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$G_{s\alpha}$-selective G protein antagonists

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ABSTRACT Suramin acts as a G protein inhibitor because it inhibits the rate-limiting step in activation of the G_α subunit, i.e., the exchange of GDP for GTP. Here, we have searched for analogues that are selective for $G_{s\alpha}$. Two compounds have been identified: NF449 (4,4′,4″,4‴-[carbonyl-bis[imino-5,1,3-benzenetriyl bis-(carbonylimino)]]tetrakis-(benzene-1,3-disulfonate) and NF503 (4,4′-[carbonylbis[imino-3,1-phenylene-(2,5-benzimidazolylene)carbonylimino]]bis-benzenesulfonate). These compounds (*i*) suppress the association rate of guanosine 5′-[γ-thio]triphosphate ([^{35}S]GTP[γS]) binding to $G_{s\alpha\text{-}s}$ but not to $G_{i\alpha\text{-}1}$, (*ii*) inhibit stimulation of adenylyl cyclase activity in S49 cyc$^-$ membranes (deficient in endogenous $G_{s\alpha}$) by exogenously added $G_{s\alpha\text{-}s}$, and (*iii*) block the coupling of β-adrenergic receptors to G_s with half-maximum effects in the low micromolar range. In contrast to suramin, which is not selective, NF503 and NF449 disrupt the interaction of the A_1-adenosine receptor with its cognate G proteins (G_i/G_o) at concentrations that are >30-fold higher than those required for uncoupling of β-adrenergic receptor/G_s tandems; similarly, the angiotensin II type-1 receptor (a prototypical G_q-coupled receptor) is barely affected by the compounds. Thus, NF503 and NF449 fulfill essential criteria for $G_{s\alpha}$-selective antagonists. The observations demonstrate the feasibility of subtype-selective G protein inhibition.

In current pharmacotherapy the input into G protein-regulated signaling is manipulated by targeting the receptor with appropriate agonists and antagonists. Several arguments, however, suggest that elements of the receptor-activated, downstream signaling cascade, in particular G proteins, may be considered as drug targets *per se*. (*i*) The molecular diversity of G protein α, β, and γ subunits is large, and the number of distinct αβγ oligomers that can be produced by combinatorial association of subunit is excessive (1). (*ii*) The interaction of a given receptor with the cellular complement of G proteins may be governed by both exquisite specificity and promiscuity; in the first case, only one defined oligomer supports the ability of a receptor to regulate an effector in an intact cell (for review see refs. 2 and 3). (*iii*) On the other hand, many receptors couple to multiple G proteins (2, 3); this is exemplified by the thyrotropin receptor (4), which can activate essentially all G protein α subunits expressed in the thyroid (i.e., members of all subfamilies of G_α other than the transducins). Thus, cellular stimulation by a receptor often results in the concerted activation of several distinct G proteins such that multiple effector pathways are recruited to produce the biological response. In theory, it may be desirable to block signaling of a receptor via one type of G protein but not via the other G proteins (resulting in "biased inhibition of receptor/G protein tandem formation"), a goal that cannot be achieved by receptor antagonists but that may be achieved by compounds that bind selectively to individual G proteins. In addition, several human diseases arise from activation of G protein α subunits by point mutations (for a brief overview, see ref. 5); appropriate G protein antagonists are desirable under these circumstances.

Suramin has been shown previously to act directly on G protein α subunits (6) and to block their activation by receptors (7–9). In the present work, we have searched for compounds that inhibit $G_{s\alpha}$ directly. Two compounds of remarkable selectivity were identified that suppress the coupling of β-adrenergic receptors to G_s, whereas they affect a prototypical G_i/G_o- and G_q-coupled receptors (A_1-adenosine and angiotensin II receptor, respectively) to a much lesser extent.

MATERIALS AND METHODS

Materials and Chemical Synthesis. Suramin, chloroglycouril, DTT, Hepes, angiotensin II, and saralasin were purchased from Sigma; [^{125}I], [^{125}I]CYP (iodocyanopindolol), [^{35}S]GTP[γS], and [α-^{32}P]ATP were from NEN. The synthesis of NF503 [$C_{41}H_{28}N_8O_9S_2Na_2$; disodium-4.4′-[carbonylbis[imino-3.1-phenylene-(2.5-benzimidazolylene)carbonylimino]]bis-benzenesulfonate)] has been described (10); NF445 [$C_{45}H_{36}N_6O_{17}S_4Na_4$; tetrasodium-4.4′.4″.4‴-[carbonylbis[imino-2.1.4-benzenetriylbis-(carbonyliminomethylene)]]tetrakis-(benzenesulfonate)] and NF449 [$C_{41}H_{24}N_6O_{29}8Na_8$; octasodium-4.4′.4″.4‴-[carbonyl-bis [imino-5.1.3-benzenetriylbis-(carbonylimino)]]tetrakis-(benzene-1.3-disulfonate)] were synthesized using methods described in detail for other suramin analogues (11). For NF445, 3-(aminomethyl)benzenesulfonic acid (50 mmol in 50 ml of H_2O) was treated at pH 8.5 and room temperature with 2-nitroterephtalic acid dichloride (30 mmol dissolved in 100 ml of toluene). The precipitate formed during the reaction was filtered, recrystallized from H_2O, and hydrogenated in aqueous solution (1%) using Pd/C as catalyst. After filtration, the concentrated filtrate (10% original volume) was reacted with a large excess of phosgene (20% in toluene) at room temperature and pH 4. The aqueous phase of the reaction mixture was evaporated to dryness. The residue was recrystallized from H_2O/CH_3OH; overall yield was 75% (yellowish powder). NF449 was synthesized in a similar way starting from aminobenezene-2.4-disulfonic acid and 5-nitroisophtalic acid dichloride; overall yield was 25% (yellowish powder). All reaction steps were followed up by TLC. The purity of the final products was >95% as determined by HPLC (12). Saralasin and angiotensin II were iodinated by incubating the peptides (7 nmol) and carrier-free Na^{125}I (1 mCi) in 40 μl of 0.1 M NaP_i, pH 7.4, in a tube precoated with 20 μg of chloroglycouril for 10 min on ice. The reaction products were resolved by HPLC (Merck Lichrospher 100 RP-C18; 4.6 × 250 mm; 5 μm) using a 35-ml linear gradient of 15 to 40% acetonitrile in 0.1% trifluoroacetic acid. Saralasin, monoiodinated saralasin, and di-

