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Differential Uncoupling of A_1 Adenosine and D_2 Dopamine Receptors by Suramin and Didemethylated Suramin (NF037)

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ABSTRACT

Suramin analogues uncouple two G_i/G_o-coupled receptors, the D_2 dopamine receptor in rat striatum and the A_1 adenosine receptor in human cerebral cortex, with distinct structure-activity relations. This discrepancy may reflect true differences in the affinity of the analogues for specific receptor/G protein complexes or may be attributable to differences in species or in the tissue source used. We addressed this question by using human embryonic kidney 293 cells that stably express the human A_1 and rat A_1 receptor and the human D_2 receptor. Suramin is 10-fold more potent than its didemethylated analogue NF037 in inhibiting the interaction between G proteins and the rat A_1 or human A_1 receptor; in contrast, both compounds are equipotent in uncoupling the D_2 receptor. These differences are observed regardless of whether (1) inhibition of high affinity agonist binding to the receptors or (2) agonist-stimulated GTPγS binding is used as readout, (3) the receptors are allowed to interact with the G protein complement in human embryonic kidney 293 cell membranes, or (4) the receptors are forced to interact with a defined G protein α subunit (i.e., after reconstituting pertussis toxin-treated membranes with exogenous $rG_{i\alpha\text{-}1}$). The apparent affinity of suramin depends in a linear manner on receptor occupancy, which shows that suramin and the receptor compete for the G protein. Finally, the affinity of the receptors for $rG_{i\alpha\text{-}1}$ (human A_1 > rat A_1 > human D_2) is inversely correlated with the potency of suramin in uncoupling ternary complexes formed by these receptors and thus determines the selectivity of the suramin analogues for specific receptor/G protein tandems.

In most cells, G protein-coupled receptors interact with multiple distinct G protein oligomers, and the overall biological response to the agonist-activated receptor results from the concerted regulation of multiple G protein-dependent effector systems (Gudermann *et al*., 1996a). The ability of a receptor to activate multiple G proteins is specified by discrete, poorly conserved regions in the intracellular loops that connect the transmembrane helices and, in some cases, within the carboxyl terminus of the receptor (Gudermann *et al*., 1996b). Based on the observation that there is more than one active conformation of rhodopsin (Arnis *et al*., 1993, 1994), it has been proposed that other G protein-coupled receptors also may adopt several active conformations that interact with distinct G proteins; these may be selected by "biased" agonists, which will favor a conformation that preferentially interacts with one type of G protein (Gudermann *et al*., 1996a; Kenakin, 1996). This hypothesis is supported by two lines of experimental evidence. (1) Point mutations in a given receptor can elicit distinct effects on its coupling properties; for example, substitution of Ile486 by phenylalanine in the human thyroid-stimulating hormone receptor produces a receptor that constitutively activates both the adenylyl cyclase and phospholipase C cascade; in contrast, substitution of Phe631 by isoleucine only raises cAMP (Parma *et al*., 1995). (2) The PACAP receptor I can be stimulated by PACAP-27 and PACAP-38; however, on heterologous expression of the receptor, PACAP-27 activates adenylyl cyclase more potently than PACAP-38, whereas the reverse is true for stimulation of inositol trisphosphate formation (Spengler *et al*., 1993). Other arguments and additional experimental evidence in support of the hypothesis that multiple R* conformations exist have been reviewed recently (Gudermann *et al*., 1996a; Kenakin, 1996). A corollary of this concept is the assumption that compounds that block the interaction of R with G may be selective for specific R/G tandems. If compared with receptor antagonists, compounds that block the interaction of receptors and G proteins over receptor antagonists offer the advantage that they should provide for an additional level of selectivity in inhibiting signal transduction; provided that inhibitors with high selectivity can be found, they will block signaling of the activated receptor via one G

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ABBREVIATIONS: PACAP, pituitary adenylyl cyclase activating polypeptide; GTPγS, guanosine-5′-(3-*O*-thio)triphosphate; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; XAC, xanthine amine congener; HEK, human embryonic kidney; SDS, sodium dodecyl sulfate; CPA, N^6-cyclopentyladenosine.

protein-regulated pathway but will not perturb other receptor-generated signals within the cell.

Earlier work showed that suramin acted as an inhibitor of receptor/G protein coupling (Butler *et al*., 1988; Huang *et al*., 1990); circumstantial evidence for selective disruption of specific receptor/G protein tandems was provided by the observation that suramin inhibited the activation of pertussis toxin-substrate G proteins by δ-opioid receptors in membranes from NG 108–15 cells, whereas the stimulation of the guanine nucleotide exchange reaction of these G_o/G_i proteins by serum factors, which acted on an unidentified receptor, was not blocked (Huang *et al*., 1990). In addition, we recently reported that the didemethylated suramin derivative NF037 discriminated between A_1 adenosine receptor/G protein tandems in the human cerebral cortex and D_2 dopamine receptor/G protein tandems in the rat striatum (Beindl *et al*., 1996). However, in rat cerebral cortex, the A_1 adenosine receptor is resistant to the uncoupling effect of suramin unless the membranes are extracted with detergent to remove an inhibitory constraint imposed by an ancillary protein (Nanoff *et al*., 1997). Thus, the distinct activity profile of suramin and NF037 on human A_1 adenosine and rat D_2 dopamine receptor may have been due to species differences or may have arisen from the expression of the receptors in distinct cell types and/or in distinct microcompartments of the plasma membrane. Here, we eliminated these confounding variables by expressing the human and rat receptors in the same cell line; furthermore, the receptors were forced to interact with the same G protein α subunit. The results show that NF037 is selective for D_2 dopamine receptor/G protein tandems even if the receptors couple to the same $G_{i\alpha}$ subtype. In addition, the potency of suramin and NF037 in uncoupling receptor/G protein complexes is inversely correlated to the affinity of the receptor for the G protein.

