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Suramin Analogues as Subtype-Selective G Protein Inhibitors

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SUMMARY

G protein α subunits expose specific binding sites that allow for the sequential, conformation-dependent binding of protein reaction partners, e.g., G protein $\beta\gamma$ dimers, receptors, and effectors. These domains represent potential sites for binding of low-molecular-weight inhibitors. We tested the following suramin analogues as G protein antagonists: 8-(3-nitrobenzamido)-1,3,5-naphtalenetrisulfonic acid (NF007), 8-(3-(3-nitrobenzamido)benzamido)-1,3,5-naphtalenetrisulfonic acid (NF018), 8,8′-(carbonylbis(imino-3,1-phenylene))bis-(1,3,5-naphtalenetrisulfonic acid) (NF023), 8,8′-(carbonylbis(imino-3,1-phenylene)carbonylimino-(3,1-phenylene))bis-(1,3,5-naphtalenetrisulfonic acid) (NF037), and suramin. The compounds suppressed [^{35}S]GTPγS binding to purified, recombinant G protein α subunits, an effect that is due to inhibition of GDP release. Suramin is selective for recombinant $G_{s\alpha\text{-}s}$ (EC_{50} values of ~240 nM; rank order of potency, suramin > NF037 > NF023 > NF018 > NF007), whereas NF023 is selective for recombinant $G_{i\alpha\text{-}1}$ and recombinant $G_{o\alpha}$ (EC_{50} value of ~300 nM; rank order of potency, NF023 ≥ NF037 > suramin > NF018 > NF007). Selectivity was also demonstrated on a cellular level. In rat sympathetic neurons, α_2-adrenergic and muscarinic receptor-dependent inhibition of the voltage-sensitive calcium current is mediated by G_i/G_o, whereas inhibition by vasoactive intestinal peptide (VIP) is mediated by G_s. Calcium current inhibition by α_2-adrenergic and muscarinic receptors was greatly reduced when 100 μM NF023 was applied intracellularly, whereas the response to VIP was unaffected; in contrast, the response to VIP was blunted only with 100 μM suramin in the recording pipette. The suramin analogues do not interfere with the interaction between α subunits and G protein $\beta\gamma$ dimer but compete with binding of the effector. The addition of purified adenylyl cyclase reverses the inhibitory effect of suramin on the rate of [^{35}S]GTPγS binding to recombinant $G_{s\alpha\text{-}s}$, indicating direct competition for a common site; similarly, immunoprecipitation by an antibody directed against an epitope of the effector binding site is inhibited by suramin. Our results show that it is possible to design G protein inhibitors that target the effector binding site on the α subunits.

The basic mechanism of G protein-mediated signal transduction is understood in considerable detail (1). During the cycle of activation and deactivation, the G protein α subunits interact with a series of ligands, including the appropriate receptor that catalyzes the release of prebound GDP; GTP, which binds to the empty guanine nucleotide pocket; magnesium, which is required for activation; the effector molecule, which propagates the biological signal; and the G protein $\beta\gamma$ dimer, with which the α subunit reassociates after hydrolytic cleavage of GTP. Additional proteins, such as components of the cytoskeleton, also interact with G proteins (2). Thus, several specific binding sites exist on the G protein α subunits that may be exploited for the design of synthetic inhibitory or stimulatory ligands. In a preceding study, we targeted the guanine nucleotide binding pocket of G protein α subunits as a potential binding site for a G protein antagonist. We characterized the 2′,3′-dialdehyde analogue oGTP as an irreversible G protein antagonist (3, 4). Although this compound and related analogues are useful experimental tools, they probably block other GTP-dependent processes, e.g., vesicular traffic, protein and RNA synthesis, cell growth, and cytoskeletal dynamics. A high degree of homology exists within the guanine nucleotide binding pocket of individual families of G proteins (5, 6). Modified guanine nucleotide analogues are thus unlikely to be useful in the search for selective G protein inhibitors.

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ABBREVIATIONS: NF007, 8-(3-nitrobenzamido)-1,3,5-naphtalenetrisulfonic acid; NF018, 8-(3-(3-nitrobenzamido)benzamido)-1,3,5-naphtalenetrisulfonic acid; NF023, 8,8′-(carbonylbis(imino-3,1-phenylene))bis-(1,3,5-naphtalenetrisulfonic acid); NF037, 8,8′-(carbonylbis(imino-3,1-phenylene)carbonylimino-(3,1-phenylene))bis-(1,3,5-naphtalenetrisulfonic acid; VIP, vasoactive intestinal peptide; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; HEDL, HEPES·NaOH/EDTA/dithiothreitol/Lubrol buffer; HED, HEPES·NaOH/EDTA/dithiothreitol buffer; UK 14,304, 5-bromo-*N*(4,5-dihydro-1*H*-imidazol-2-yl)-6-quinoxalinamine.

The peptide wasp venom mastoparan is known to directly activate G proteins of the G_o/G_i class (7) and thus acts as a receptomimetic. Similarly, short peptides, derived in particular from the third intracellular loop of various G protein-coupled receptors, stimulate the guanine nucleotide exchange reaction of purified G proteins (8–14). Appropriate substitutions lead to inhibitory peptides (15). Although the precise structural determinants are still a matter of debate, an excess of positive charges and a lipophilic surface seem to be important. This assumption is supported by the observation that benzalkonium as well as other amphiphilic compounds characterized by a positive charge and an aromatic ring can directly activate G proteins (9, 15). The trypanocidal drug suramin contains several aromatic rings and is highly negatively charged. Furthermore, suramin suppresses guanine nucleotide-dependent activation of adenylyl cyclase (16) and receptor-dependent stimulation of GTPase activity in membranes (17) as well as formation of the high affinity ternary complex formed by agonist, receptor, and G protein (18). Suramin may thus represent a starting point in the search for direct G protein inhibitors. In the present study, we therefore tested several suramin analogues for an inhibitory action on defined G protein α subunits. Using three G protein α subunits ($G_{s\alpha\text{-}s}$, $G_{i\alpha\text{-}1}$, and $G_{o\alpha}$), which spontaneously exchange guanine nucleotides at measurable rates, we showed that a direct interaction occurs with the effector binding domain on the G protein α subunit and that subtype-selective compounds exist with affinities in the submicromolar range.

Experimental Procedures

Materials. [^{35}S]GTPγS, [α-^{32}P]GTP, and [α-^{32}P]ATP were purchased from NEN (Boston, MA); nucleotides and adenosine deaminase were purchased from Boehringer Mannheim (Mannheim, Germany); forskolin, rolipram, and protein A-Sepharose were purchased from Sigma Chemical Co. (St. Louis, MO); and nitrocellulose BA85 filters were purchased from Schleicher & Schuell. Suramin was a generous gift from Bayer AG (Wuppertal, Germany); the synthesis of the suramin analogues NF007, NF018, NF023, and NF037 has been described previously (19). The structure of suramin is given in Figure 1.

Membrane preparation and protein purification. Human platelet membranes were prepared as described (4). Recombinant $G_{s\alpha\text{-}s}$ was expressed in *Escherichia coli* and purified from bacterial lysates (20); myristoylated recombinant $G_{i\alpha\text{-}1}$ and recombinant $G_{o\alpha}$ were obtained as described previously (21). G protein $\beta\gamma$ dimer were purified from bovine brain membranes and chromatographically resolved from α subunits (22). Adenylyl cyclase was purified from bovine brain membranes by affinity chromatography on forskolin/agarose according to the method of Pfeuffer *et al.* (23) with modifications as outlined in Graziano *et al.* (20).