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Abbreviations: [^{125}I]HPIA, N^6-(3-iodo-4-hydroxyphenylisopropyl)adenosine; [^{35}S]GTP[γS], guanosine 5′-[γ-thio]triphosphate.
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iodinated saralasin were eluted at 6, 14, and 18 ml, respectively; angiotensin II, monoiodinated angiotensin II, and di-iodinated angiotensin II were eluted at 20, 24, and 27 ml, respectively. N^6-(3-Iodo-4-hydroxyphenylisopropyl)adenosine ([^{125}I]HPIA) was synthesized as described (13).

Protein Purification and Membrane Preparations. Recombinant $G_{s\alpha\text{-s}}$ and $rG_{i\alpha\text{-1}}$ were expressed in *Escherichia coli* and purified from bacterial lysates (14, 15). Membranes were prepared from rat heart ventricles as described (16). Rat glomeruli were isolated by a sieving procedure (17); for membrane preparation the glomeruli were lysed by two freeze–thaw cycles in hypotonic TEM buffer (in mM: 50 Tris·HCl, pH 7.5, 1 EDTA, 5 $MgCl_2$) and homogenized with an Ultra-Turrax. The particulate material was sedimented at 50,000 × *g* for 10 min and taken up at a protein level of 5 mg/ml. Human brain membranes were prepared as described (18). 293 cells stably expressing the human A_1-adenosine receptor (19) were a generous gift of M. J. Lohse (University of Würzburg, FRG). For membrane preparation, cells were scraped off their plastic support and lysed by a freeze–thaw cycle in TEM buffer followed by brief sonication; the particulate material was collected by centrifugation as described above.

Binding Assays. The binding of [^{35}S]GTP[γS] to $rG_{s\alpha\text{-s}}$ and $rG_{i\alpha\text{-1}}$ (2–4 pmol/assay) was carried out as described (6). β-Adrenergic receptors were labeled with the antagonist [^{125}I]CYP; rat cardiac membranes (8–12 μg/assay) or S49 cyc$^-$ membranes (3–6 μg/assay) were incubated in TEMA buffer (in mM: 50 Tris·HCl, pH 7.5, 5 $MgCl_2$, 1 EDTA, 1 ascorbic acid) and the concentrations of [^{125}I]CYP, isoproterenol, suramin analogues, and GTP[γS] indicated in the figure legends. High-affinity agonist binding was reconstituted in S49 cyc$^-$ membranes with oligomeric G_s (a combination of 3 pmol of $rG_{s\alpha\text{-s}}$ and 10 pmol of purified $\beta\gamma$ dimers/reaction) as outlined in ref. 20. Nonspecific binding was determined in the presence of 100 μM isoproterenol (<15% of total binding). A_1-adenosine receptors were labeled with the agonist [^{125}I]HPIA. The binding reaction was carried out in 50 μl containing TEMA buffer, 8 units/ml adenosine deaminase, human brain membranes (~6–9 μg), or membranes from stably transfected 293 cells (12–15 μg), 1 nM [^{125}I]HPIA in the absence and presence of increasing concentrations of suramin analogues. Nonspecific binding (<10% of total binding) was determined in the presence of 1 μM CPA (N^6-cyclopentyladenosine). Angiotensin II type 1 receptors were labeled with the antagonist [^{125}I]saralasin II or the agonist [^{125}I]angiotensin II. The binding assay was carried out in 30 μl containing TEMA buffer, 12.5 μg/ml truncated human ACTH$^{11-24}$, 100 μM bacitracin, glomerular membranes (5 μg), 0.5 nM [^{125}I]saralasin II, or [^{125}I]angiotensin II in the absence and presence of increasing concentrations of GTP[γS] and of suramin analogues. Nonspecific binding ($\leq$20% of total binding) was determined in the presence of 1 μM unlabeled saralasin ([^{125}I]angiotensin II) or angiotensin II ([^{125}I]saralasin). After 60 min at 30°C, the binding reactions were stopped by filtration over glass-fiber filters (presoaked in 1% BSA for angiotensin receptor binding).

