Dynamics of Atrial Natriuretic Factor-Guanylate Cyclase Receptors and Receptor-Ligand Complexes in Cultured Glomerular Mesangial and Renomedullary Interstitial Cells*  

(Received for publication, December 13, 1991)  

Gou Y. Koh†, Daniel R. Nussenzveig†, Juraj Okolicany‡, Deborah A. Price‡, and Thomas Maack§$§  
From the †Department of Physiology and Biophysics and the §Department of Medicine, Cardiovascular Center, Cornell University Medical College, New York, New York 10021  

The dynamics of the guanylate cyclase receptor of atrial natriuretic factor (GC_A-ANF receptor) were investigated in cultured glomerular mesangial and renomedullary interstitial cells from the rat. In these cells, the GC_A-ANF receptor did not mediate internalization and lysosomal hydrolysis of 125I-ANF_1-28 and did not undergo ligand-induced endocytosis. Glomerular mesangial cells were able, however, to mediate internalization and lysosomal hydrolysis of 125I-ANF_1-28 via clearance ANF (C-ANF) receptors and to promote rapid receptor-mediated internalization and lysosomal hydrolysis of 125I-ANF (Ser^I) angiotensin II. Radioligand specifically bound to surface GC_A-ANF receptors was rapidly dissociated at 37°C (k_{off} > 0.8 min^{-1}), with a Q_{10}(30-37°C) > 6. The dissociation was markedly slower at subphysiological temperatures (Q_{10}(4-30°C), 2-3) or in the presence of 0.5 mm amiloride. The results demonstrate that the GC_A-ANF receptor, contrary to C-ANF receptors and most other polypeptide hormone receptors, is a membrane resident protein that does not mediate internalization and lysosomal hydrolysis of ligand. The termination of the interaction of ANF with GC_A-ANF receptors results from a physiological process that leads to rapid dissociation of receptor-ligand complexes. The unique dynamics of GC_A-ANF receptor-ligand complexes are likely to contribute importantly to stimulus-response homeostasis of ANF.  

There are two biochemically and functionally distinct classes of atrial natriuretic factor (ANF) receptors. The biological receptor proper (originally named type B, or type I (R1), and more recently, guanylate cyclase (GC) receptor) is a 120-130-kDa protein that contains in its cytoplasmic domain guanylate cyclase- and tyrosine kinase-like sequences (1). Two subtypes of GC receptors, named GC_A and GC_B, have been identified (2, 3). GC_A receptors are unlikely to be physiological receptors of ANF because they display a very low affinity for the native hormone but have a relatively high affinity for CNP, another member of the natriuretic peptide family (4). GC_A-ANF receptors mediate most if not all functional effects of ANF and have as their main known second messenger cGMP (5-8). The other major class of ANF receptors is named clearance (C) or type II (R2) receptor. The C-ANF receptor is a homodimer of 120-130 kDa (60 kDa under reducing conditions) which has a very short cytoplasmic domain, a property that is shared by other clearance or transport receptors (8-11). C-ANF receptors do not mediate any of the known end-organ effects of the hormone in renal and vascular tissues (e.g. natriuresis, vasorelaxation) but have an important role in the removal of ANF from the circulation (12-16). C-ANF receptors are by far the most abundant class of ANF receptors, comprising more than 95% of the total ANF receptor population in kidney cortex and vascular tissues (14, 15, 17).  

Receptor-mediated endocytosis of ligand is a common property of polypeptide hormone receptors which contributes to ligand metabolism, termination of receptor-ligand interactions, and, in many instances, to the regulation of receptor cell surface density (18, 19). We have shown previously that C-ANF receptors exert their clearance function by receptor-ligand internalization, lysosomal hydrolysis of endocytosed ANF, and recycling of internalized C-ANF receptors to the cell surface (15). Very little is known, however, regarding the dynamics of membrane GC-ANF receptors and of GC-ANF receptor-ligand complexes. In the present study we determined these processes in cultured glomerular mesangial and renomedullary interstitial cells from the rat. The results will show that the GC_A-ANF receptor is a membrane resident protein that, contrary to C-ANF receptors, and most other polypeptide hormone receptors, undergoes minimal, if any, endocytosis. Consequently, GC_A-ANF receptors do not mediate internalization and lysosomal hydrolysis of ANF. The data will further show that the interaction of ANF with GC_A-ANF receptors is rapidly terminated by a process that leads to a marked increase in the dissociation of receptor-ligand complexes at physiological temperatures.  

EXPERIMENTAL PROCEDURES  

Materials—Rat ANF_1-28 (ANF_{1-28}) and des[Gln^6, Ser^16, Gly^32, Leu^37, Gly^40]ANF_{1-28}-NH_2 (C-ANF_{1-28}) were generous gifts from Dr. John Lewicki, California Biotechnology Inc., Mountain View, CA. The
following materials and substances were purchased commercially: culture flasks and six-well culture plates (Falcon-Becton Dickinson Co., Lincoln Park, NJ), defined fetal bovine serum (HyClone Laboratories Inc., Logan, UT), carrier-free NaI\(^{125}\)I (Amersharm Corp., Arlington Heights, IL), insulin, amiloride, and trifluoracetic acid (Pierce Chemical Co.). (Sar\(^1\))-angiotensin II (Sar\(^2\)-ATII), Dubecro's modified Eagle's medium, RPMI 1640 medium, Hanks' balanced salt solution, bovine serum albumin, amiloride, and all other reagents were obtained from Sigma.

