 °C until used.

**Fate and Dissociation of Specifically Bound 125I-ANP-(1–28) and Determination of Dissociation Constants in Intact Cells and Isolated Membranes—**The fate of specifically bound 125I-ANP-(1–28) in intact cells was determined by chase experiments as previously described (10, 12). Briefly, CHO cells stably transfected with GCAYCT or mutant GCA receptors were grown to near confluence in six-well plates. Before the plates were washed with ice-cold washing solution and incubated for 2 h at 4 °C with binding solution containing 0.1–0.5 μCi/ml 125I-ANP-(1–28). The wells were washed three times with ice-cold washing solution, and the cells of four wells were incubated with ice-cold binding solution containing 0.1 μM unlabeled ANP-(1–28). The cells in the remaining two wells were incubated with 0.5 μM ANP-(1–28) for non-specific binding determination. The plates were then immediately transferred to a shaking water bath kept at 4, 22, or 37 °C. Samples of supernatant were collected at several time intervals from 0.5 to 30 min. At the end of this period, cells were rapidly washed twice with ice-cold washing solution, and the radioactivity remaining bound to the surface membrane was determined by further incubation with the hypertonic acid solution as described above. Recovery experiments showed that, in all instances, the sum of 125I-ANP-(1–28) radioactivity released to the medium and that remaining at the cell surface by the end of the experiment was >95% of the total radioactivity. More than 95% of the radioactivity released to the medium was precipitated by 10% trichloroacetic acid, and high performance liquid chromatography revealed that this radioactivity comigrated with 125I-ANP-(1–28).

The fate of specifically bound 125I-ANP-(1–28) was also evaluated in membranes obtained from transfected CHO-GCAYCT and CHOGCAYCT cells. For this purpose, membranes were first diluted with 10 mM Tris (pH 7.4) to give a final protein concentration of 100 μg/ml. 125I-ANP-(1–28) (0.5–1 μCi/ml) was added to the incubation reaction, and equilibrium binding was attained after incubation at room temperature for 90 min. ANP-(1–28) (1 μM) was then added to the incubation reaction, and the tubes were immediately transferred to a shaking water
bath at 37 °C. Aliquots (125 μl) were taken at several time intervals from 0.5 to 30 min and immediately filtered through a Whatman GF/C filter precoated with 1% polyethyleneimine using a vacuum manifold. The filters were then washed three times with 10 ml Tris (pH 7.4). The radioactivity remaining in the filters was counted using a γ-counter.

**Cell Membranes**—Transfected CHO cells were plated in six-well plates and grown to near confluence. Washing and incubation solutions were the same as the binding solution described above without BSA. Cell monolayers were washed twice and preincubated for 15 min at 37 °C with 1 ml of incubation solution to which 3-isobutyl-1-methylxanthine was added to a final concentration of 0.25 mM. Cell monolayers were washed again, and incubation was initiated by adding 1 ml of incubation containing 0.25 mM 3-isobutyl-1-methylxanthine with or without 0.1 μM ANP-1–28. Incubation was carried out for 5 min at 37 °C. At the end of this period, cGMP was extracted by adding 5% trichloroacetic acid to the incubation mixture. Water-saturated diethyl ether was used to remove trichloroacetic acid from the supernatant, and cGMP was determined by the [3H]cGMP radioimmunoassay kit from Amersham Pharmacia Biotech using the procedure recommended by the vendor. Cells were lysed in 0.2% SDS, and the amount of protein was measured by the Bradford procedure (23). Using these assay conditions, >95% of cGMP generated in 5 min of incubation period was present inside the cells.

**Guanylyl Cyclase Assay in Membranes**—GC activity in crude membranes was assayed as described (24). Briefly, the reaction was initiated by addition of 30 μg of membrane protein in a final volume of 0.3 ml of solution containing 50 mM Tris-HCl (pH 7.5), 2 mM 3-isobutyl-1-methylxanthine, 1 mM GTP, 4 mM MgCl₂, 15 units/ml creatine phosphokinase (250 units/mg of protein). After incubation for 3 min at 37 °C, the reaction was terminated by addition of 10 μl of 1.5 M sodium acetate (pH 4.7), followed by boiling for 3 min in a water bath. Finally, the reaction was centrifuged at 12,000 rpm for 3 min, and cGMP generation was quantified by radioimmunoassay. To determine the effect of ANP (1–28) and/or ATP, these substances were added directly to the reaction at concentrations of 0.1 μM and 0.5 mM, respectively. All experiments were done in triplicates and repeated at least twice.