Determination of Adenylyl Cyclase Activity. Adenylyl cyclase activity was reconstituted to S49 cyc$^-$ membranes by addition of $rG_{s\alpha\text{-s}}$ as described (21) with minor modifications; $rG_{s\alpha\text{-s}}$ (0.1 mg/ml) was preactivated in buffer (in mM: Hepes·NaOH, pH 7.6, 1 EDTA, 1 DTT, 0.01 GTP[γS], 10 $MgSO_4$, 0.1% Lubrol) for 30 min at 30°C and diluted to give the appropriate amounts of $rG_{s\alpha\text{-s}}$. Alternatively, inactive

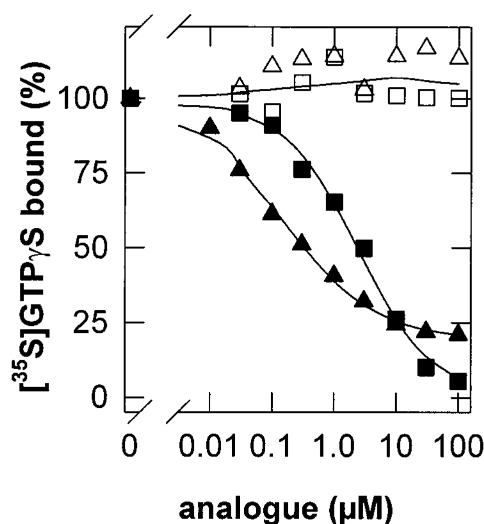

FIG. 1. Binding of [^{35}S]GTP[γS] to $rG_{s\alpha\text{-s}}$ and $rG_{i\alpha\text{-1}}$ in the presence of NF449 and of NF503. Binding of [35S]GTP[γS] was determined as described under *Materials and Methods* using 2–4 pmol of $rG_{s\alpha\text{-s}}$ (▲, ■) and $rG_{i\alpha\text{-1}}$ (△, □) and the indicated concentrations of NF449 (△, ▲) and of NF503 (□, ■); the incubation lasted for 3 and 15 min for $rG_{s\alpha\text{-s}}$ and $rG_{i\alpha\text{-1}}$, respectively (<1*$t_{1/2}$ of the GDP/GTP[γS] exchange reaction). Binding in the absence of any added compound (~0.8–1.6 pmol) was set 100%.

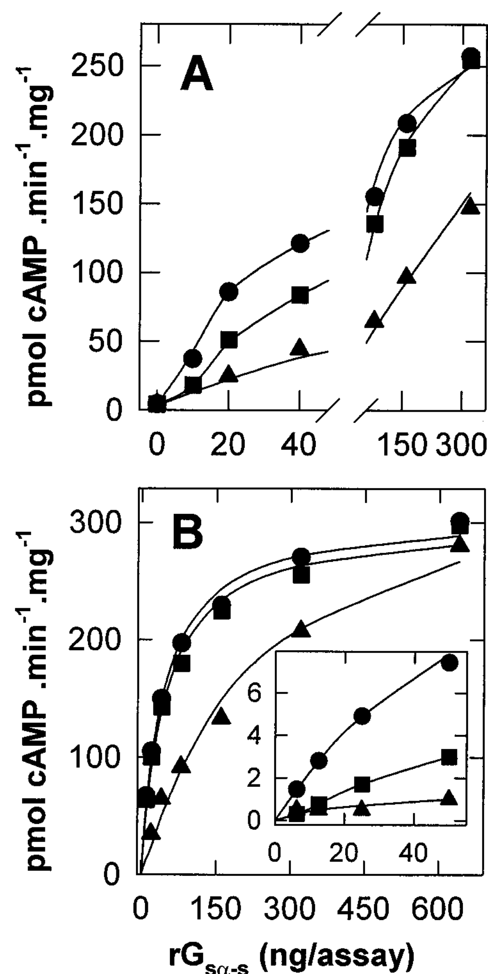

FIG. 2. Reconstitution of the adenylyl cyclase activity in $G_{s\alpha}$-deficient S49 cyc$^-$ membranes with $rG_{s\alpha\text{-s}}$ (*A*) or $rG_{s\alpha\text{-s}}$ preactivated with GTP[γS] (*B*). (*A*) cyc$^-$ membranes (12.5 μg) were preincubated with the indicated amounts of $rG_{s\alpha\text{-s}}$; cAMP formation was started by adding a prewarmed substrate solution containing 1 μM GTP[γS] in the absence (●) and presence of 10 μM NF449 (▲) or of 10 μM NF503 (■). (*B*) $rG_{s\alpha\text{-s}}$ was preactivated as described under *Materials and Methods*; appropriate dilutions were added to cyc$^-$ membranes; the assay was done as described for *A*. (*Inset*) cAMP formation was determined with a substrate solution containing 10 μM GTP.