**Culture Media and Binding Solution**—The composition of culture media and binding solutions was as follows. Solution A was Hanks' balanced salt solution supplemented with 3.6 g/liter HEPES, 100 units/ml penicillin, 0.1 mg/ml streptomycin, and 0.25 mg/ml amphotericin B, pH 7.4. Solution B was RPMI 1640 supplemented with 20% fetal bovine serum, 2 g/liter NaHCO\(_3\), 3.6 g/liter HEPES, 0.6 units/ml insulin, 100 units/ml penicillin, 0.1 mg/ml streptomycin, and 0.25 mg/ml amphotericin B. Solution C was Dubecro's modified Eagle's medium (supplemented with 10% fetal bovine serum, 3.7 g/liter NaHCO\(_3\), HEPES, 0.6 unit/ml insulin, and the antibiotics and antifungal agents indicated above) conditioned with 3T3 Swiss Albino mouse fibroblasts (ATCC) during their log phase of growth. Binding solution was RPMI 1640 supplemented with HEPES (3.6 g/liter) to a pH of 7.4, bovine serum albumin (2 mg/ml), and insulin (0.6 unit/ml).

\[125\]I-\(^{125}\)I-ANF, -The fate of specifically bound \(^{125}\)I-ANF, was determined by chase experiments, as described previously (15). Briefly, cultured EGM, LGM or RMI cells grown to confluence in six-well dishes, were washed three times with 2 ml of ice-cold serum-free binding solution. Then, cells were incubated for 2 h at 4°C with 1 ml of binding solution containing or not unlabeled ANF, to a specific activity of approximately 500 Ci/mmol, to give a saturating concentration of 2 nM (15, 23). After washing with ice-cold binding solution to remove unbound ligand, cells were further incubated at 37°C with fresh binding solution in the absence of added radioligand for several time intervals. In some experiments in LGM and RMI cells, 0.3 μM C-ANF, was added to the medium to prevent binding of radioligand to the small, if any, amount of C-ANF receptors present in these cells. The results of these experiments in LGM and RMI cells did not differ from those in which C-ANF, was not added. Experiments were performed in the presence or absence of the lysosomotropic weak bases NILC (10 mM) or chloroquine (0.1 mM). At these concentrations, the lysosomotropic agents were previously shown to block lysosomal hydrolysis of internalized \(^{125}\)I-ANF, in BAVSM cells (15). In separate experiments, amiloride was added to the medium to a final concentration of 0.5 mM, 5 min before the incubation at 37°C, to determine its effect on the fate of bound ligand.

The temperature dependence of the dissociation of surface GC-ANF receptor-ligand complexes was determined in LGM cells preloaded with saturating concentrations of \(^{125}\)I-ANF, (2 nM) at 4°C. Trypsinization of these cells was performed by adding 1 mg/ml trypsin to 1 ml of cell suspension, followed by incubation for 30 min at 37°C. Monolayers were further incubated for time intervals ranging from 0.5 to 60 min at 4, 10, 20, 30, or 37°C in fresh binding medium containing 1 μM unlabeled ANF, to preclude rebinding of the radioligand.

**Determination of Radioactivity at the Cell Surface, in Intracellular Compartments, and in Medium**—At the end of incubation, cells were washed three times with 2 ml of ice-cold solution A. Bound ligand was removed from the cell surface by incubating the cells with an hypotonic acid solution (0.2 M acid in 0.5 M NaCl) at 4°C for 15 min. Intracellular radioactivity was determined in acid-washed cells as described above. After incubation of the cells with 4°C, acid washed cells were dissolved by incubation with a 2 N NaOH solution for 30 min at 37°C. For the determination of intact and degraded ligand in medium, the incubation medium was mixed with ice-cold trichloroacetic acid/phosphotungstic acid solution to a final concentration 6%/1% (w/v), and precipitate (intact ligand) and supernatant (degraded ligand) were separated by centrifugation. Radioactivity counts were determined in a γ-spectrometer. Nonspecific binding, receptor-independent internalization, and receptor-independent hydrolysis of radioligand were determined in parallel, by adding a 150-fold molar excess of unlabeled ligand (0.3 μM). Results for each time of incubation were calculated as the percentage of total internalized radioactivity decayed at 4°C.

**Trypsinization of Surface Receptors and Solubilization of Cells**—Total cell and intracellular concentrations of receptors in LGM cells were determined by a trypsinization-solubilization protocol, as described previously (15). Cell monolayers were preloaded with excess ANF, (0.1 μM) at 4°C for 2 h. After washing, cell monolayers were warmed to 37°C in fresh medium for several time intervals to elicit internalization of receptors. Then, monolayers were washed three times with Ca\(^{2+}\)-Mg\(^{2+}\)-free solution A and incubated for 30 min at 4°C with 0.5 ml of the same solution containing 5 mM EDTA and 1 mg/ml trypsin. At the end of incubation, the trypsin effect was neutralized by adding soybean trypsin inhibitor and calf serum to final concentrations of 1 mg/ml and 10%, respectively. Control cells were treated in an identical manner, except that trypsin was not added to the incubation mixture.