[^{35}S]GTPγS and [α-^{32}P]GDP binding. Binding of GTPγS to G protein α subunits (1–3 pmol) was carried out in 30–50 μl of HEDL (consisting of 50 mM HEPES·NaOH, pH 7.6, 1 mM EDTA, 1 mM dithiothreitol, 0.01% Lubrol) containing 10 mM $MgSO_4$ and 1 μM [^{35}S]GTPγS (20–100 cpm/fmol) in the absence and presence of suramin and its analogues at 20° (30° for $G_{i\alpha\text{-}1}$). In experiments in which the effect was determined of purified adenylyl cyclase on the kinetics of [^{35}S]GTPγS binding, the concentration of [^{35}S]GTPγS was 0.1 μM (200–1000 cpm/fmol), and 0.1–0.2 pmol of recombinant $G_{s\alpha\text{-}s}$ was used. Purified adenylyl cyclase was stored in HED (50 mM HEPES·NaOH, pH 7.6, 1 mM EDTA, 1 mM dithiothreitol) containing 0.05% Lubrol and 200 mM KP_i, pH 8. At the highest concentration of adenylyl cyclase used (Fig. 2B), Lubrol and KP_i were diluted ≥12-fold by carry-over into the [^{35}S]GTPγS binding reaction mixture. At the time points indicated in the figure legends, the reaction was stopped by the addition of 2 ml of ice-cold buffer (consisting of 10 mM Tris·HCl, pH 8.0, 25 mM $MgCl_2$, 100 mM NaCl, 0.1 mM GTP). Bound [^{35}S]GTPγS was trapped on BA85 filters, which were washed with 15 ml of GTP-free stop buffer and quantified with the use of liquid scintillation counting. For the determination of [α-^{32}P]GDP release, G protein α subunits (1–2 pmol/assay) were prelabeled in the presence of 1 μM [α-^{32}P]GTP (30 cpm/fmol) for 30 min at 20° in HEDL plus 10 mM $MgSO_4$. Because the catalytic rate of GTP hydrolysis exceeds the rate of GDP release by a factor of >10 (20), the nucleotide

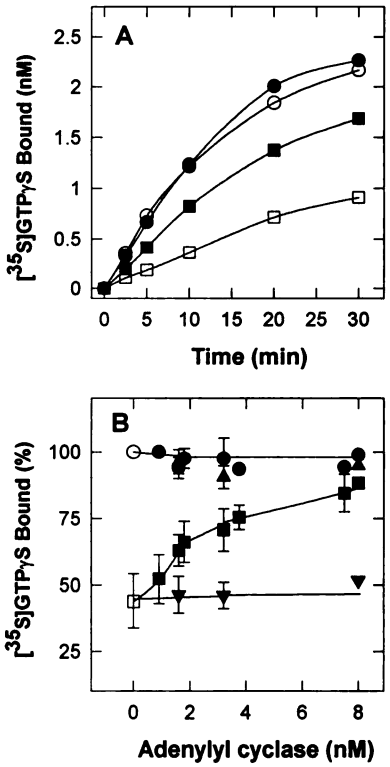

Fig. 2. Reversal of the inhibitory effect of suramin on the association rate of [^{35}S]GTPγS binding to recombinant $G_{s\alpha\text{-}s}$ by purified bovine brain adenylyl cyclase. A, Recombinant $G_{s\alpha\text{-}s}$ (0.13 pmol) was incubated in the absence (○, □) and presence (●, ■) of purified bovine brain adenylyl cyclase (0.15 pmol) in HED containing 0.1% Lubrol for 30 min on ice in a final volume of 10 μl. The binding reaction was initiated by the addition of 20 μl of HED prewarmed to 20° and containing 0.75 mM ATP, 7.5 mM $MgSO_4$, 0.15 μM [^{35}S]GTPγS (200 cpm/fmol; ○, ●) or the combination of [^{35}S]GTPγS and 1.5 μM suramin (□, ■). B, recombinant $G_{s\alpha\text{-}s}$ (0.1–0.15 pmol) was preincubated in the absence (○, □) or presence of increasing concentrations of adenylyl cyclase (●), ■) or heat-denatured adenylyl cyclase (10 min at 70°; ▲, ▼), and the binding reaction was initiated as described for A. The incubation was carried out in the absence (○, ●, ▲) and presence (□, ■, ▼) of 1 μM suramin for 15 min at 20°. Control binding in the absence of any addition was set at 100% to account for differences in the amounts of recombinant $G_{s\alpha}$ used in individual experiments. Data are mean values from three experiments carried out in duplicate; *error bars*, standard deviation.

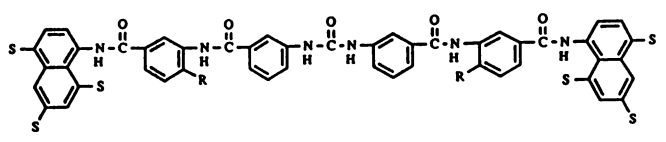

Fig. 1. Structure of suramin (S═SO_3Na; R═CH3 in suramin and H in all analogues. NF037 is didemethylated suramin. NF023 corresponds to NF037, but lacks two benzamide rings on each side of the central urea bridge. NF007 and NF018 are "half-molecules" of NF023 and NF037, respectively.

bound at equilibrium is [α-^{32}P]GDP. Dissociation was subsequently initiated by the addition of 100 μM unlabeled GTP in the presence or absence of suramin. At the time points indicated in Fig. 3, the reaction was quenched by the addition of buffer consisting of 10 mM Tris·HCl, pH 8.0, 25 mM $MgCl_2$, 100 mM NaCl, 0.01 mM NaF, and 20 μM $AlCl_3$; the samples were processed as described for the binding of [^{35}S]GTPγS.

Determination of adenylyl cyclase activity. Adenylyl cyclase activity in human platelet membranes was assayed in a 0.1-ml reaction containing 50 mM HEPES-NaOH, pH 8.0, 0.05 mM [α-^{32}P]ATP (~200 cpm/pmol), 10 mM $MgCl_2$, 0.1 mM rolipram, 10 mM creatine phosphate, 1 mg/ml creatine kinase, 5 μg/ml adenosine deaminase, concentrations of suramin analogues and GTPγS as indicated in Figs. 4 and 5, and 10–40 μg of membrane protein. Inhibitory regulation of adenylyl cyclase was determined in the presence of 10 μM forskolin and 2 mM $MgCl_2$. If not indicated otherwise, the incubation was terminated after 20 min at 25°, and [^{32}P]cAMP was separated with the use of double-column chromatography (24). The activity of purified adenylyl cyclase was determined as described previously (25). Recombinant $G_{s\alpha\text{-}s}$ was incubated with 10–50 ng of adenylyl cyclase for 30 min on ice in 10 μl of HEDL (0.1% Lubrol); afterward, the reaction was started by the addition of 40 μl of prewarmed substrate solution containing 0.6 mM[α-^{32}P]ATP (20 cpm/pmol) and 10 mM $MgSO_4$ and carried out for the time intervals shown in Fig. 6 at 30°.

Immunoprecipitation. Recombinant $G_{s\alpha\text{-}s}$ (50–200 ng/assay) was preactivated for 30 min at 20° in 25 μl of buffer containing 50 mM HEPES-NaOH, pH 7.6, 1 mM EDTA, 10 mM $MgSO_4$, 0.1 mM GTPγS, and 0.01% Lubrol; thereafter, 5 μl of buffer containing 0.1–2 μg of affinity-purified antiserum 584 (directed against amino acids 311–325 of $G_{s\alpha\text{-}s}$; Ref. 26) or CS1 (directed against the carboxyl-terminal decapeptide of $G_{s\alpha}$; Ref. 27) was added, and the volume was adjusted with buffer to maintain a constant concentration of recombinant $G_{s\alpha\text{-}s}$ (1 ng/μl). The incubation was continued in the absence and presence of 10 μM suramin for 2 hr on ice. Control incubations with heat-denatured recombinant $G_{s\alpha\text{-}s}$ (15 min at 60°) were carried out to rule out nonspecific effects of suramin. Subsequently, protein A-Sepharose was added, and the immunoprecipitate was recovered by centrifugation after 2 hr on ice. After three washes, the bound proteins were released by being boiled in Laemmli sample buffer (50 mM Tris·HCl, pH 6.8, 10% glycerol, 0.1% sodium dodecyl sulfate, 0.01% bromphenol blue) containing 80 mM dithiothreitol and applied to 9% sodium dodecyl sulfate-polyacrylamide gels. After electrophoretic transfer to nitrocellulose membranes, recombinant $G_{s\alpha\text{-}s}$ was visualized with the use of antiserum CS1 followed by a second antibody coupled to horseradish peroxidase with the use of the Amersham ECL reagents.