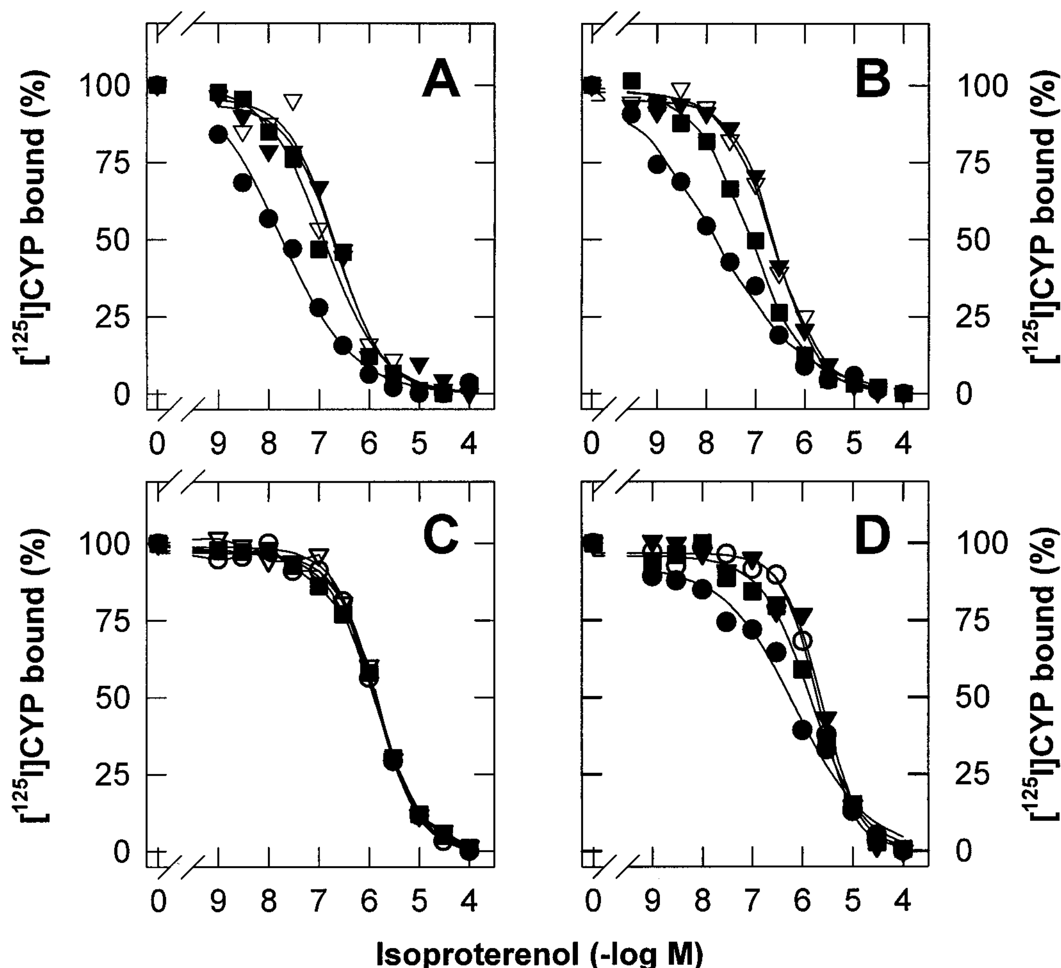

FIG. 3. Competition of isoproterenol for [^{125}I]CYP binding to rat cardiac membranes (*A* and *B*) and S49 cyc$^-$ membranes (*C* and *D*). (*A* and *B*) Cardiac membranes (10 μg) were incubated with 75 pM of [^{125}I]CYP and isoproterenol in the absence (●) and presence of 100 μM suramin (■, *A*), 10 μM NF503 (■, *B*), 100 μM GTP[γS] (▽), the combination of GTP[γS] + suramin (▼, *A*), or GTP[γS] + NF503 (▼, *B*). (*C* and *D*) S49 cyc$^-$ membranes (~3 μg/25 μl) were preincubated in the absence (C; ○, *D*) or presence of 3 pmol of rG$_{s\alpha\text{-s}}$ and 10 pmol of $\beta\gamma$ dimer (●, ▼, and ■, *D*). The reaction was started with 75 μl of buffer containing [^{125}I]CYP (to give 150 pM) and isoproterenol in the absence (○, ●) or presence of 100 μM GTP[γS] (▽, *C*; ▼, *D*) or of 10 μM NF503 (■). Specific binding in the absence of isoproterenol (~1.2 and 1.4 fmol of [^{125}I]CYP bound in cardiac and cyc$^-$ membranes, respectively) was set 100%.

rG$_{s\alpha\text{-s}}$ was diluted in buffer lacking GTP[γS] and $MgSO_4$. S49 cyc$^-$ membranes (12.5 μg) were preincubated with rG$_{s\alpha\text{-s}}$ in 20 μl for 20 min on ice; the reaction was started by adding 30 μl of substrate solution to yield (in mM) 50 Hepes·NaOH, pH 7.6, 1 EDTA, 0.1 DTT, 0.05 [α-^{32}P]ATP (~400 cpm/pmol), 9 $MgCl_2$, 1 $MgSO_4$, 1 μM GTP[γS] or 10 μM GTP in the absence and presence of 10 μM NF449 or of 10 μM NF503. The incubation lasted for 30 min at 20°C.

Experiments were carried out in duplicate; if not otherwise indicated, representative experiments are shown, which were repeated at least twice.