**Data Analysis Statistics**—A nonlinear exponential curve fit was performed to determine the dissociation rate constant (K_d) of ANP from wild-type or mutant GCA receptors. The decay curves of specifically bound [125]I-ANP (1–28) from the cell surface or from isolated membranes fitted a single-phase exponential decay with a high degree of reliability (r² > 0.95). In several of the experiments, the decay curves fitted equally well a two-phase exponential decay. However, in this case, the major component accounted for >80% of the total dissociation. Thus, for simplicity, we chose to calculate an overall rate of dissociation using a single-phase exponential curve fit. Statistics were performed by analysis of variance with the Tukey-Kramer post-test. Differences were considered statistically significant when p < 0.05.

**RESULTS**

Fig. 1A depicts the time course of dissociation of specifically bound [125]I-ANP (1–28) from transfected CHOCAWT, CHOCGACYT−, CHOGCATK−, and CHOGCAGC− cells at 37 °C. Fig. 1B shows the corresponding appearance of intact [125]I-ANP (1–28) in the medium. Practically all specifically bound [125]I-ANP (1–28) that dissociated from surface membrane receptors was released in intact form to the medium, demonstrating that there was minimal, if any, receptor-mediated internalization or hydrolysis of ANP.

The dissociation of [125]I-ANP (1–28) from CHOCAWT cells at physiological temperatures was very fast, with K_d = 0.49 ± 0.02 min⁻¹, a value similar to that found for native GCA receptors in cultured glomerular and remodelling interstitial cells (18). In CHOCGACYT− cells, the rate of dissociation decreased by >20-fold to 0.018 ± 0.001 min⁻¹. Removal of the kinase-like and catalytic guanylyl cyclase domains also decreased receptor-ligand dissociation to the slow rates of 0.064 ± 0.006 and 0.043 ± 0.004 min⁻¹ for CHOGCATK− and CHOGCAGC− cells, respectively. These results show that the cytoplasmic domain of GCA is involved in the mediation of the fast receptor-ligand dissociation at physiological temperatures and that major deletions within the cytoplasmic domain markedly reduce the ability of GCA receptors to physiologically modulate receptor-ligand dissociation.

Fig. 2 shows that in isolated membranes prepared from...
transfected cells, contrary to intact cells, the rate of dissociation of ANP from CHOGCACYT \((K_{off} = 0.038 \pm 0.002 \text{ min}^{-1})\) was not different from that from CHOGCAWT \((K_{off} = 0.035 \pm 0.006 \text{ min}^{-1})\). This is due to the major decrease in the rate of dissociation of ANP from GCAWT in membrane preparations compared with intact cells at 37 °C. In isolated membrane preparations, receptor-ligand dissociation was very slow and was not regulated by the cytoplasmic domain. This result suggests that factor(s) present in the intact cell and absent in the isolated membranes are responsible for interacting with the cytoplasmic domain of GCA to effectuate a fast dissociation rate of surface receptor-ligand complexes at 37 °C.

Fig. 3 summarizes the \(K_{off}\) values of ANP from wild-type and mutant GCA receptors in intact cells at 37 °C. In addition to the major decrease in ANP dissociation when the cytoplasmic domain of GCA is deleted or when the TK and catalytic GC domains are truncated (see Fig. 1), some point mutations in a putative ATP-binding site in Hanks’ subdomain I of the TK domain resulted in significant (albeit relatively small) decreases in the receptor-ligand dissociation rate. Although the \(K_{off}\) of ANP in CHOGCA505 cells (0.50 ± 0.06 min\(^{-1}\)) was practically identical to that in CHOGCAWT cells (0.49 ± 0.02 min\(^{-1}\)), substitution of Ser\(^{505}\) by Ala and particularly the double substitution of Gly\(^{505}\) and Ser\(^{506}\) by Ala resulted in significant decrease in \(K_{off}\) to 0.36 ± 0.03 and 0.24 ± 0.02 min\(^{-1}\), respectively \((p < 0.01 \text{ versus CHOGCAWT})\). In no instance, however, did the \(K_{off}\) value of these point mutants approach the low value observed in the cytoplasmic domain-truncated receptors.