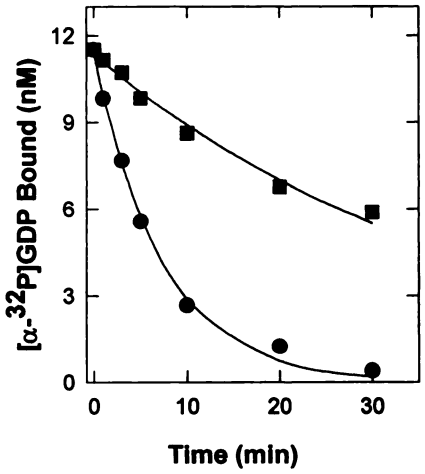

Fig. 3. Inhibition of [α-^{32}P]GDP release from recombinant $G_{s\alpha\text{-}s}$ by suramin. Recombinant $G_{s\alpha\text{-}s}$ (1.2 pmol/assay) was prelabeled in the presence of 1 μM [α-^{32}P]GTP (specific activity, 30 cpm/fmol) for 30 min at 20° in HEDL + 10 mM $MgSO_4$; dissociation was initiated (time = 0) by the addition of 100 μM unlabeled GTP in the absence (●) or presence (■) of 1 μM suramin. At the time points indicated, the reaction was quenched by the addition of stop buffer containing 20 μM $AlCl_3$ and 10 mM NaF. The on-rate for [^{35}S]GTPγS binding was determined in parallel and gave rates that were identical to the off-rate of GDP (0.14 and 0.02 min^{-1} in the absence and presence of 1 μM suramin, respectively). Data represent mean values from duplicate determinations.

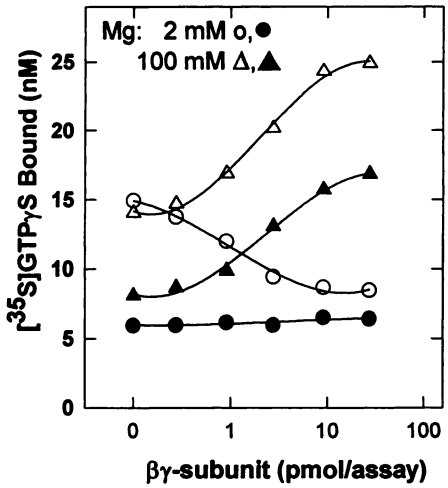

Fig. 4. Effect of the G protein βγ subunit on the rate of [^{35}S]GTPγS binding to recombinant $G_{o\alpha}$ in the presence of low and high magnesium. Recombinant $G_{o\alpha}$ (1 pmol/assay) was incubated in the presence of increasing concentrations of βγ dimer in a final volume of 30 μl of HEDL containing and 1 μM [^{35}S]GTPγS (specific activity, 20 cpm/fmol), 2 mM (○, ●), or 100 mM $MgSO_4$ (△, ▲) in the absence (○, △) and presence (●, ▲) of 20 μM NF023 for 2 min at 20°. Data represent mean values from duplicate determinations. Similar results were obtained with NF037, and a similar effect of NF023 was obtained with $G_{i\alpha\text{-}1}$.

Preparation of sympathetic neurons. Superior cervical ganglia were dissected from 2–6-day-old Sprague-Dawley rat pups and dissociated as described previously (28). Briefly, ganglia were freed from adhering connective tissue and blood vessels, cut into two pieces, and incubated in collagenase (1.5 mg/ml; No. 9891, Sigma) and dispase (3.0 mg/ml; No. 165859, Boehringer Mannheim) for 20 min at 36°. Subsequently, the ganglia were trypsinized (0.25% trypsin; No. 3703, Worthington) for 15 min at 36°, dissociated by trituration, and plated onto culture dishes coated with poly-D-lysine. The cells were used for patch-clamp recordings within 24 hr after plating.

Electrophysiological methods. The methods for the determination of the effects of suramin analogues on G protein-mediated inhibition of neuronal Ca^{2+} currents were essentially identical to the procedures used for the characterization of oGTP (4). In summary, whole-cell currents were recorded from neurons either without or with only a few, short processes at room temperature (20–24°) with use of the patch-clamp technique (29). Ca^{2+} currents were elicited by 30-msec depolarizing voltage steps from a holding potential of −80 mV to 0 mV, at a frequency of 4/min. The bathing solution consisted of 120 mM NaCl, 3.0 mM KCl, 5.0 mM $CaCl_2$, 2.0 mM $MgCl_2$, 20 mM glucose, and 10 mM HEPES-NaOH, pH 7.4, and contained 1 μM tetrodotoxin to block voltage-sensitive Na^+ channels. The internal solution contained 115 mM *N*-methyl-D-glucamine, 20 mM tetraethylammonium chloride, 1.6 mM $CaCl_2$, 10 mM EGTA, 10 mM glucose, 20 mM HEPES, 2 mM Mg-ATP, and 2 mM Li-GTP, adjusted to pH 7.3 with HCl, in the absence and presence of suramin or NF023. Receptor agonists (UK 14,304, VIP, and oxotremorine-M) were applied sequentially via the bathing solution for 30 sec. The effect of each agonist was evaluated sequentially (in a random order of agonist addition) on each cell with intermittent washout, thus allowing for direct comparison of suramin- and NF023-mediated effects on channel regulation by receptor agonists. Washout periods lasted 30 sec for

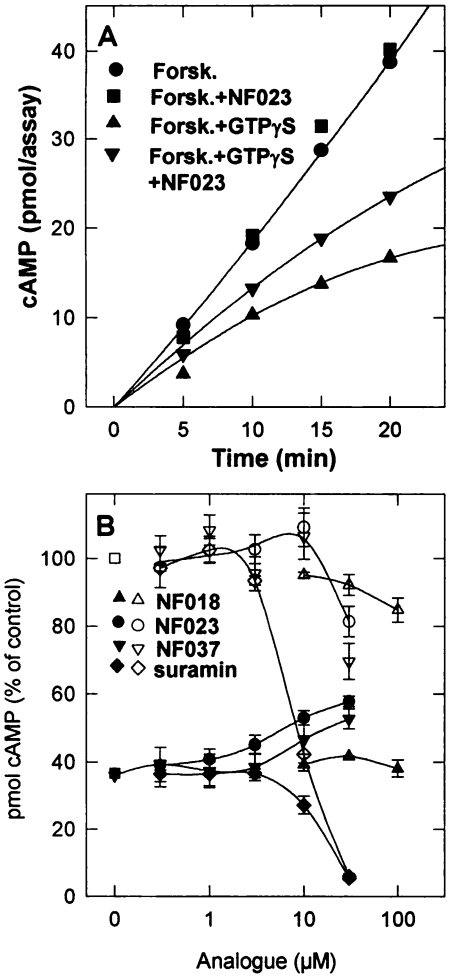

Fig. 5. Partial reversal of GTPγS-dependent adenylyl cyclase inhibition in human platelet membranes by NF023 and NF037. A, Platelet membranes (10 μg) were incubated in the presence of 10 μM forskolin (●), the combination of 10 μM forskolin plus 0.1 μM GTPγS (▲), 10 μM forskolin plus 10 μM NF023 (■), or 10 μM forskolin plus 0.1 μM GTPγS plus 10 μM NF023 (▼) at 25°. Data represent mean values from duplicate determinations. B, Platelet membranes (10 μg) were incubated with 10 μM forskolin in the absence (□) and presence of NF023 (○), NF037 (▽), suramin (◇), or NF018 (△). Inhibition of cAMP formation was measured in the presence of forskolin plus 0.1 μM GTPγS (■), a combination to which NF023 (●), NF037 (▼), suramin (◆), or NF018 (▲) was added. Forskolin-stimulated activity (225 ± 10 pmol of cAMP/mg/min) was set at 100%. Data are mean values from three experiments carried out in duplicate; *error bars*, standard deviation.