RESULTS AND DISCUSSION

G Protein Selectivity. The association rate of [^{35}S]GTP[γS] binding to G protein α subunits is determined by the release of prebound GDP, which is the rate-limiting step in G protein activation (22, 23). The inhibitory effect of compounds on the initial rate of [^{35}S]GTP[γS] binding to G$_\alpha$ subunits can thus be used as experimental readout to screen for G protein inhibitors. In our previous work, we had only investigated suramin analogues of varying size (6). We have expanded our search by testing analogues, which are substituted with sulfonates at distinct positions of different aromatic rings, on rG$_{s\alpha\text{-s}}$ and rG$_{i\alpha\text{-1}}$. This approach led to the identification of NF449 and of NF503, which suppressed the rate of GTP[γS] binding to rG$_{s\alpha\text{-s}}$ while barely affecting binding to rG$_{i\alpha\text{-1}}$ (Fig. 1). NF449, which has eight negative charges, was more potent (IC_{50} = 0.14 ± 0.04 μM); NF503 is nevertheless an interesting compound because it is a reasonably good inhibitor of rG$_{s\alpha\text{-s}}$ (IC_{50} = 3.1 ± 0.9 μM) but only carries two negative charges.

G$_{s\alpha}$-Dependent Stimulation of Adenylyl Cyclase in the Presence of NF449 and of NF503. Because NF449 and NF503 inhibit the association rate for the binding of GTP[γS], they are expected to reduce the G protein-dependent regulation of an effector. G$_{s\alpha}$-dependent effector regulation was assessed in the cyc$^-$ reconstitution assay. S49 cyc$^-$ cells lack G$_{s\alpha}$; their membranes are therefore devoid of basal adenylyl cyclase activity; addition of active G$_{s\alpha}$ restores enzymatic activity. Three different assay conditions were used (Fig. 2). (*i*) Inactive rG$_{s\alpha\text{-s}}$ was added to the membranes, and the reaction was started by the concomitant addition of substrate (ATP) and GTP[γS] in the absence and presence of NF503 or of NF449 (Fig. 2*A*). Under these conditions, GTP[γS]-liganded rG$_{s\alpha\text{-s}}$ is formed over the time course of the assay, and this leads to adenylyl cyclase stimulation. A delay in the release of GDP (and binding of GTP[γS]) results in a reduced activation of adenylyl cyclase by rG$_{s\alpha\text{-s}}$; a stronger relative inhibition is expected at low concentrations of rG$_{s\alpha\text{-s}}$ where the amount of active protein is limiting. At high concentration of G$_{s\alpha}$, the amount of

GTP[γS]-liganded $G_{s\alpha}$ that has been formed will eventually suffice to cause full enzyme activation. As can be seen in Fig. 2*A*, these observations were made. In addition, the effect of NF449 was more pronounced than that exerted by NF503; this is consistent with the substantially higher affinity of NF449 for $rG_{s\alpha\text{-s}}$ (see Fig. 1). (*ii*) If $rG_{s\alpha\text{-s}}$ is first preincubated with GTP[γS] in the presence of Mg^{2+}, the rate-limiting GDP–GTP[γS] exchange reaction is eliminated, and the apparent affinity of $rG_{s\alpha\text{-s}}$ for adenylyl cyclase increases (*cf.* control curve in Fig. 2 *A* and *B*). Under these assay conditions, the effect of NF503 was abolished, whereas that of NF449 was greatly reduced (Fig. 2*B*). This decrease in the efficacy of suramin analogues is to be expected for the following reasons. Because suramin analogues augment the affinity of GDP for the α-subunit, GDP-bound G_α is predicted to have a higher affinity for suramin analogues than GTP[γS]-bound G_α. This was verified by measuring the affinity of GDP-liganded (K_d = 0.17 ± 0.06 μM) and of GTP[γS]-liganded $rG_{s\alpha\text{-s}}$ (K_d = 1.24 ± 0.06 μM) for [^{3}H]suramin by equilibrium dialysis. In contrast, GTP[γS]-bound $rG_{s\alpha\text{-s}}$ has a higher affinity for the effector. Finally, the inhibitory effect of suramin on the binding of [^{35}S]GTP[γS] to $rG_{s\alpha\text{-s}}$ is reversed by the addition of purified adenylyl cyclase, indicating that the binding site for suramin analogues overlaps with the effector binding region on the G protein α subunit (6). (*iii*) If $rG_{s\alpha\text{-s}}$ was activated by GTP (Fig. 2*B*, *Inset*), the inhibitory effect of NF503 and of NF449 was more pronounced than that observed in the presence of GTP[γS]. This is due to the fact that, in the presence of GTP, stimulation of cAMP formation by $G_{s\alpha}$ involves multiple rounds of GDP release, which are subject to inhibition by the suramin analogues. The stimulation of adenylyl cyclase via the β_2-adrenergic receptor (endogenous to the cyc$^-$ membranes) was also profoundly suppressed (data not shown).