To determine whether changes in receptor-ligand interactions were related to receptor activity, we measured basal and ANP-stimulated guanylyl cyclase activity or cGMP levels in CHO cells stably transfected with wild-type or mutant GCA receptors. Fig. 4A shows basal, ATP-, ANP-, and (ANP + ATP)-stimulated guanylyl cyclase activity in isolated membranes obtained from CHOGCAWT and CHOGCATK cells. Basal guanylyl cyclase in CHOGCAWT cells was very low, and ANP + ATP produced a major (>30-fold) activation of guanylyl cyclase. ATP alone was without effect, and ANP alone had only a modest effect. In membranes obtained from CHOGCATK cells, basal levels of guanylyl cyclase activity were markedly elevated, reaching levels similar to those in isolated membranes from CHOGCAWT cells maximally stimulated with ANP + ATP. In this truncated mutant, ATP slightly but consistently decreased guanylyl cyclase activity \((p < 0.01 \text{ versus CHOGCAWT})\), whereas ANP or ANP + ATP did not further increase guanylyl cyclase activity from its high basal level. As expected, CHOGCACYT and CHOGCAGC cells showed no significant ANP-(1–28)-stimulated guanylyl cyclase activity or increases in cellular cGMP levels (data not shown).

Fig. 4B shows the results of the studies on cGMP levels in intact CHOGCAWT and CHOGCATK cells. Deletion of the kinase-like domain also led to marked increases in basal cGMP levels and unresponsiveness to ANP. However, in intact cells, contrary to the observation in isolated membranes, the basal levels of cGMP in CHOGCATK cells were significantly lower than the maximally ANP-stimulated levels of cGMP in CHOGCAWT cells, even when the results were normalized by the density of surface receptors. We interpret this finding to suggest that under sustained constitutive activation of GCA in intact cells, there are adaptive mechanisms that increase the hydrolysis or extrusion of cGMP from the cells. It is of interest that despite the chronically elevated basal levels of cGMP, we could not detect a change in growth rate of cultured CHOGCATK cells compared with CHOGCAWT cells (data not shown).

Fig. 5 shows the effects of point mutations in Hanks’ subdo-
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Fig. 4. Effect of deletion of the kinase-like domain on guanylyl cyclase activity and cGMP levels. A, basal and ANP-stimulated guanylyl cyclase activities in isolated membranes obtained from transfected CHOGCAWT and CHOGCATK cells. ANP and ATP were added to the medium at concentrations of 0.1 μM and 0.5 mM, respectively. Results are means ± S.E. of four to five wells obtained in two separate experiments. ATP alone did not significantly increase guanylyl cyclase activity; ANP alone had a significant (albeit small) effect; and ANP + ATP dramatically increased guanylyl cyclase activity to >30-fold basal activity (bars labeled B). Deletion of the kinase-like domain led to full constitutive activation of guanylyl cyclase with basal levels similar to those obtained in wild-type receptors exposed to a maximal concentration of ANP. Under these conditions, ATP had a small but consistent inhibitory effect, and the TK domain-deleted receptor became unresponsive to ANP. B, effect of ANP (0.1 μM) on cGMP levels in transfected CHOGCAWT and CHOGCATK cells. Results are means ± S.E. of three experiments in triplicate. Deletion of the kinase-like domain led to constitutive increases in cGMP levels, and the mutant receptors became unresponsive to ANP. The results in A and B were normalized by the density of surface receptors, as determined by the density of surface receptors (see “Experimental Procedures”). The values for the density of surface receptors were as follows: CHOGCAWT, 82,700 ± 5000 and 29,200 ± 3200 receptors/cell for CHOGCAWT and CHOGCATK, respectively.

main I on basal and ANP-stimulated cGMP levels. Basal levels of cGMP were similar in all transfected cells, except in CHOGCA505/A506 cells, in which there was an ~5-fold increase compared with CHOGCAWT cells (p < 0.01). However, this higher basal level of cGMP in CHOGCA505/A506 was still far lower than that observed in CHOGCATK (see also Fig. 4B). Maximal ANP-stimulated cGMP was significantly decreased in CHOGCAWT and, in a more pronounced manner, in the double mutant CHOGCA505/A506.