To determine the degree by which the trypsin treatment removed specific binding sites of ANF, trypsinized and control cells were incubated for 4 h at 4°C with saturating concentration of \(^{125}\)I-ANF, in the presence and absence of trypsin. It was found that the trypsin treatment for 30 min removed 90-95% of the specific surface binding sites for \(^{125}\)I-ANF, but resulted in some detachment of cells from the monolayers. Consequently, the 30-min incubation time with 1 mg/ml trypsin was chosen.

After trypsinization, cells were scrapped from the wells and washed...
three times with solution A containing 10% calf serum. Trypsinized
control cells were solubilized by a slight modification of the
method of Marshall et al. (24). Briefly, the cells were suspended
incubated at 1 h at 4 °C in 500 μl of Ca2+-Mg2+-free Hanks' balanced
salt solution containing 35 mM HEPES (pH 7.4), 0.4% octylace-
hydrolydodecyl ether (C1208), 1 mM EDTA, 1 mM EGTA, 1 mg/ml
soybean trypsin inhibitor, 20 μg/ml leupeptin, 1 mg/ml bacitracin,
and 0.5 mM phenylmethylsulfonl fluoride. At the end of incubation,
50 μl of 2% bovine γ-globulin and 550 μl of 30% w/v polyethylene
glycol (molecular weight, 8,000) were added to the suspension.
The cells were centrifuged, and after washing with binding solution, the
pellet was reconstituted in Hanks' balanced salt solution (supple-
mented with 35 mM HEPES to a pH of 7.4) containing 0.025% (21208,
0.2% bovine serum albumin, 0.6 mg/ml egg white lysozyme, and
soybean trypsin inhibitor, leupeptin, bacitracin, and phenylmethyl-
sulfonl fluoride at the above indicated concentrations.

The concentration of solubilized ANF receptors was determined as
reported previously (15). Briefly, 80 μl of the solubilized receptor
solution was mixed with 35 mM HEPES to a final concentration of 2 nm.
Equilibrium was allowed to proceed in microcentrifuge tubes for 24 h
at 4 °C. Receptor-ligand complexes were precipitated with 50 μl of
0.2% bovine γ-globulin and 350 μl of 30% w/v polyethylene glycol,
and the microtubes were centrifuged for 5 min at 12,000 x g. Super-
natants were aspirated, and the pellets were counted for radioactivity.
Nonspecific binding (125I-ANF1-28 bound in the presence of 0.3 μM
unlabeled ANF1-28) was subtracted from total binding, and a 1:1
stoichiometry for receptor-ligand interaction was assumed to
calculate the concentration of receptors.

RESULTS

The values for the density (Bmax) and equilibrium dissociation
constant (Kd) at 4 °C of the total surface ANF receptor population
in LGM, EGM, and RMI cells used in the present experiments are summarized in Table I. The table also gives the relative distribution of ANF receptor subtypes, and the ANF1-28-induced generation of cGMP in these cell lines. EGM, LGM, and RMI cells have 8,000-30,000 ANF receptors/cell. Competition binding studies using C-ANF3-23 or
satura
tion binding experiments with 125I-(Y3)-C-ANF3-23, specific ligands of C-ANF receptors which do not bind to gua-
nylate cyclase receptors (12, 17, 23), revealed that >85% of the total population of ANF receptors in LGM and RMI cells
is constituted by GC-ANF receptors. Cross-linking experiments in LGM cells with 125I-ANF1-28 revealed a single auto-
radiographic band that migrated at approximately 130 kDa in
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sodium dodecyl sulfate-polyacrylamide gel electrophoresis un-
derlying conditions (not shown), confirming that LGM cells
express GC-ANF receptors and only a minimal amount, if any, of C-ANF receptors. The very high affinity of these receptors for ANF1-28 at 4 °C (Kd < 0.1 nM) indicates that they belong to the GCα, relative to the GCβ subtype (4). For
unknown reasons EGM cells, albeit derived from the same
clones as LGM cells, also express C-ANF receptors in variable
proportions (10-40% of the total ANF receptor population).
The particular clone of EGM cells used in the experiments was
previously reported to occur in cultured BAVSM cells, a cell type
that has only a very small proportion of guanylate cyclase receptors
(7, 15).

Fig. 1 depicts data on the fate of 125I-ANF1-28 initially bound to
surface GC-ANF receptors in LGM cells. Panel A shows that specifically bound radioactivity disappeared rapidly from the
surface cell with a half-time of <3 min when cells were warmed
to 4 °C. However, this disappearance was accompanied by only minimal (<10%/60 min) internalization of
radioactivity (panel B) or hydrolysis of the radioligand (panel C). Practically all radioactivity that disappeared from the
surface cell appeared as intact 125I-ANF1-28 (as assessed by trichloroacetic acid precipitability) in the medium, with a
time course that precisely matched its disappearance from the
cell surface (panel D). HPLC analysis of the medium dem-
strated that the bulk of the radioactivity co-eluted with intact 125I-ANF1-28 and that minimal amounts eluted as la-
beled metabolites (not shown). Fig. 1 further shows that NH4Cl, a lysosomotropic weak base that blocks lysosomal
hydrolase of ANF (16), had no detectable effects on binding,
dissociation, or hydrolysis of 125I-ANF1-28 in LGM cells.