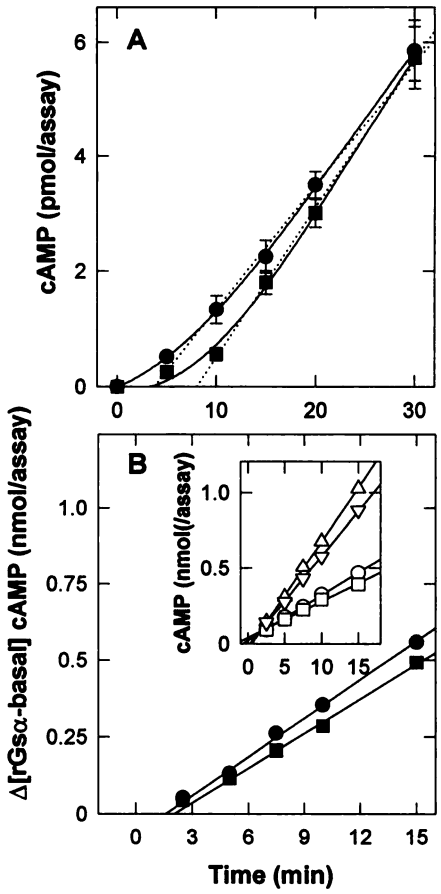

Fig. 6. A, Effect of suramin on the lag phase for GTPγS-dependent adenylyl cyclase activation in human platelet membranes. Platelet membranes (12 μg) were incubated with 10 μM GTPγS in the absence (●) and presence (■) of 1 μM suramin at 30°. At the time points indicated, the reaction was stopped, and the [^{32}P]cAMP that was formed was quantified. *Intercepts of the dotted lines with the time axis*, lag phase of activation in the absence and presence of suramin. Data are mean values from five experiments carried out in duplicate; *error bars*, standard deviation. B, Effect of suramin on the lag phase for activation of purified adenylyl cyclase by the combination of recombinant $G_{s\alpha\text{-}s}$ and GTPγS. Purified adenylyl cyclase (20 ng ≅ 0.15 pmol) was incubated in the absence (*inset*; ○, □) and presence (*inset*; △, ▽) of 2.5 pmol recombinant $G_{s\alpha\text{-}s}$ for 30 min on ice in 10 μl of HED containing 0.1% Lubrol. Thereafter, the reaction was started by the addition of 40 μl of prewarmed substrate solution containing 0.6 mM [α-^{32}P]ATP, 12.5 μM GTPγS (*inset*; △, ▽), and 1.25 μM suramin (*inset*; ▽, □). The difference is shown between recombinant $G_{s\alpha\text{-}s}$ plus GTPγS-stimulated and basal activity in the absence (●) and presence (■ of 1 μM suramin. Data are mean values from duplicate determinations.

oxotremorine-M, 90 sec for UK 14,304, and 150 sec for VIP as these periods of time guaranteed complete recovery from the agonist-induced inhibition. Currents were quantified through the measurement of peak current amplitudes. To account for the variable run-down of Ca^{2+} currents during the time course of a recording, currents obtained in the presence of agonists at α_2-adrenergic, muscarinic, or VIP receptors (*B*) were set in relation to control currents recorded before (*A*) and after (*C*) the application of receptor agonists according to the following equation: percent inhibition = 100 * {1 − 2*B*/(*A* + *C*)}.

Data analysis. Data from kinetic experiments were subjected to nonlinear, least-squares curve fitting with the use of equations describing a pseudo-first order association or monophasic exponential decay. Similarly, concentration-response curves were fitted to a three-parameter logistic equation for recombinant $G_{s\alpha\text{-}s}$ and a four-parameter logistic equation (with the fourth term describing residual binding in the presence of suramin analogues) for recombinant $G_{i\alpha\text{-}1}$ and for recombinant $G_{o\alpha}$. Coefficients of variation for the calculated parameters were 5–25%. If not indicated otherwise, each experiment was performed three times with at least two different protein preparations.

Results

Inhibition of guanine nucleotide exchange. The rate-limiting step for binding of GTP (or of the hydrolysis-resistant analogue GTPγS) to G protein α subunits is the release of prebound GDP. Suramin inhibited [^{35}S]GTPγS binding to purified G protein α subunits, an effect that was more pronounced for recombinant $G_{s\alpha\text{-}s}$ (Fig. 7A) than for recombinant $G_{o\alpha}$ (Fig. 7B) or recombinant $G_{i\alpha\text{-}1}$ (data not shown). We ruled out that this inhibition was due to competition for the gua-

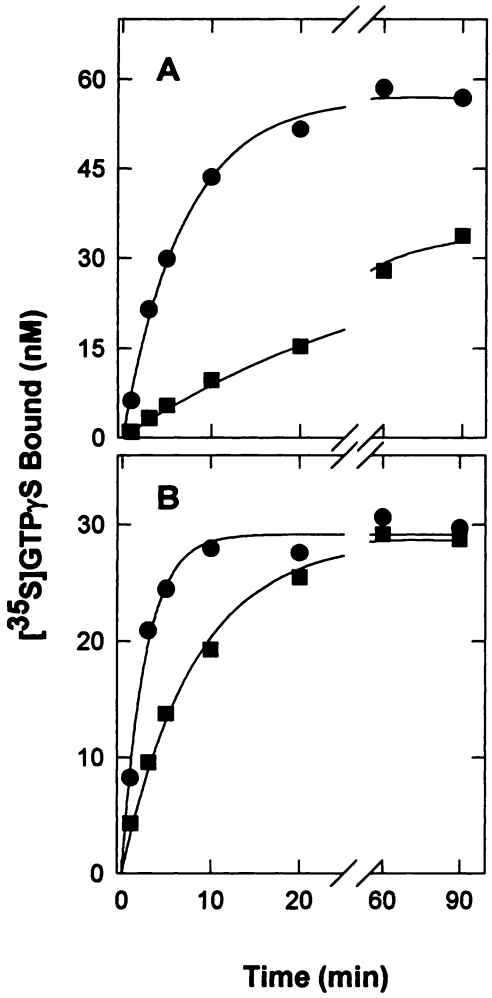

Fig. 7. Inhibition by suramin of [^{35}S]GTPγS binding to recombinant $G_{s\alpha\text{-}s}$ (A) and to recombinant $G_{o\alpha}$ (B). Purified recombinant $G_{s\alpha\text{-}s}$ (3 pmol/assay) and $G_{o\alpha}$ (1.5 pmol/assay) were incubated in a final volume of 50 μl of HEDL containing 10 mM $MgSO_4$ and 1 μM [^{35}S]GTPγS (specific activity, 20 cpm/fmol) in the absence (●) and presence (■ of 1 μM suramin at 20°. At the time points indicated, the reaction was stopped, and bound and free [^{35}S]GTPγS were separated over nitrocellulose filters. Data represent mean values from duplicate determinations.