We also tested receptor-ligand dissociation and receptor activity (cGMP generation) in cells stably transfected with GCA receptors that had point mutations in other conserved Hanks’ subdomains within the TK domain. GCA535, GCA551, and GCA646 are mutants in which Lys535 (subdomain II), Glu551 (subdomain III), and Asp646 (subdomain VII) were mutated to Ala, respectively. In this series, maximal ANP-induced generation of cGMP in CHOGCAWT was 19.8 ± 4.3 pmol/5 min/10⁹ surface GCA receptors. The values for the mutants were as follows: CHOGCA535, 1.8 ± 0.6 pmol/5 min/10⁹ receptors (p < 0.001 versus CHOGCAWT); CHOGCA646, 6.1 ± 0.4 (p < 0.05 versus CHOGCAWT); and CHOGCA551, 10.1 ± 2.0 (not significantly different from CHOGCAWT, p > 0.05). The dissociation of ¹²⁵I-ANP (1–28) from these mutants was determined in intact cells at 37 °C in the same manner as described above (see Fig. 1A). The K_d values of ANP from CHOGCA535 (0.64 ± 0.05 min⁻¹), CHOGCA551 (0.53 ± 0.14 min⁻¹), and CHOGCA646 (0.57 ± 0.05 min⁻¹) were not significantly different (p > 0.05) from the K_d of ANP from CHOGCAWT (0.48 ± 0.04 min⁻¹). Finally, we tested the effects of the dele-
tion of an amino acid sequence between Hanks' subdomains I and II (from Val120 to Lys126). This deletion was completely unable to generate cGMP upon maximal stimulation with ANP, whereas the dissociation of 125I-ANP-(1–28) was decreased by only ~40% to 0.30 ± 0.02 min⁻¹ (p < 0.01 versus CHO-GCAWT).

Fig. 6 shows the temperature dependence of the rate of dissociation of 125I-ANP-(1–28) from wild-type and mutant GCA receptors stably transfected into CHO cells. Between 22 and 37 °C, the dissociation rate increased from 0.063 ± 0.005 to 0.49 ± 0.04 min⁻¹ (Q₁₀ = 5.2), whereas between 4 and 22 °C, the increase was only from 0.04 ± 0.001 to 0.063 ± 0.005 min⁻¹ (Q₁₀ = 1.0). Fig. 6A shows that this remarkable increase in dissociation of ANP from CHO-GCAWT at near physiological temperatures was abolished in CHO-GCACYT⁻, CHO-GCATK⁻, and CHO-GCCACG⁻ cells. Fig. 6B shows the temperature dependence of the dissociation of ANP from transfected CHO cells expressing GCA receptors with point mutations in Hanks' subdomain I. Although the Kₐ values of ANP from CHO-GCAA506 and CHO-GCAA505/506 at 37 °C were significantly lower than those from CHO-GCAWT and CHO-GCAA505, all point mutant receptors were still able to show the disproportionate increase in receptor-ligand dissociation rates at near physiological temperatures.

It is noteworthy that the major differences in the rate of dissociation of ANP from wild-type and cytoplasmic domain-truncated receptors at 37 °C virtually disappeared at 4 °C, a temperature at which dissociation from all receptors was similar and very slow (Fig. 6). Accordingly, the measured apparent equilibrium dissociation constants (Kᵈ) in transfected cells at 4 °C were similarly low in wild-type and cytoplasmic domain-truncated receptors, amounting to 0.54 ± 0.06, 0.26 ± 0.12, 0.69 ± 0.44, and 0.68 ± 0.58 nM for CHO-GCAWT, CHO-GCACYT⁻, CHO-GCATK⁻, and CHO-GCCACG⁻, respectively.