The results above demonstrate that the disappearance of
specifically bound radioligand from the cell surface of LGM cells is caused by a rapid dissociation of 125I-ANF1-28 from
GCα-ANF receptors when cells are warmed from 4 to 37 °C. Because amidarone has been previously shown to increase the
affinity of GC-ANF receptors in membranes from bovine adrenal glomerulosa (25), we tested the effects of this com-
pound on the dynamic of GCα-ANF receptor-radioligand com-
plexes in LGM cells. The results of these experiments are also shown in Fig. 1 (diamond symbols). As can be seen,
amidarone markedly decreased the disappearance rate of radi-
oligand from the cell surface by more than 3-fold (panel A) and correspondingly decreased the rate of appearance of
radioligand in the medium (panel D). The half-time of residence of specifically bound 125I-ANF1-28 at the membrane surface of amidarone-treated cells was approximately 9 min, whereas it
was <3 min in controls (panel A). In spite of this marked increase in resident time, there was no evidence for internal-
ization (panel B) and for NH4Cl-sensitive (lysosomal) hy-

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### Table I

| ANF receptors and cGMP generation in cultured EGM, LGM, and RMI cells |
|-----------------|-----------------|-----------------|-----------------|
|                  | Binding parameters* | cGMP levelsa |
| ANF receptors/cell | Bmax (pmol/mg protein) | Kd (nM) | GCα-ANF receptors | C-ANF receptors |
| EGM              | 15,652 ± 1,130 | 73 ± 9 | 60 | 40 |
| LGM              | 11,250 ± 930 | 81 ± 11 | >90 | <10 |
| RMI              | 7,856 ± 1,643 | 74 ± 37 | >85 | <15 |

* Density (Bmax) and equilibrium dissociation constant (Kd) of the total population of ANF receptors were determined in confluent EGM, LGM, and RMI cell monolayers by saturation binding experiments at 4 °C with 125I-ANF1-28 (see “Experimental Procedures”). Bmax and Kd values for RMI cells between the 10th and 17th passages were taken from Fontoura et al. (23). The proportion of C-ANF receptors (in percent of the total population of ANF receptors) was determined by competition binding experiments at 4 °C between 125I-ANF1-28 and saturating concentrations (0.3-1 μM) of C-ANF1-28, a specific ligand of C-ANF receptors, as reported previously (15, 23).

a C-ANF1-28 was determined in confluent cell monolayers of EGM, LGM, and RMI cells at 37 °C in the presence of 0.5 mM 3-isobutyl-1-methoxanthine and in the absence of ANF1-28 (10-11-10-7M), as reported previously (23).
drolysis of radioligand (panel C).

When LGM cells were preloaded with trace concentrations (10 PM) of [125I]-ANF_{1-28} instead of saturating concentrations of radioligand, the results were indistinguishable from those depicted in Fig. 1 (not shown). The HPLC profiles shown in Fig. 2 demonstrate that there was only a minimal appearance of hydrolytic products in the medium when LGM cells were incubated for 2 h at 37 °C in the continuous presence of 20 PM [125I]-ANF_{1-28} with or without 10 mM NH_4Cl or 0.1 μM unlabeled ANF_{1-28}.

Fig. 3 demonstrates that the dynamics of receptor-radioligand complexes in RMI cells, a cell type in which the overwhelming majority of ANF receptors is also constituted by GC_{α}-ANF receptors (Table I), is indistinguishable from that observed in LGM cells.

The above results show that at physiological temperatures the offset of radioligand from GC_{α}-ANF receptors is very fast. The temperature dependence of the off-rate of [125I]-ANF_{1-28} from GC_{α}-ANF receptors in LGM cells is summarized in Table II. In these experiments the k_{off} was determined from the initial monoexponential release of specifically bound radioligand to the medium which, at the higher temperatures (30, 37 °C), accounted > 85% of the dissociation of [125I]-ANF_{1-28} from GC_{α}-ANF receptors. At lower temperatures, a second sluggish exponential component, which accounted for > 50% of radioligand dissociation, was too slow to be determined with accuracy in the time (1 h) of the experiments. The initial k_{off} at 4 °C (0.015 min^{-1}) is very slow, consistent with the very low equilibrium dissociation constant (K_{D}) at this temperature (Table I). As expected, k_{off} increases with temperature (Q_{10}, 2-3) from 4 to 30 °C. Between the temperatures of 30 and 37 °C, however, there is an unexpected disproportionate 4-fold increase in the k_{off} from 0.18 to 0.85 min^{-1} (Q_{10} > 6). At 37 °C, 51% of specifically bound radioligand had already been released to the medium by 0.5 min, the first sampling time. Therefore, 0.85 min^{-1} represents a minimal value for the dissociation rate constant at 37 °C. The disproportionate increase in the off-rate at 37 °C, but not at subphysiological temperatures, indicates that this phenomenon depends on the metabolic integrity of the cells, probably on the generation of ATP (see "Discussion").