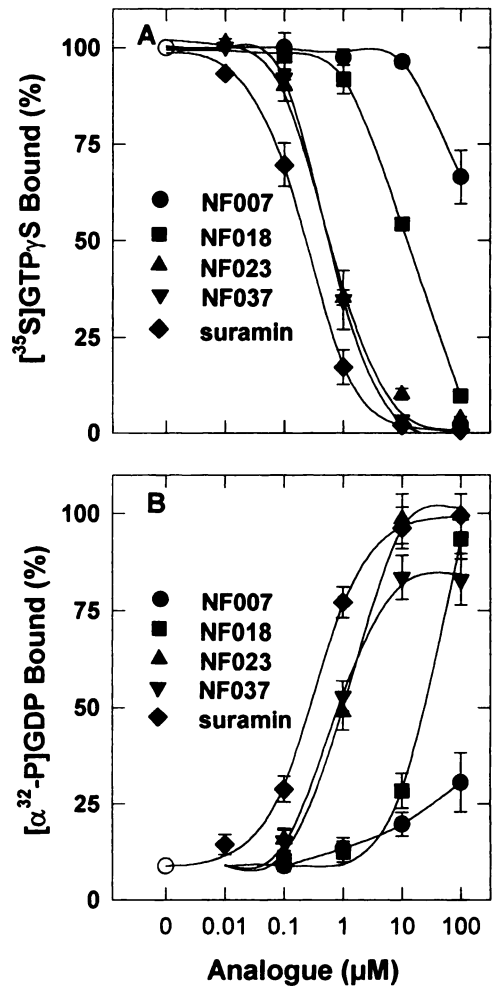

Fig. 8. Inhibition by suramin analogues of [^{35}S]GTPγS binding to $G_{s\alpha\text{-}s}$ (A) and of [α-^{32}P]GDP release from recombinant $G_{s\alpha\text{-}s}$ (B). Assay conditions were as outlined in the legends to Figs. 3 and 7. The reaction was allowed to proceed for 2 min (A) and 10 min (B) in the absence (○) and presence of the indicated concentrations of NF007 (●), NF018 (■), NF023 (▲), NF037 (▼), and suramin (◆). Data are mean values from three experiments performed in duplicate; *error bars*, standard deviation. To normalize for the different amount of proteins used in individual experiments, binding in the absence of any analogue was set at 100% (A), whereas binding in the presence of 100 μM suramin was set at 100% (B).

nine nucleotide binding site. This was directly verified by determining the rate of dissociation of prebound GDP after prelabeling of recombinant $G_{s\alpha\text{-}s}$ with [α-^{32}P]GDP in the presence and absence of suramin (Fig. 3). The calculated kinetic parameters clearly show that the ability of suramin to block the binding of [^{35}S]GTPγS (Fig. 7A) is fully accounted for by the suppression of GDP release. Similar results were obtained with $G_{o\alpha}$ (data not shown). We further tested this interpretation, i.e., that the ability of suramin to inhibit binding of GTPγS is due to the suppression of GDP release, by comparing the effect of various suramin analogues on both reactions. As can be seen from Fig. 8, the structure-activity relationship for the analogues used was identical regardless of which reaction was determined.

Potential G protein antagonists are of interest if they discriminate among G protein species. We therefore tested whether the structure-activity relationships for the suramin analogues used differed among G protein α subunits. These experiments are summarized in Fig. 9 and Table 1. Suramin was the most potent analogue when tested on recombinant $G_{s\alpha}$ (see Fig. 8). In contrast, the apparent affinity of suramin for recombinant $G_{i\alpha\text{-}1}$ was ~20-fold lower, and NF023 was the most potent analogue for recombinant $G_{i\alpha\text{-}1}$ and recombinant $G_{o\alpha}$. Apart from the difference in apparent affinity, the extent of inhibition was also dissimilar; for recombinant $G_{s\alpha\text{-}s}$, almost complete suppression of guanine nucleotide exchange was achieved with the suramin analogues, whereas the effect was less pronounced for recombinant $G_{i\alpha\text{-}1}$ and recombinant $G_{o\alpha}$. This discrepancy cannot be attributed to the differences in the intrinsic rates of guanine nucleotide exchange of the proteins because the rates of GTPγS binding to recombinant $G_{o\alpha}$. and recombinant $G_{i\alpha\text{-}1}$ are faster and slower, respectively, than that of recombinant $G_{s\alpha\text{-}s}$. In addition, the effect of suramin analogues is independent of the free Mg^{2+} concentration over a range of 0.1–100 mM (data not shown; see also Fig. 4).

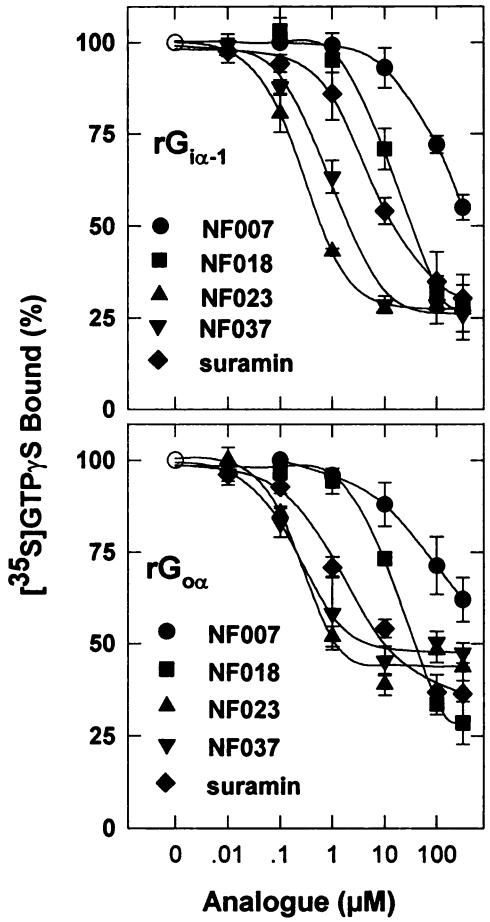

Fig. 9. Inhibition of [^{35}S]GTPγS binding to recombinant $G_{i\alpha-1}$ and recombinant $G_{o\alpha}$ by suramin analogues. Assay conditions were as outlined in the legends to Fig. 8 with the use of 2–4 pmol of purified α subunits/assay. The incubation lasted 1 and 15 min for recombinant $G_{o\alpha}$ and recombinant $G_{i\alpha-1}$, respectively, in the absence (○) and presence of the indicated concentrations of NF007 (●), NF018 (■), NF023 (▲), NF037 (▼), and suramin ([diaf). Data are mean values from three to five experiments performed in duplicate; *error bars*, standard deviation. Binding in the absence of any analogue was set at 100% to normalize for the different amount of proteins used in individual experiments.

Interaction with the βγ subunit in the presence of suramin analogues. The inhibitory effect on GDP release by suramin and its analogues is phenomenologically similar to the effect of the G protein βγ dimer, which increases the affinity of the G protein for GDP by a reduction in its dissociation rate (30). However, suramin and its analogues act on a site of the α subunit that is distinct from the domain interacting with the βγ dimer. This conclusion is based on the experiment depicted in Fig. 4. The addition of increasing concentrations of purified bovine brain βγ dimer to recombinant $G_{o\alpha}$ decreased the initial [^{35}S]GTPγS binding rate because the βγ subunit complex increases the affinity of the α subunit for prebound GDP (30). The addition of 20 μM NF023 results in maximal inhibition of GDP release, which cannot be inhibited further by the addition of βγ dimers. Under these conditions, it was not possible to differentiate between a common and a distinct site of action for these two ligands. However, in the presence of high magnesium concentrations, βγ dimer are known to accelerate rather than decrease the release of prebound GDP (30). This phenomenon can also be seen in Fig. 4. It was exploited to test whether βγ dimer and suramin analogues compete for a common site of action on the α subunit. Although NF023 still inhibited the basal rate of [^{35}S]GTPγS binding, it does not prevent the βγ subunit complex from accelerating the rate of [^{35}S]GTPγS binding in the presence of 100 mM magnesium, and half-maximum activation is observed at ~100 nM βγ dimer. Therefore, we conclude that suramin analogues and βγ dimer bind at distinct sites on the α subunit.