Importantly, the maximal activity (V_{max}) of adenylyl cyclase was not altered by NF503 or NF449. Hence, in contrast to suramin, which blocks cAMP formation directly at ≥10 μM (6), NF503 and NF449 do not affect catalysis *per se*. Similarly, an effect on G_i-mediated inhibition of adenylyl cyclase is also unlikely; adenylyl cyclase activity in cyc$^-$ membranes is inhibited by G_i even in the absence of $G_{s\alpha}$ (24); G_i is activated by GTP[γS] in the cyc$^-$ reconstitution assay. Any reversal of G_i-dependent inhibition by NF503 or NF449 ought to have been detectable as increase in V_{max} (14). We have also assessed GTP[γS]-dependent inhibition of forskolin-stimulated adenylyl cyclase, which was unaffected (e.g., EC_{50} = 42 ± 21 and 45 ± 17 nM, maximal inhibition = 29 ± 4 and 27 ± 4% for GTP[γS] in the absence and presence of NF503, respectively); this is predicted from the inability of the compounds to modify the association rate of GTP[γS] to $rG_{i\alpha\text{-}1}$ (see Fig. 1).

Uncoupling of β-Adrenergic Receptors from G_s by Suramin Analogues. Suramin analogues disrupt the interaction between receptors and G proteins (7–9). Hence, NF503 and NF449 ought to impede the interaction of G_s-coupled receptors with their cognate G protein in membranes. To address this question, we have used the β-adrenergic receptors, an extensively characterized paradigm (25). The receptors in rat cardiac membranes and in S49 cyc$^-$ membranes were labeled with the antagonist [^{125}I]CYP. The suramin analogues did not interfere with the ligand binding pocket of the receptor as they did not inhibit [^{125}I]CYP binding; in saturation experiments with rat cardiac membranes, a modest increase in B_{max} was observed (~20%; e.g., K_d = 9 ± 2 and 9 ± 2 pM, B_{max} = 149 ± 6 and 181 ± 11 fmol/mg in the absence and presence of 100 μM NF503, respectively). This is consistent with uncoupling of the receptor from the G protein, as antagonists bind preferentially to the receptor that is uncoupled from its G protein (26, 27). In contrast, binding of [^{125}I]CYP to cyc$^-$ membranes remained unaffected (e.g., K_d = 6.0 ± 2.3 and 6.2 ± 2.0 pM, B_{max} = 682 ± 60 and 707 ± 55 fmol/mg in the absence and presence of 100 μM NF503, respectively).

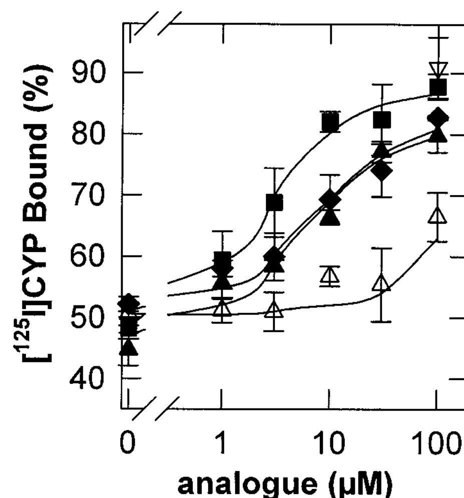

FIG. 4. Structure–activity relation of suramin analogues for reversing the isoproterenol-induced inhibition of [^{125}I]CYP binding to rat cardiac membranes. Rat cardiac membranes (12 μg) were incubated with 75 pM of [^{125}I]CYP in the absence and presence of suramin (●), NF503 (■), NF449 (▲), NF445 (△), and 100 μM GTP[γS] (▽). At each concentration of the compounds, specific[^{125}I]CYP binding was determined in the absence and presence of 30 nM isoproterenol, and the ratio of these two values is given as % (to account for the ~1.2-fold increase in [^{125}I]CYP binding induced by the suramin analogues and GTP[γS]). Data are means of four independent experiments; error bars indicate SD.

As expected, the agonist isoproterenol competed for binding of [^{125}I]CYP to rat cardiac membranes with a shallow curve (Fig. 3 *A* and *B*); with an appropriate curvilinear regression, two sites can be resolved that bind the agonist with high and with low affinity (~50% each in the experiments shown in Fig. 3 *A* and *B*). The high-affinity sites reflect the agonist-specific ternary complex (agonist/receptor/G protein), whereas the low affinity sites correspond to the interaction of the agonist with the uncoupled form of the receptor. Addition of GTP[γS] allows the receptor to complete the cycle of G protein activation (22, 23); the activated α subunit is formed, which leads to dissociation of the G protein subunits and, hence, of the ternary complex. This is reflected by a rightward shift and a steep slope of the agonist competition curve (Fig. 3 *A* and *B*). Addition of 100 μM suramin (Fig. 3*A*) or of 10 μM NF503 (Fig. 3*B*) mimicked the effect of GTP[γS]; the combination of GTP[γS] + suramin (Fig. 3*A*) or GTP[γS] + NF503 (Fig. 3*B*) did not cause an additional shift. This indicated that suramin and NF503 uncoupled the receptor from the G protein. This interpretation was verified by carrying out similar experiments with S49 cyc$^-$ membranes. In the absence of added $rG_{s\alpha\text{-s}}$, all competition curves were steep and superimposable irrespective of whether the assay was done in the absence or presence of GTP[γS] or of NF503 (Fig. 3*C*). Addition of $rG_{s\alpha\text{-s}}$ (in combination with βγ dimers) reconstituted high-affinity binding of isoproterenol (Fig. 3*D*), which was abolished by GTP[γS] (Fig. 3*D*), NF503 (Fig. 3*D*), and suramin (not shown). This showed that agonist binding to β-adrenergic receptors was only affected by the suramin analogues if the receptors were allowed to couple to G_s.