DISCUSSION

The present results demonstrate that GCA receptors stably transfected into CHO cells are not endocytosed at appreciable rates and do not mediate lysosomal hydrolysis of ANP. Extremely rapid receptor-ligand dissociation in intact cells at 37 °C, but not at subphysiological temperatures, terminates the interaction of ANP with GCA. These results are in full agreement with our previous observation with native GCA receptors in primary cultures of glomerular mesangial and renomedullary interstitial cells (18). In contrast, they are at variance with reported studies showing receptor internalization for GCA in Leydig tumor cells and, more recently, for transiently transfected GCA in COS-7 cells, an SV40-transformed cell line (25, 26). Although we do not have a definitive explanation for this apparent discrepancy, it is not surprising that tumor cells or SV40-transformed (COS-7) cells would have enhanced endocytosis, resulting in internalization of constitutive membrane proteins nonspecifically entrapped in coated pits or other endocytic regions of the cell. In this regard, it is noteworthy that GCA lacks key internalization signals in its cytoplasmic tail, a feature consistent with our finding that the receptors do not undergo specific endocytosis (18). Our results cannot be attributed to a putative defect of CHO cells to effectuate receptor endocytosis or to an intrinsic decrease in receptor affinity in these cells. Indeed, we have previously shown that clearance receptors of natriuretic peptides stably transfected into CHO cells undergo robust endocytosis and have very low receptor-ligand dissociation at 37 °C (12). Thus, the lack of appreciable endocytosis and the rapid receptor-ligand dissociation of native GCA in primary culture cells or of GCA stably transfected into CHO cells are likely to reflect the dynamics of this receptor under physiological conditions.

The dissociation of ANP from wild-type GCA receptors at 37 °C is markedly slower in isolated membranes than in intact cells. On one hand, this novel finding demonstrates that temperature per se cannot account for the dramatic increase in ANP dissociation from GCA receptors in intact cells at near
membranes at 37 °C in the presence of ATP, the dissociation rate of ANP in isolated membranes preparations or in intact cells at subphysiological temperatures, the extracellular domain of GCA loses its conformational control by the cytoplasmic domain, resulting in exceedingly slow rates of ligand dissociation.

As previously shown by several investigators (14, 27–33) and confirmed in this study (Fig. 4), signaling of GCA is dependent on ATP. Studies by several laboratories, including our own, indicated that ATP is also involved in the modulation of receptor-ligand dissociation and in receptor affinity (18, 27, 28, 34–36). In isolated membranes from bovine zona glomerulosa cells, addition of ATP or ATP-SiS significantly increases the half-time of a fast component of the dissociation of ANP from GCA, whereas amiloride competitively counteracts this effect (35, 36). On the basis of these results, it was postulated that ATP interacts with the cytoplasmic domain of GCA and by an allosteric effect switches these receptors from a high affinity to a low affinity state. In our previous study (18), we also found that amiloride markedly decreased the dissociation of ANP from GCA in intact glomerular mesangial and renomedullary interstitial cells, a finding consistent with the above interpretation.

As a whole, the studies referred to above strongly indicate that ATP participates in the modulation of receptor-ligand dissociation. However, this study shows for the first time that ATP alone cannot fully account for rapid GCA receptor-ligand dissociation. The dissociation of ANP from wild-type GCA receptors in intact cells at 37 °C was ~10-fold faster than in isolated membranes at 37 °C. Addition of 1 mM ATP to the isolated membranes approximately doubled the overall dissociation rate (data not shown) to values similar to those reported for the effect of ATP on the fast component of ANP dissociation reported in the earlier work by Larose et al. (35). Thus, even in the presence of ATP, the dissociation rate of ANP in isolated membranes at 37 °C is 4–5 times slower than in intact cells at physiological temperatures. Moreover, it is unlikely that differences in cellular concentration of ATP between 22 and 37 °C are of such magnitude as to explain the major increase in receptor-ligand dissociation between these temperatures (Fig. 6). The nature of cytoplasmic factors other than ATP that contribute to the rapid receptor-ligand dissociation remains to be elucidated. It is possible, even likely, that temperature-dependent interactions between GCA and cytosolic components such as the cytoskeleton and chaperone proteins contribute to the physiological regulation of receptor-ligand dissociation. In this regard, the recent findings demonstrating that hsp90 interacts with the cytoplasmic domain of GCA and that geldanamycin, an inhibitor of hsp90, significantly decreases ANP-induced activation of GCA (37) suggest intriguing new possibilities to explain not only the modulation of GCA activity, but also the unique properties of GCA receptor-ligand dissociation described in the present study. Further studies are needed to test this hypothesis.