Effector regulation in the presence of suramin analogues. Suramin analogues inhibit the rate-limiting step in the activation of G protein α subunits, namely, the release of prebound GDP. They should thus interfere with G protein-dependent regulation of effectors in membranes and partially reverse the effect of guanine nucleotide. This was assessed by measuring the bidirectional regulation of adenylyl cyclase activity in human platelet membranes. NF023 did not significantly affect forskolin-stimulated adenylyl cyclase activity at a concentration of ≤10 μM, whereas concentrations of ≥30 μM were inhibitory (Fig. 5). This inhibition probably resulted from direct action of NF023 on the catalytic moiety as suramin analogues are known to inhibit ATP-binding enzymes (19). More importantly, however, NF023 partially reversed the GTPγS-induced inhibition of adenylyl cyclase in a concentration range in which the compound did not affect forskolin-stimulated activity (inhibition by 0.1 μM GTPγS and the combination of GTPγS and NF023 = 62 ± 2% and 40 ± 2% of the forskolin-stimulated activity, respectively; three experiments). Higher concentrations of NF037, which is less potent in interaction with $G_{i\alpha-1}$, were required to reverse the GTPγS-induced inhibition of adenylyl cyclase (Fig. 5B). The analogue NF018, which has a ~40-fold lower affinity for recombinant $G_{i\alpha-1}$ (see Table 1), failed to reverse the GTPγS-dependent inhibition at a concentration of ≤100 μM (Fig. 5B). At concentrations exceeding 3 μM, suramin per se strongly inhibited forskolin-stimulated adenylyl cyclase activity so that reversal of GTPγS-dependent inhibition by suramin could not be evaluated (Fig. 5B).

As mentioned above, NF023 did not completely suppress the guanine nucleotide exchange reaction of recombinant $G_{i\alpha-1}$ (see Fig. 9). Therefore, the fact that guanine nucleotide-dependent inhibition of platelet adenylyl cyclase was only partially reversed by NF023 was predictable. However, it is evident that substantially higher (~20-fold) concentrations of NF023 and NF037 were required for half-maximal reversal of adenylyl cyclase inhibition (EC_{50} = 6.02 ± 0.3 and 12.7 ± 2 μM for NF023 and NF037, respectively; three experiments) than for direct inhibition of GTPγS binding to recombinant $G_{i\alpha-1}$.

For the determination of guanine nucleotide-dependent stimulation of adenylyl cyclase, we used suramin as this was the most potent analogue in inhibiting guanine nucleotide exchange of recombinant $G_{s\alpha-s}$ (see Table 1). Activation of adenylyl cyclase by GTPγS and other hydrolysis-resistant analogues displays a characteristic hysteresis. This lag reflects the dissociation of GDP from $G_{s\alpha}$. As shown in Fig. 6A, 1 μM suramin only modestly prolonged the lag phase for GTPγS-induced adenylyl cyclase activation (1.6 ± 0.2-fold, five experiments). At a concentration of 1 μM, NF037 and NF023 were even less effective in prolonging the lag phase (1.3 ± 0.1-fold and 1.1 ± 0.2-fold, respectively; three experiments). This finding was in marked contrast to the profound inhibition seen on purified recombinant $G_{s\alpha-s}$ (see Fig. 7A),

TABLE 1

Inhibition of GTPγS binding to G protein α subunits: affinity estimates for suramin analogues

Parameter estimates (mean ± standard deviation) were calculated by fitting the data summarized in Fig. 4 to a three-parameter (Fig. 8) and four-parameter (Fig. 9) logistic equation, which yields estimates for the concentration at which half-maximal inhibition occurs (IC_{50}) and for the slope of the inhibition curve (n_H) (and a term estimating residual binding insensitive to suramin analogues; see Fig. 9). Because the inhibitory potency of NF007 was low, reliable affinity estimates could not be derived.

	$rG_{s\alpha\text{-}s}$		$rG_{i\alpha\text{-}1}$		$rG_{o\alpha}$	
	IC_{50} μM	n_H	IC_{50} μM	n_H	IC_{50} μM	n_H
NF018	11.9 ± 0.6	1.03 ± 0.04	13.2 ± 3.2	1.28 ± 0.21	14.3 ± 1.13	1.27 ± 0.16
NF023	0.69 ± 0.11	0.81 ± 0.05	0.28 ± 0.06	1.04 ± 0.06	0.32 ± 0.05	1.11 ± 0.09
NF037	0.66 ± 0.15	1.30 ± 0.18	0.87 ± 0.12	1.03 ± 0.03	0.30 ± 0.12	1.09 ± 0.14
Suramin	0.24 ± 0.08	1.09 ± 0.04	4.75 ± 2.24	0.95 ± 0.09	2.53 ± 0.39	0.74 ± 0.08

which predicted a 7-fold increase in the lag phase of activation. We ruled out that this discrepancy arose from the inability of suramin to interact with $G_{s\alpha}$ in the membrane due to the presence of phospholipids or other unknown components. If recombinant $G_{s\alpha\text{-}s}$ was premixed with purified bovine brain adenylyl cyclase in detergent solution, suramin (1 μM) barely affected the lag phase of activation (Fig. 6B). A substantial prolongation of the lag phase was observed at higher concentrations of suramin (≥10 μM); these concentrations, however, also resulted in profound direct inhibition of adenylyl cyclase (data not shown; see also Fig. 5B).

Interaction of suramin with the effector binding site. Taken together, these observations (i.e., the 20-fold higher EC_{50} value of NF023 in reversing GTPγS-dependent adenylyl cyclase inhibition and the modest effect of suramin on GTPγS-dependent adenylyl cyclase activation in membranes and in detergent solution) are consistent with the interpretation that the interaction between suramin analogues and G protein α subunits is impeded in the presence of effectors. We determined the kinetics of guanine nucleotide exchange of recombinant $G_{s\alpha\text{-}s}$ in the presence of the effector adenylyl cyclase. The addition of purified bovine brain adenylyl cyclase did not affect the rate of [^{35}S]GTPγS binding to recombinant $G_{s\alpha\text{-}s}$, whereas it blunted the inhibitory effect of suramin (Fig. 2A). The ability of increasing concentrations of adenylyl cyclase to relieve the suramin-induced suppression of guanine nucleotide exchange was examined by determining the amount of [^{35}S]GTPγS bound to recombinant $G_{s\alpha\text{-}s}$ after 20 min (Fig. 2B). The effect of adenylyl cyclase was concentration dependent, and a half-maximum reversal was observed at ~4 nM. Heat inactivation of adenylyl cyclase (10 min at 70°) abolished its ability to reverse the inhibition by suramin, whereas the heat-inactivated enzyme had no effect on GTPγS-binding in the absence of suramin (Fig. 2B). The addition of adenylyl cyclase resulted in a modest carry-over of additional detergent (≤0.004% Lubrol at the highest concentration of enzyme). The effect of suramin on the rate of GTPγS binding to recombinant $G_{s\alpha\text{-}s}$, however, is independent of the Lubrol concentration (range tested, 0–0.1%; data not shown). The ability of adenylyl cyclase to reverse the action of suramin seems counterintuitive because it requires adenylyl cyclase to interact with inactive, GDP-liganded $G_{s\alpha}$ to account for this effect. However, earlier kinetic analyses provided evidence that in membranes, G_s and cyclase existed as a preformed complex under basal conditions, i.e., before activation of $G_{s\alpha}$ by guanine nucleotides, which is consistent with the "collision coupling" model (31, 32). In addition, the complex between inactive G_s and adenylyl cyclase is sufficiently stable to remain associated in detergent solution and throughout a ~400-fold purification (33).