Chase experiments with [125I]-ANF_{1-28} were also performed in EGM cells, a cell type that was derived from the same clone as the LGM cells (see "Experimental Procedures") but has a significant proportion (40%) of C-ANF receptors in addition to GC_{α}-ANF receptors (Table I). Fig. 4 shows the results of these experiments. The decay of specifically bound radioligand from the cell surface (panel A) is clearly bimodal, with a fast component similar to that observed in LGM and RMI cells (Figs. 1 and 3) and a slower component that is similar to that reported previously for C-ANF receptors in BAVSM cells (15). The appearance of trichloroacetic acid-precipitable radioactivity in the medium (panel D) has a single component that corresponds precisely to the fast component of radioligand decay from the cell surface. This component is similar to that observed in LGM and RMI cells (Figs. 1 and 3) and sharply contrasts with that observed previously in BAVSM cells, in which the appearance of trichloroacetic acid-precipitable radioactivity in medium is neg-

FIG. 1. Fate of [125I]-ANF_{1-28} specifically bound to GC_{α}-ANF receptors in LGM cells: effects of NH_4Cl and amiloride. Surface GC-ANF receptors of LGM cell monolayers were preloaded with saturating concentrations of [125I]-ANF_{1-28} (2 nM) at 4 °C in the presence or absence of 10 mM NH_4Cl or 0.5 mM amiloride. After washing at 4 °C to remove unbound radioligand, cells were incubated at 37 °C for the indicated times with fresh binding solution in control conditions (circles) or in the presence of 10 mM NH_4Cl (triangles) or 0.5 mM amiloride (diamonds). At the end of each incubation time, cells and medium were processed as described under "Experimental Procedures." Panel A, cell surface radioactivity; panel B, intracellular radioactivity; panel C, trichloroacetic acid-soluble radioactivity in the medium; and panel D, trichloroacetic acid-precipitable radioactivity in the medium. The results are expressed as a percentage of [125I]-ANF_{1-28} specifically bound to the surface of control cells at time 0. Results are means of three separate experiments in duplicate or triplicate for control and NH_4Cl-treated cells (S.E. < 10%) and of two separate experiments in duplicate for amiloride-treated cells.

FIG. 2. Lack of hydrolysis of [125I]-ANF_{1-28} after prolonged exposure of LGM cells to the radioligand: HPLC profiles of radioactivity in the medium. LGM cell monolayers were incubated for 2 h at 37 °C in binding medium containing 20 PM [125I]-ANF_{1-28} alone (panel A) or 20 PM [125I]-ANF_{1-28} in the presence of 10 mM NH_4Cl (panel B) or 0.1 μM unlabeled ANF_{1-28} (panel C). At the end of the incubation, 100-μl samples of medium were injected onto a 7.8 × 30-mm reverse-phase C_{18} μBondapak column and eluted with a linear gradient of 10-60% acetonitrile (CH_3CN) over 60 min (dotted line) at a flow rate of 1 ml/min. The gradient was formed by a mixture of CH_3CN and 0.1% trifluoroacetic acid. The column was standardized previously with [125I]-ANF_{1-28}. The major peaks in the HPLC elution profiles correspond to intact [125I]-ANF_{1-28}, and the very minor earlier elution peaks correspond to labeled hydrolytic products. Note the almost complete absence of hydrolysis of ANF.
ligible (15). Similar to the earlier observation on C-ANF receptor-mediated endocytosis and lysosomal hydrolysis of ANF in BAVSM cells (15), there is significant internalization of radioactivity in EGM cells, which is magnified in the presence of the lysosomotropic agent chloroquine (panel B), and is accompanied by chloroquine-sensitive appearance of hydrolytic products in medium (panel C). Similar results were obtained when 10 mM NH4Cl was used instead of 0.1 mM chloroquine (not shown). Thus, consistent with the almost equivalent proportion of GCα-ANF and C-ANF receptors, the dynamics of receptor-ligand complexes in EGM cells are intermediate between those of LGM or RMI cells (GCα-ANF receptors) and BAVSM cells (C-ANF receptors).

We investigated further whether the lack of internalization and lysosomal hydrolysis of ANF in LGM was caused by a generalized defect of receptor-mediated endocytosis and/or lysosomal function in these cells. For this purpose we determined the dynamics of AT1 receptors using 125I-(Sar1)-ATII as a radioligand and the same protocol as that used to investigate the dynamics of GCα-ANF receptors in these cells. Fig. 5 illustrates the results of these experiments. The radioligand disappeared rapidly from the cell surface (panel A), and this disappearance can be entirely accounted by internalization (panel B) with subsequent NH4Cl-sensitive (lysosomal) hydrolysis of 125I-(Sar1)-ATII (panel C). Contrary to the observation with 125I-ANF1-28 (Fig. 1), the appearance of intact 125I-(Sar1)-ATII in medium was negligible (panel D). NH4Cl did not affect the rate of internalization (panel A) but led to the accumulation of radioligand within the cell (panel B) and a marked blunted hydrolysis of the ligand (panel C). Thus, LGM cells are able to promote fast receptor-mediated inter-

**TABLE II**

| Temperature °C | kobs min⁻¹ | Q10 |
|---------------|------------|-----|
| 4             | 0.015      | 3.3 |
| 19            | 0.036      | 1.0 |
| 20            | 0.067      | 2.7 |
| 30            | 0.178      | >6.8|
| 37            | >0.845     |     |

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Fig. 3. Fate of 125I-ANF1-28 specifically bound to GC-ANF receptors in RMI cells. Experiments were performed in RMI cell monolayers as described for control LGM cells in the legend of Fig. 1. **Panel A**, radioactivity specifically bound to the cell surface; **panel B**, intracellular radioactivity; **panel C**, trichloroacetic acid-soluble radioactivity in the medium; **panel D**, trichloroacetic acid-precipitable radioactivity in the medium. Results, expressed as a percentage of 125I-ANF1-28 specifically bound to the cell surface at time 0, are mean of three experiments in duplicate (S.E. < 10%).