At 30 nM, isoproterenol displaced about 50% of [^{125}I]CYP binding to rat cardiac membranes in the absence of GTP[γS] (or of suramin), whereas only a modest inhibition is observed in the presence of GTP[γS] (see Fig. 3 *A* and *B*). Thus, reversal of the isoproterenol-induced displacement by suramin analogues was employed as an assay to determine their structure–activity relation in uncoupling the cardiac β-adrenergic receptors from G_s. NF445, a structural analogue of NF449, was used as a negative control because of its low apparent affinity for $rG_{s\alpha\text{-s}}$ (~30% decrease in [^{35}S]GTP[γS] binding to $rG_{s\alpha\text{-s}}$ at 100 μM NF445). NF445 had only a minor effect on the displace-

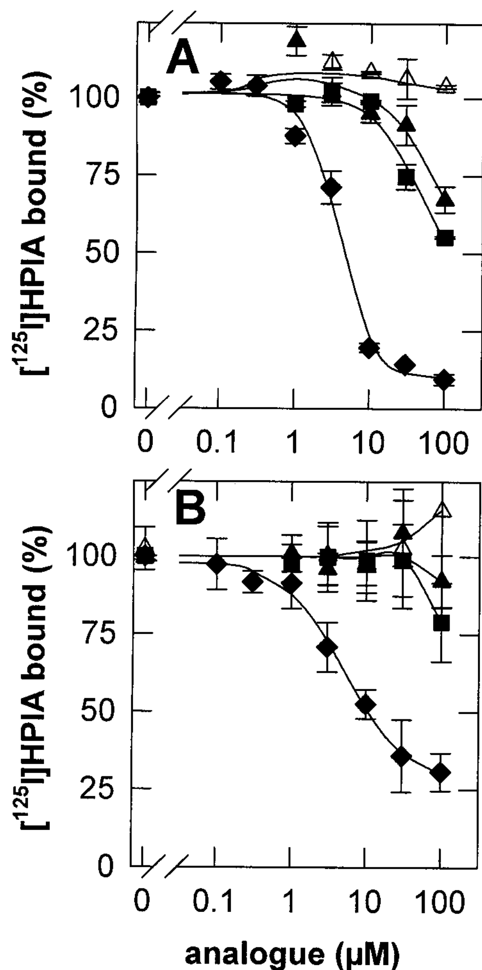

FIG. 5. Inhibition of [^{125}I]HPIA binding to membranes prepared from human cerebral cortex (*A*) and from 293 cell stably expressing the human A_1-adenosine receptor (*B*) by suramin analogues. Human brain membranes (~6–9 µg; *A*) or from 293 cell membranes (~12–15 µg; *B*) were incubated with 1 nM [^{125}I]HPIA in the absence and presence of suramin (◆), NF503 (■), NF449 (▲), and NF445 (△). Specific binding in the absence of any compound was set 100% and corresponded to ~1.6–1.8 and 1.4–1.7 fmol per assay for membranes from human brain and 293 cells, respectively. Data are means of three independent experiments; error bars indicate SD.

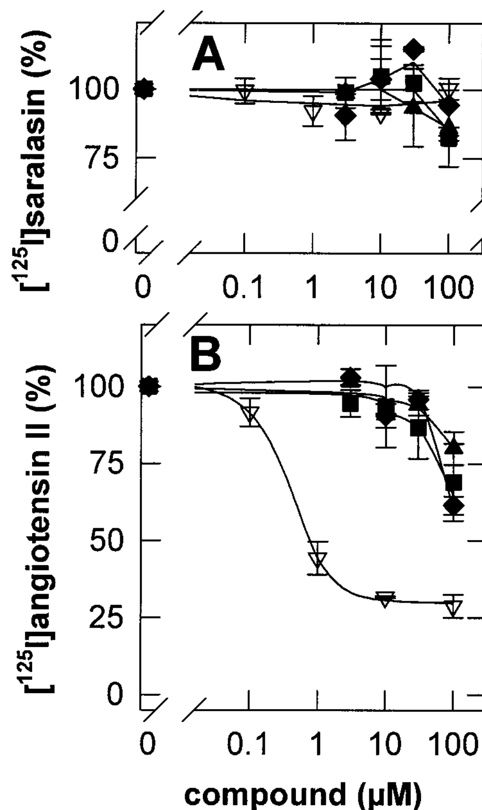

FIG. 6. Inhibition of [^{125}I]saralasin (*A*) and [^{125}I]angiotensin II binding (*B*) to membranes prepared from rat renal glomeruli by suramin analogues. Rat glomerular membranes (~5 µg) were incubated with 0.5 nM [^{125}I]saralasin (*A*) or 0.5 nM [^{125}I]angiotensin II (*B*) in the absence and presence of suramin (●), NF503 (■), NF449 (▲), and GTP[γS] (▽). Specific binding in the absence of any compound (~1.5 fmol bound ligand) was set 100%. Data are means of three independent experiments; error bars indicate SD.