To obtain additional evidence for an interaction of suramin in the vicinity of the effector binding site, we tested its ability to suppress the immunoprecipitation by two antisera raised against peptides from the sequence of $G_{s\alpha}$; antisera 584 and CS1 are directed against amino acids 311–325 of $G_{s\alpha\text{-}s}$ (26) and against the carboxyl-terminal decapeptide of $G_{s\alpha}$ (27), respectively (see Fig. 10C). These regions contribute to the effector and receptor binding site, respectively (34, 35). The immunoprecipitation was carried out under conditions that protect recombinant $G_{s\alpha\text{-}s}$ against denaturation (preactivation with GTPγS in the presence of 10 mM $MgSO_4$) and with the heat-inactivated protein. The addition of suramin inhibits immunoprecipitation of native recombinant $G_{s\alpha\text{-}s}$ by antiserum 584 (Fig. 10, *lanes 584− and 584+*). This effect is

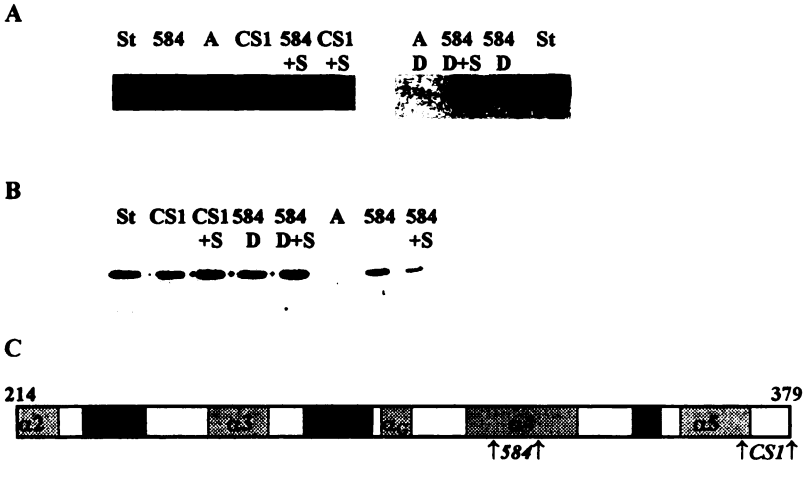

Fig. 10. A and B, Immunoprecipitation of recombinant $G_{s\alpha\text{-}s}$ in the presence and absence of suramin. GTPγS-preactivated recombinant $G_{s\alpha\text{-}s}$ (A, 200 ng/200 μl; B, 50 ng/50 μl) was immunoprecipitated by affinity-purified antiserum 584 (*lane 584*: 1 μg in A, 2 μg in B) or CS1 (*lane CS1*: 1 μg in A, 0.25 μg in B) in the absence and presence of 10 μM suramin (+*S*). As a control, recombinant $G_{s\alpha\text{-}s}$ was denatured and immunoprecipitated with antiserum 584 in the absence (*584 D*) and presence (*584 D+S*) of 10 μM suramin. *Lanes A and A D*, adsorption of GTPγS-liganded and denatured recombinant $G_{s\alpha\text{-}s}$, respectively, to protein A-Sepharose in the absence of added antibody. *Lane St.*, 200 and 50 ng recombinant $G_{s\alpha\text{-}s}$. The immunoprecipitated protein was detected by blotting with CS1 with the Amersham ECL system. C, Schematic overview over the carboxyl-terminal part of $G_{s\alpha\text{-}s}$ starting with amino acid 214 (invariant arginine). The secondary structure elements as deduced from the structure of $G_{t\alpha}$ and $G_{i\alpha\text{-}1}$ are indicated. *Arrows*, regions identified by the antisera 584 and CS1.

specific and not due to an effect of suramin on the antibody according to the following criteria: (i) if the protein was denatured before immunoprecipitation, suramin failed to impede the ability of antiserum 584 to bind recombinant $G_{s\alpha\text{-}s}$ (Fig. 10, *lanes 584D+ and 584D−*). (ii) Suramin does not affect the ability of antiserum CS1 to interact with recombinant $G_{s\alpha\text{-}s}$ (Fig. 10, *lanes CS1− and CS1+*). (iii) Antiserum 584 has an intrinsically lesser ability to immunoprecipitate native recombinant $G_{s\alpha\text{-}s}$ than CS1. If the concentration of epitope binding sites of antiserum 584 was increased by 8-fold, the difference in immunoprecipitation was greatly reduced. Nevertheless, inhibition by suramin was still detectable (Fig. 10B).

Calcium channel regulation in the presence of suramin analogues. The results presented so far indicate that suramin analogues discriminate among G protein α subunits via interaction with the effector binding domain. In particular, NF023 was selective for α subunits of the G_o/G_i group, whereas suramin was selective for $G_{s\alpha}$. We tested whether this selectivity could also be observed in a cellular environment by applying suramin and NF023 to the cytoplasm of rat superior cervical ganglion neurons via the recording pipette in the whole-cell configuration of the patch-clamp technique (29). In these cells, activation of muscarinic (36) and α_2-adrenergic (37) agonists reduces voltage-activated Ca^{2+} currents via pertussis toxin-sensitive G proteins. The signaling pathway activated by α_2-adrenergic agonists was shown to specifically involve $G_{o\alpha}$ (37). Under experimental conditions identical to those of the current study, the effects of muscarinic and α_2-adrenergic agonists on Ca^{2+} currents of rat sympathetic neurons were completely abolished by pretreatment of the neurons with 100 ng/ml pertussis toxin for 24 hr (38). VIP, on the other hand, inhibited these currents via G_s (39). Therefore, the muscarinic agonist oxotremorine-M, the α_2-adrenergic agonist UK 14,304, and VIP (all at 10 μM) were applied to neurons that had been dialyzed for 10 min with control solution or with either 100 μM suramin or 100 μM NF023 (Fig. 11A). Under control conditions, inhibition of Ca^{2+} currents by each of the three agonists was virtually the same and amounted to ~50%. Intracellular suramin exclusively reduced the inhibitory effect of VIP (mediated via G_s), whereas NF023 attenuated only the inhibition via muscarinic and α_2-adrenergic receptors (mediated via G_i/G_o; Fig. 11B).