Fig. 4. Fate of 125I-ANF1-28 specifically bound to ANF receptors in EGM cells. Experiments were performed as those in Fig. 1 for LGM cells (see the legend of Fig. 1) in controls (circles) or in the presence of 0.1 mM chloroquine (triangles). **Panel A**, radioactivity specifically bound to the cell surface; **panel B**, intracellular radioactivity; **panel C**, trichloroacetic acid-soluble radioactivity in the medium; **panel D**, trichloroacetic acid-precipitable radioactivity in the medium. Results (average of duplicates of one experiment) are expressed as a percentage of 125I-ANF1-28 specifically bound to the cell surface of control cells at time 0. A repeat experiment gave similar results.

Fig. 5. Internalization and metabolism of 125I-(Sar1)-ATII specifically bound to ATII receptors in LGM cells. Experiments were performed as described in the legend of Fig. 1, except that 125I-(Sar1)-ATII (1 nM) was used as a radioligand for ATII receptors. Experiments were performed in control conditions (circles) or in the presence of 10 mM NH4Cl (triangles). **Panel A**, radioactivity specifically bound to the cell surface; **panel B**, intracellular radioactivity; **panel C**, trichloroacetic acid-soluble radioactivity in the medium; **panel D**, trichloroacetic acid-precipitable radioactivity in the medium. Results (average of duplicates of one experiment) are expressed as a percentage of 125I-(Sar1)-ATII specifically bound to the cell surface of control cells at time 0.
nalization and lysosomal hydrolysis of $^{125}$I-Sar$^2$-ATII, indicating that these cells do not have a defective endocytic-lysosomal apparatus.

The very rapid dissociation of $^{125}$I-ANF$_{28}$ when LGM or RMI cells are warmed from 4 to 37 °C precludes a conclusion on whether there is ligand-induced internalization of unoccupied GC$_\alpha$-ANF receptors in the experiments depicted in Figs. 1 and 3. To investigate this issue we directly determined the time-dependent changes in the intracellular concentration of GC$_\alpha$-ANF receptors after exposure of LGM cells to saturating concentrations of ligand (see “Experimental Procedures”). Fig. 6 shows that after exposure to ANF, the total as well as the intracellular concentration of GC$_\alpha$-ANF receptors in LGM cells remain constant throughout the experiment. This result contrasts with that reported previously for BAVSM cells exposed to ANF, in which there is first an increase in the intracellular concentration of C-ANF receptors (internalization) and then a decrease toward control values (recycling) (15). The lack of change in the intracellular concentration of GC$_\alpha$-ANF receptors in LGM cells further demonstrates that ANF does not induce the internalization of occupied or unoccupied GC$_\alpha$-ANF receptors in these cells.

**DISCUSSION**

The present study unveils two major properties of the dynamics of membrane GC$_\alpha$-ANF receptors and of GC$_\alpha$-ANF receptor-ligand complexes in cultured renal cells. First, in these cells GC$_\alpha$-ANF receptors do not internalize at detectable rates and do not mediate internalization and subsequent lysosomal hydrolysis of ANF. Second, the termination of the interaction between GC$_\alpha$-ANF receptors and ANF at the cell surface results from a very rapid dissociation when the ligand binds to the receptor at physiological temperatures.

The lack of internalization of GC$_\alpha$-ANF receptors is not caused by characteristics of a single specific cell type or by a generalized defect of the endocytic-lysosomal apparatus in the cultured renal cells used in the present experiments. Indeed, the dynamics of GC$_\alpha$-ANF receptors in two different species of cultured renal cells (LGM and RMI cells) were practically the same (Figs. 1 and 3). Furthermore, LGM cells, although failing to endocytose and hydrolyze ANF, conserve the ability to promote rapid receptor-mediated internalization and lysosomal hydrolysis of ATII (Fig. 5). Finally, EGM cells, which express both GC$_\alpha$-ANF and C-ANF receptors in near equal proportions, handle ANF in a manner consistent with the presence of the two subtypes of receptors, namely internalization and lysosomal hydrolysis via C-ANF receptors and rapid off-rate via GC$_\alpha$-ANF receptors. It is noteworthy that the relative degree of internalization and offset of specifically bound radioligand in EGM cells is similar to the relative proportion of C-ANF and GC$_\alpha$-ANF receptors in these cells.