ment of [^{125}I]CYP by isoproterenol (Fig. 4). NF503 was the most potent analogue (EC_{50} = 2.8 ± 0.8 µM) in suppressing high-affinity binding of isoproterenol and, its efficacy was comparable with GTP[γS] (Fig. 4). Suramin and NF449 (EC_{50} = 7.2 ± 0.4 and 7.9 ± 1.5 µM, respectively) were less potent than NF503, although NF449 (see Fig. 1) and suramin (IC_{50} ~0.2 µM; see ref. 6) are better inhibitors of the nucleotide exchange reaction of $rG_{s\alpha\text{-}s}$. This does not necessarily indicate that there are two binding sites for suramin analogues on $G_{s\alpha}$. In saturation experiments with [^{3}H]suramin, we observed 1:1 stoichiometry of binding to $rG_{s\alpha\text{-}s}$ (data not shown). Distinct rank orders of potencies were also noted previously if the ability of suramin analogues to suppress [^{35}S]GTP[γS] binding to $rG_{i\alpha\text{-}1}$ and $rG_{o\alpha}$ was compared with their effect on the interaction of $G_i/_o$-coupled receptors with their cognate G proteins (6, 9). These earlier and the present findings suggest that there is no strict correlation between the extent to which a suramin analogue can act as a "plug" preventing GDP release from the α subunit and as an inhibitor of receptor docking.

Effect of Suramin Analogues on High Affinity Agonist Binding to the G_i/G_o-Coupled A_1-Adenosine and the G_q-Coupled Angiotensin II-Type 1 Receptor. To verify that NF503 and NF449 selectively uncoupled G_s-coupled receptors, we determined their effect on high-affinity agonist binding to the A_1-adenosine receptor and to the angiotensin II type 1 receptor. The A_1-adenosine receptor is a prototypical G_i/G_o-coupled receptor (13, 18); suramin analogues do not inhibit binding of antagonists to the receptor (9). We used human brain membranes (Fig. 5*A*), where the A_1-adenosine receptor is expressed at high levels in the presence of abundant amounts of $G_{o\alpha}$, and membranes of 293 cells (Fig. 5*B*), which stably expressed the receptor and which contain only a modest amount of $G_{i\alpha}$ subtypes. In both membranes, suramin (Fig. 5) inhibited high affinity binding of the agonist [^{125}I]HPIA with an IC_{50} in the micromolar range. In contrast, the IC_{50} of NF503 (Fig. 5) and of NF449 were in the range of ≥100 µM. Hence, we conclude that NF449 and NF503 are ≥30-fold selective for the G_s-coupled β-adrenergic receptors and that suramin does not discriminate between ternary complexes formed by G_i/G_o-coupled receptors and by G_s-coupled receptors. The latter conclusion was also reached by Huang *et al.* (8) who found essentially no difference in the potency of suramin for uncoupling the human β_2- and α_2-adrenergic receptors.

Angiotensin II-type 1 receptors interact with α subunits of the G_i and G_q family (28, 29). In the renal cortex, angiotensin II type 1 receptors stimulate inositol trisphosphate formation in a pertussis toxin-insensitive manner (30) and are highly enriched in glomeruli (31); we have therefore labeled the angiotensin receptors in membranes prepared from rat renal glomeruli either with the antagonist [^{125}I]saralasin (Fig. 6*A*) or with the agonist [^{125}I]angiotensin II (Fig. 6*B*) and determined the effect of the suramin analogues on high-affinity agonist

binding. GTP[γS] was used as an internal control and had no effect on [^{125}I]saralasin binding (Fig. 6*A*) but potently reduced binding of the agonist (Fig. 6*B*). In contrast, suramin, NF449, and NF503 were ineffective up to 10 μM and modestly inhibited agonist binding at higher concentrations (Fig. 6*B*); however, at 100 μM, NF449 and NF503 also inhibited antagonist binding to some extent (by ~20%; see Fig. 6*A*). This suggests that inhibition of agonist binding at 100 μM was at least in part due to an interaction of the compounds with the ligand binding pocket of the receptor. Regardless of the mechanism underlying the inhibition, it was only seen at concentrations that were substantially higher than those required for uncoupling β-adrenergic receptors from G_s.

Taken together, our observations show that NF503 and NF449 are selective $G_{s\alpha}$ antagonists; the compounds may be useful as experimental tools. We believe that NF503, which has only two negative charges, is of interest as a lead compound in the search for cell-permeable G protein inhibitors. These are required to test the physiological implications of direct G protein inhibition. Domains are readily exchanged between $G_{i\alpha}$ and $G_{s\alpha}$, and the chimeric proteins are functionally active (32, 33). Hence, the selectivity of NF449 may be exploited to map the binding site(s) of suramin analogues by determining which domain(s) of $G_{s\alpha}$ confer(s) NF449 sensitivity to $G_{i\alpha\text{-}1}$. Two G_α subunits ($G_{i\alpha\text{-}1}$ and transducin) have been crystallized, and five different conformations have been studied (for review see ref. 34). Hence, it ought to be possible to generate the information required for the design of G protein inhibitors that may eventually be relevant to human pharmacotherapy.

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