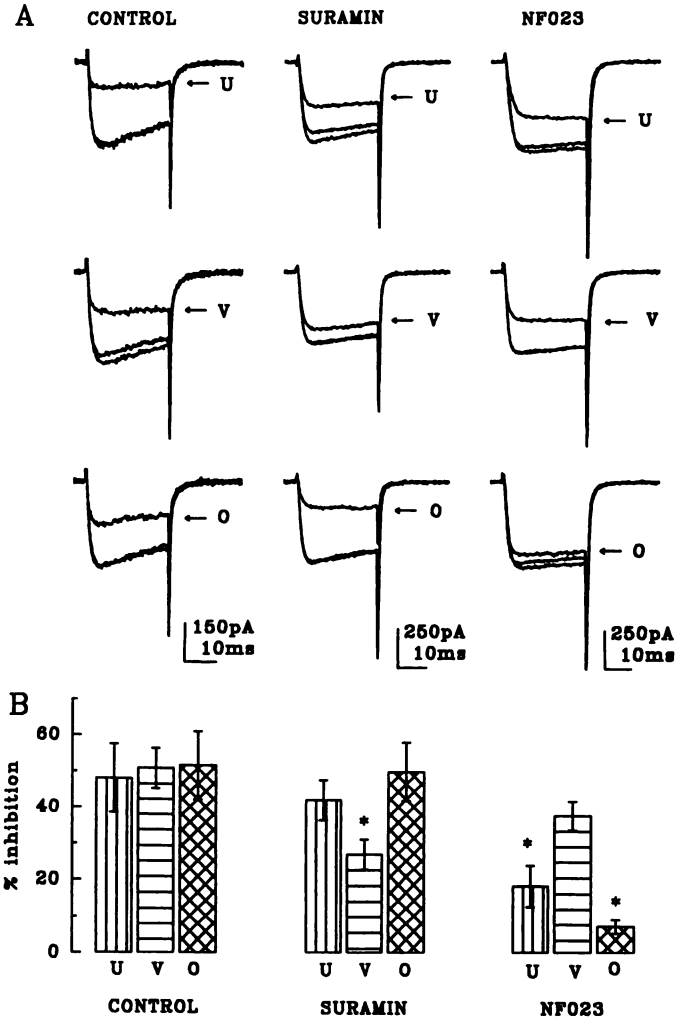

Fig. 11. Attenuation of G protein-mediated inhibition of Ca^{2+} currents in rat sympathetic neurons by suramin and NF023. A, Whole-cell Ca^{2+} currents of three neurons; one was dialyzed with control solution (*left*), one was dialyzed with 100 μM suramin (*middle*), and one was dialyzed with 100 μM NF023 (*right*). Currents were elicited by depolarizations from −80 to 0 mV in 15-sec intervals at least 10 min after formation of the whole-cell configuration. UK 14,304 (*U*), VIP (*V*), or oxotremorine-M (*O*) (all at 10 μM) were applied via the bathing solution for 30 sec. Traces show currents recorded before and after 30 sec of agonist exposure, as well as after washout of the agonists. Washout periods lasted 30 sec for oxotremorine-M, 90 sec for UK 14,304, and 150 sec for VIP as these periods of time guaranteed complete recovery from the agonist-induced inhibition. B, Reduction of peak current amplitudes induced by the above agonists under control conditions or with 100 μM of either suramin or NF023 applied intracellularly. Results are shown as percent inhibition and represent arithmetic mean ± standard error of five cells. *, Significant differences ($p < 0.05$) versus the inhibition caused by the same agonist under control conditions, as determined with the Mann-Whitney U test.

Discussion

The kinetics of [^{35}S]GTP[gama]S binding to G protein subunits directly reflects the transition from the inactive to the active species; alterations in the association rate caused by test compounds reveal their ability to act as G protein activators or G protein antagonists. Our experiments show that suramin and its analogues meet important criteria for direct G protein antagonists. (i) They directly inhibit the rate-limiting step in the activation of G protein subunits, i.e., the release of prebound GDP. The affinities of several analogues are reasonably high, that is, in the submicromolar range. (ii) A distinct structure-activity relationship is observed for individual subunits, resulting in compounds that are modestly selective for $G_{i\alpha}/G_{o\alpha}$ (NF023) or for $G_{s\alpha}$ (suramin). (iii) If the permeation barrier imposed by the cell membrane is overcome by intracellular application of suramin analogues, a selective disruption of the receptor-dependent regulation of an effector can also be demonstrated in intact cells. In rat sympathetic neurons, NF023 blocks the modulation of Ca^{2+} channels by α_2-adrenergic and muscarinic receptor activation, effects that are mediated by a subtype of $G_{i\alpha}$ and/or $G_{o\alpha}$ (36, 37). In contrast, NF023 does not suppress the response to VIP, which results from the apparently direct interaction of $G_{s\alpha}$ with voltage-activated Ca^{2+} channels (39). Conversely, the effect of VIP is partially reversed by suramin, which does not cause appreciable attenuation of the α_2-adrenergic and

muscarinic responses. However, under these conditions, it obviously is not possible to determine whether this selectivity arises from inhibition at the level of receptor/G protein coupling or G protein/effector interaction. Previously, suramin and related polysulfonated anions have been tested for their ability to reduce high affinity agonist binding to α_2-adrenergic (G_i/G_o-coupled) and β_2-adrenergic (G_s-coupled) receptors in membranes (18). The authors failed to find any selectivity in the uncoupling effect of suramin, observations that are in contrast to previous work in which suramin was found to be capable of discriminating between two G_i-coupled receptors (17). Our experiments were not designed to address this discrepancy, but preliminary evidence suggests that a distinct structure-activity relationship exists for the suramin analogues used to uncouple distinct ternary complexes of agonist, receptor, and G protein.[1]

A minimum of five reaction partners of the G protein α subunit are required to generate the secondary cellular message after stimulation by an agonist: activated receptor; effector; G protein $\beta\gamma$ dimer, which contributes to the receptor binding site on the oligomeric G protein; magnesium; and GTP (1, 5, 6). The interaction of the latter three ligands with the α subunit is not subtype selective and therefore provides little, if any, potential for discrimination. All α subunits bind GTP with very high affinity and require low amounts of magnesium for the transition to the active conformation. Of all possible combinations of β- and γ subunits, only the combination existing in the transducin $\beta\gamma$ dimer($\beta1\gamma1$) displays a modest ability to discriminate among α subunits (22, 40). Our results rule out a direct interaction of suramin analogues with the guanine nucleotide binding pocket. This is also true for the magnesium binding site of the α subunit, as similar effects of suramin analogues are observed over a wide range of magnesium concentrations (see Fig. 4). Binding of $\beta\gamma$ dimer to α subunits requires the amino terminus of the G protein α subunit (34) and the helix $\alpha2$ (35, 41). Helix $\alpha2$ is also the first of the three contact sites involved in binding the effector that have been defined by mutagenesis and peptide inhibition (34, 35, 42–44). Suramin analogues do not impede the interaction between α subunit and the $\beta\gamma$ subunit complex. Therefore, $\beta\gamma$ binding sites and the first effector binding site on the α subunit are not involved in binding suramin.

Other than helix $\alpha2$ (effector binding site 1), the effector binding surface on the α subunit is formed by helix $\alpha3$ and the loop connecting $\alpha3/\beta5$ (effector binding site 2), as well as helix $\alpha4$ and the loop connecting $\alpha4/\beta6$ (effector binding site 3) (34, 35, 42–44). Our experiments indicate that suramin analogues interact within the effector binding region because the effect of suramin on $G_{s\alpha}$ can be competitively blocked by the appropriate effector, i.e., purified adenylyl cyclase. This is also supported by the ability of suramin to inhibit the interaction between antiserum 584 and recombinant $G_{s\alpha\text{-}s}$ in its native conformation. The epitope of the antibody is an insert in helix $\alpha4$, which is unique to $G_{s\alpha}$. It undergoes a conformational switch on activation by GTPγS (3) and is thought to be part of or at least to lie adjacent to the third effector binding site on $G_{s\alpha}$ (34). Helix $\alpha4$ of $G_{t\alpha}$ binds the γ subunit of its effector, the cGMP-phosphodiesterase (35, 43).

In contrast, suramin does not interfere with binding of antiserum CS1 to recombinant $G_{s\alpha\text{-}s}$. The epitope of this antibody is the carboxyl terminus of $G_{s\alpha}$, which is one of the two receptor binding sites; the second ($\beta6/\alpha5$) is adjacent to and in part overlapping with the third effector binding site ($\alpha4/\beta6$) (34, 35). Thus, if suramin and its analogues bind in the vicinity of the third effector binding site, they are predicted to impede the interaction between G protein and receptor; earlier observations (17, 18) and our preliminary observations[2] support this interpretation.

The effector binding domain is diverse among individual G protein α subunits. Therefore, the selectivity that we observed in the interaction of suramin analogues with α subunit is also consistent with their binding to the effector domain. It is noteworthy that the "half-molecules" were considerably less potent (NF018) or essentially inactive (NF007). This stresses the importance of multiple ionic centers for activity. In addition, suramin provides a bulky hydrophobic surface, whereas NF023 is considerably smaller (lacking two benzamide rings). This may also be an important determinant of affinity. When tested directly on the purified G protein α subunits, the affinity of the most active compounds was in the submicromolar range. However, α subunits bind their effectors with affinities that range from picomolar to nanomolar (45). It is therefore not surprising that in the presence of effectors, substantially higher amounts of the suramin analogues are required to block the regulatory action of the α subunit. This highlights that it is the relative affinity, i.e., the ratio of K_D of the α subunit for the effector to K_D for the compound, that determines the usefulness of a direct G protein antagonist *in vivo*.

Our work demonstrates that suramin analogues can be used as templates to search for low-molecular-weight compounds that target the effector binding site on G protein α subunits. The currently observed selectivities are modest; however, a large array of suramin analogues are available (19), and the structures of G protein α subunits are known (35, 43, 44). Therefore, it should be possible to generate the information required for the design of high affinity ligands that act as selective G protein antagonists.

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[2] W. Beindl, unpublished observations.

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