The dynamics of surface GC$_\alpha$-ANF receptors were followed by using its specific radioligand as a marker. In view of the very rapid off-rate of $^{125}$I-ANF$_{28}$ at 37 °C, it was possible that internalization of unoccupied GC-ANF receptors could have remained undetected. However, even when the off-rate of the radioligand was markedly decreased by amiloride, no significant internalization of receptor-ligand complexes or of lysosomal hydrolysis of $^{125}$I-ANF$_{28}$ was detectable (Fig. 1). It could be argued that amiloride itself, perhaps by acidifying the cells, may have blocked internalization. The following additional evidence, however, strongly supports the conclusion that GC$_\alpha$-ANF receptors of LGM cells are not endocytosed: (i) there is lack of significant hydrolysis of $^{125}$I-ANF$_{28}$ even when LGM cells are incubated in the continuous presence of saturating or trace amounts of radioligand for up to 2 h at 37 °C (Fig. 2); and (ii) ANF does not induce a time-dependent change in intracellular concentration of GC-ANF receptors in EGM cells (Fig. 6).

Membrane receptors that are constitutively concentrated in coated pits or are clustered in these or other endocytic regions when complexed with their ligands are internalized at very fast rates, which in some instances may exceed 100% of surface receptors/min (16). Receptors for low density lipoprotein (19, 27), several other clearance and/or transport receptors (18, 28–32), and polypeptide-hormone receptors such as insulin, epidermal growth factor (26, 33–35), and ATII (Fig. 5) are examples of this category of receptors that undergo rapid endocytosis. Naturally occurring or experimentally induced mutations in which “internalization signals” in the cytoplasmic domain of these receptors are deleted or altered markedly reduce the endocytic rate to less than 10%/min but do not abolish endocytosis (26, 27, 29, 32, 35). In the case of the low density lipoprotein receptor, only the deletion of the entire cytoplasmic domain abolishes detectable internalization (27). It is generally assumed that relatively slow (<10%/min) but significant rates of endocytosis correspond to the entrapment of highly mobile membrane receptors in coated pits or other endocytic regions. On the other hand, rapid receptor-mediated endocytosis depends on specific biochemical interactions between the cytoplasmic domain of receptors with constitutive proteins of the endocytic apparatus (28).
Dynamics of ANF-Guanylate Cyclase Receptors

We have demonstrated previously that C-ANF receptors in cultured BAVSM cells are constitutively internalized at a relatively slow rate of 4-5%/min and that ligand binding does not increase this rate (15). This would suggest that C-ANF receptors are mobile membrane proteins that, with or without their ligand, are internalized as they become entrapped in endocytic regions. However, the present study demonstrates that endocytosis of GC-ANF receptors in GM and RMI cells is minimal (<0.1%/min) even in presence of ligand. These dynamics, characteristic of bona fide membrane resident proteins (36), are highly uncommon for polypeptide-hormone receptors. The only other known example in this regard is the insulin receptor in IM-9 human lymphocyte cells, which, contrary to insulin receptors in other cell types, does undergo only minimal, if any, detectable internalization (33, 34).

The present experiments do not exclude the possibility that GC-ANF receptors may internalize in cells other than GM and RMI cells. Thus, it has been reported that GC-ANF receptors of Leydig tumor and pheochromocytoma (PC12) cells mediate internalization and lysosomal hydrolysis of ANF (37, 38). In these studies the degree of constitutive internalization of GC-ANF receptors was not determined. It is possible, even likely, that tumor cells behave abnormally with respect to the dynamics of receptors and receptor-ligand complexes. Ligate-induced internalization of most biological receptors is accompanied by a prolonged decrease in cell surface receptor density (down-regulation). When receptors fail to internalize, as exemplified by insulin receptors in human lymphocyte IM-9 cells, homologous (ligand-induced) down-regulation does not occur (34). The reported lack of detectable in vivo regulation of GC-ANF receptors in glomeruli and renal papillae of the rat, when plasma levels of ANF are altered by salt diet (39, 40), is consistent with the present evidence that these receptors do not internalize at appreciable rates. Further experiments are needed, however, to test whether GC-ANF receptors in intact or freshly isolated cells and tissues have the same dynamics as those observed in cultured LGM and RMI cells.

Known molecular features of GC-ANF receptors are also consistent with its characteristics as a membrane resident protein. The binding domain of GC-ANF receptors and the enzyme that generates the second messenger (guanylate cyclase) are part of a same molecule (1). The "mobile receptor theory," which states that ligand effects depend on the coupling of different molecular species, a mobile acceptor (the receptor proper) and an enzyme effector (41), is not applicable to GC-ANF receptors. Thus, mobility of GC-ANF receptors is, theoretically, not necessary for coupling ANF binding to activation of guanylate cyclase. Moreover, the cytoplasmic domain of GC-ANF receptors does not contain sequences of amino acids that have been associated with the internalization of many other receptors, including the NPY/β-F (where X is any aminio acid) sequence, nor does it contain an aromatic amino acid (tyrosine or phenylalanine) near the transmembrane domain (26, 27, 29, 32, 35). The nearest aromatic amino acid in the cytoplasmic domain of GC-ANF receptors is a tyrosine, separated from the transmembrane domain by 42 amino acids (1). As pointed out above, however, deletion or substitution of internalization signals reduces but does not preclude internalization. Thus, it is unlikely that the absence of internalization signals that confer a high endocytic rate account entirely for the behavior of GC-ANF receptors as a membrane resident protein. Further studies are needed to elucidate this question.