Deletion of the kinase-like domain leads to a major constitutive activation of GCA, which then becomes insensitive to further stimulation with ANP (14, 17). We confirmed and extended this observation by showing that in the kinase-truncated receptor, when the results were normalized by the number of surface receptors, guanylyl cyclase activity was enhanced to levels similar to those obtained with a maximal stimulation of wild-type GCA receptors with ANP (Fig. 4). Deletion of the kinase-like like domain also markedly slowed receptor-ligand dissociation in intact cells at 37 °C to the same level as observed in cells at 4 °C or in isolated membranes at 37 °C and abolished the temperature dependence of this process. Surprisingly, deletion of the guanylyl cyclase sequence decreased receptor-ligand dissociation to the same extent as deletion of the kinase-like domain. None of the point mutations performed in this study had such a dramatic effect on receptor-ligand dissociation as the deletions of the kinase-like domain and the catalytic guanylyl cyclase sequence (see below). This suggests that structural integrity and/or receptor oligomerization rather than specific sites within the intracellular domain of GCA determines the interaction of cytosolic factors that modulate receptor-ligand dissociation. It is also possible that the common feature of the two major truncations in the cytoplasmic domain of GCA is the disruption of the hinge region between the kinase-like sequence and the guanylyl cyclase domain, a region that has been shown to participate in GCA oligomerization (15). In performing the deletion of the guanylyl cyclase domain, we also deleted the major portion of the hinge region. In the deletion of the kinase-like domain, this region was preserved, but we cannot rule out that the mutation led to a conformational change that impeded a putative participation of the hinge region in the modulation of receptor-ligand dissociation. Whatever the case, loss of rapid receptor-ligand dissociation is not directly related to the state of activation of the receptor since deletion of the kinase-like domain leads to full constitutive activation, whereas deletion of the guanylyl cyclase domain precludes activation of GCA.

In an attempt to further test the relationship between receptor activation and receptor-ligand dissociation, we also performed discrete mutations within the kinase-like domain that were previously reported to markedly reduce ANP-induced generation of cGMP (30, 38–40). We mutated Gly505 and Ser506, which belong to a putative ATP-binding site in Hanks’ subdomain I of the kinase-like domain, the so-called ATP-regulated module (28). Ser506 is a constitutively phosphorylated residue that, once dephosphorylated, is involved in the desensitization of GCA receptors (39, 40). Substitution of Ser506 and particularly the double substitution of Gly505 and Ser506 by Ala markedly decreased ANP-induced cGMP generation (Fig. 5), whereas the effect of these mutations on receptor-ligand dissociation was relatively modest, albeit significant (Fig. 3). Moreover, the remarkable temperature dependence of receptor-ligand dissociation was retained in point mutant GCA receptors (Fig. 6). The deletion of an eight-amino acid sequence between Hanks’ subdomains I and II had previously been shown to be a splice variant of GCA in Anguilla japonica and led to complete unresponsiveness to ANP (41). This study confirms this finding and shows for the first time that receptor-ligand dissociation rate is significantly decreased in this mutant. However, in this mutant, the off-rate of ANP from GCA is not nearly as slow as in the mutant with deletion of the entire kinase-like domain. Several point mutations within the kinase-like domain also inhibited ANP-induced increases in cGMP, but were without effect on receptor-ligand dissociation (see “Results”). As a whole, these results further indicate that receptor activation and receptor-ligand dissociation are at least to some extent independently modulated.

These results point out the importance of using near physiological conditions to study the dynamics of interaction of ANP with GCA receptors. Cell integrity, physiological temperatures, and integrity of the cytoplasmic domain of GCA receptors are all essential for an appropriate stimulus-response homeostasis of atrial natriuretic peptides in the regulation of cardiovascular and renal functions. The results support the notion that GCA receptors function in a “staccato” mode (2). The rapid dissociation of ANP from GCA receptors, combined with the removal
of dissociated ligand by the abundant clearance receptors, assures the availability of unoccupied receptors for prompt responses as plasma levels of the hormone rise and rapid termination of responses as plasma levels of ANP fall and impedes sustained desensitization of GCA receptors under physiological conditions.

Acknowledgments—We thank Dr. John Lewicki for the generous gift of cloned guanylyl cyclase receptors and Dr. Xin-Yun Huang for critically reading the manuscript.

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J. Biol. Chem. 2001, 276:36438-36445.
doi: 10.1074/jbc.M102208200 originally published online July 30, 2001

Access the most updated version of this article at doi: 10.1074/jbc.M102208200

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