Internalization of receptor-ligand complexes may determine, at least in part, the termination of biological effects of polypeptide hormones. In the absence of internalization, other processes must be invoked to explain the termination of GC-ANF receptor-mediated responses. Several complex ligand-induced receptor and/or postreceptor events, encompassed in the generic term "desensitization," may also contribute to the termination of hormonal responses. In the case of GC-ANF receptors, the present results indicate that the rapid dissociation of membrane GC-ANF receptor-ANF complexes at 37 °C may account at least in part for the termination of the cellular effects of ANF. The findings that there is a disproportionate increase in the off-rate of 125-I-ANF from GC-ANF receptors at subphysiological temperatures (Table II) and that amiloride blocks this event (Fig. 1) suggest that cellular metabolic events are involved in the regulation of the receptor-ligand dissociation. In membranes of bovine adrenal zona glomerulosa, amiloride has been shown to decrease the equilibrium dissociation constant (k_d) of GC-ANF receptor at subphysiological temperatures, an effect that is opposite to and partially competitive with that of ATP (25, 42). In addition, in the same preparation, ATP was shown to increase the k_cat of 125-I-ANF from GC-ANF receptors to a greater extent that it increases the k_on (43). Finally, ATP and amiloride have been postulated to interact with the kinase domain of GC-ANF receptors, which exerts a modulatory effect on guanylate cyclase activity and on receptor affinity (43, 44). These data, as a whole, suggest that ATP is likely to have an important role in the very rapid dissociation of ANF from GC-ANF receptors in intact cells. Although the contribution of other metabolic factors cannot be ruled out by the present data, a role for ATP in this process is supported by the finding that at subphysiological temperatures, in which there is limited production of ATP, the off-rate of ligand from GC-ANF receptors has a Q_10 that is 2-3-fold lower than that between 30 and 37 °C (Table II).

To our knowledge, there have been no previous systematic studies on the temperature dependence of the dissociation of membrane receptor-ligand complexes in intact cells. In most instances such studies would be hampered by the internalization of these complexes, precluding a precise determination of the dissociation rate. It is noteworthy, however, that a very high off-rate of ligand at 37 °C, similar in value to that described presently for GC-ANF receptors, has been reported for polymeric IgA receptor-IgA complexes in Madin Darby canine kidney cells when internalization is blocked by hypertonic sucrose medium (30) and for noninternalizing insulin receptors in IM-9 lymphocytes (33). At 4 °C the dissociation rates of ANF from GC-ANF and C-ANF receptors are similar and very slow, approximately 1%/min or less (Table II) (15, 45). However, at 37 °C there is a major disparity between the dissociation of ANF from C-ANF and GC-ANF receptors. Results of previous studies have shown that at 37 °C the offset of ligand from surface C-ANF receptors in BAVSM and RC2 cells is < 0.05 min⁻¹, the approximate internalization rate of C-ANF receptor-ligand complexes in these cells (15, 45). In the present study, the slower component of the decay of radioligand from the surface of EGM cells (Fig. 4, panel A) suggests that the off-rate from surface C-ANF receptors at 37 °C in these cells is also very slow. In contrast, the k_off of ANF from GC-ANF receptors in LGM cells at 37 °C is > 0.8 min⁻¹ (Table II). Unfortunately, because of the very high k_on, the equilibrium dissociation constant (k_d) or the onset rate (k_on) of ligand binding to GC-ANF receptors at 37 °C cannot be experimentally determined. In all likelihood, however, the equilibrium dissociation constant at 37 °C is much higher for GC-ANF than for C-ANF receptors. Consistent with this conclusion is the observation that at subphysiological temp-
temperatures, ATP increases the measured equilibrium dissociation constant (k_d) of radioligand binding to GC-ANF receptors in membranes from bovine adrenal zona glomerulosa (43). The present results point out that equilibrium or kinetic parameters of binding determined at subphysiological temperatures and/or outside the natural environment of the cell do not necessarily reflect those in intact cells at physiological temperatures.

The major differences in the dynamics of GC_A-ANF and C-ANF receptors at physiological temperatures in intact cells are consistent with the specialized role of these receptors. The effectiveness of the removal of ANF from the circulation, a major role of C-ANF receptors, is determined by receptor-mediated internalization and lysosomal hydrolysis of ligand. This process is favored by a slow dissociation of surface receptor-ligand complexes. Indeed, a sluggish dissociation increases the availability of unoccupied C-ANF receptors at physiological temperatures by the very high density of these receptors in vascular and renal tissues and the recycling of internalized C-ANF receptors to the membrane surface (12–16). However, mediation of the functional effects of ANF, the major role of GC_A-ANF receptors, does not depend on internalization. Under this condition, a fast dissociation of ANF from membrane GC_A-ANF receptors will contribute to hormonal stimulus-response homeostasis. When the free concentration of ANF falls, a faster dissociation favors a rapid termination of the response. The dissociated hormone can then be removed rapidly by the abundant C-ANF receptors. Conversely, a faster dissociation increases the availability of unoccupied GC_A-ANF receptors for ligand binding, favoring a heightened response when the free concentration of ANF raises. As discussed above, ATP is likely to play a major role in this homeostatic process because it increases ligand offset (and to a smaller extent onset) and magnifies the ANF-induced increase in guanylate cyclase activity. In combination, these events are likely to result in a higher efficacy of ANF effects when plasma concentration raises and a prompt termination of these effects when plasma concentration of ANF falls.

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