Experimental Procedures

Materials. [^{35}S]GTPγS, [^{125}I]OH-PIPAT [(+)-*trans*-7-hydroxy-2[*N*-propyl-*N*-3-[^{125}I]iodo-2′-propenyl)aminotetralin] and [^{125}I] were purchased from New England Nuclear Research Products (Boston, MA). [^{125}I]HPIA [(−)N^6-3[^{125}I](iodo-4-hydroxyphenyl-isopropyl)adenosine] was synthesized according to Linden (1984). Guanine nucleotides and adenosine deaminase were from Boehringer-Mannheim Biochemica (Mannheim, Germany). 1-*O*-*n*-Octyl-β-D-glucopyranoside (octylglucoside), CHAPS, and HEPES were from BIOMOL (Munich, FRG). Suramin, sulpiride, and XAC were obtained from Research Biochemicals (Natick, MA). The materials required for SDS-polyacrylamide gel electrophoresis were from Bio-Rad (Richmond, CA). Fetal calf serum was from PAA Laboratories (Linz, Austria). Dulbecco's modified Eagle medium, nonessential amino acids, β-mercaptoethanol, and G418 (geneticin) were obtained from GIBCO-BRL (Grand Island, NY). CPA, pertussis toxin, L-glutamine, penicillin G, and streptomycin were purchased from Sigma Chemical (St. Louis, MO). Buffers and salts were from Merck (Darmstadt, FRG). The cDNA coding for the rat A_1 adenosine receptor in the plasmid vector pBC-A1R (Freund *et al*., 1994) and the HEK 293 cell clone expressing the human A_1 adenosine receptor were kindly provided by M. J. Lohse (University of Würzburg). The human $D_{2\,(\text{short isoform})}$ plasmid vector and NF037 were generous gifts of C. Pifl (Institute of Biochemical Pharmacology, Vienna University) and of P. Nickel (Institut of Pharmaceutical Chemistry, University of Bonn), respectively. The vectors pEGFP-C1 and pRc-CMV were obtained from Clontech (Palo Alto, CA).

Generation of transient and stable cell lines. COS-7 (African green monkey kidney fibroblasts) cells were plated at a density of 3×10^6 cells/10-cm dish and transiently transfected with 5 μg of the cDNAs pBC-A1dhfr containing the rat A_1 adenosine receptor cDNA insert (Freund *et al*., 1994) and pCMV5 plasmid vector containing the $D_{2\text{short}}$ receptor cDNA using the calcium phosphate precipitation method (Chen and Okayama, 1988). The cells were harvested 48 hr after transfection; plasma membranes were prepared and used for radioligand binding assays. HEK 293 cells were plated at a density of 2.5×10^6 cells/10-cm dish and transfected with 7.5 μg of the plasmid pBC-A1dhfr (encoding the rat A_1 adenosine receptor) and 0.75 μg of the resistance marker plasmid pRc-CMV carrying the neomycin phosphotransferase gene. Similarly, the plasmid encoding the short splice variant of the human D_2 dopamine receptor was cotransfected with either pRc-CMV or pEGFP-C1, a vector carrying a red-shifted variant of wild-type green fluorescent protein cDNA from the jellyfish *Aequoria victoria* and a neomycin resistance cassette. The cells were grown in Dulbecco's modified Eagle medium containing 10% fetal calf serum, 2 mM L-glutamine, β-mercaptoethanol, nonessential amino acids, 100 units/ml penicillin G, and 100 μg/ml streptomycin at 5% CO_2 and 37° for 16 hr. Thereafter, the medium was removed, and the cells were subjected to an osmotic shock by adding 15% glycerol in phosphate-buffered saline for a few seconds. Cells were grown for another 24 hr and subsequently selected by adding G418 (0.8 mg/ml) to the medium for 4–6 days. pEGFP-positive clones were identified by fluorescence microscopy. Positive clones appeared in bright green and were subjected to further selection to obtain clones with different expression levels. Three clones were selected that differed in D_2 dopamine receptor density (ranging from ~0.3 to 4 pmol/mg membrane protein).

Membrane preparation and protein purification. Cells were grown to confluency in 10-cm tissue culture dishes, washed once with ice-cold phosphate-buffered saline, and scraped off their plastic support in HME buffer (25 mM HEPES·NaOH, pH 7.5, 2 mM $MgCl_2$, 1 mM EDTA). After centrifugation at 20,000 × *g* for 10 min, the cell pellet was resuspended in HME, subjected to a freeze/thaw cycle with liquid nitrogen, and further homogenized by sonication. Membranes were sedimented by centrifugation (38,000 × *g* for 10 min) and resuspended in HME at a protein concentration of 8–10 mg/ml and stored in aliquots at −80°. Recombinant (R) $G_{i\alpha\text{-}1}$ and $rG_{i\alpha\text{-}2}$ were expressed in *Escherichia coli* BL21DE3 harboring a plasmid-encoding yeast myristoyl-CoA transferase and purified from bacterial lysates (Mumby and Linder, 1994). Oligomeric G proteins were purified from bovine or porcine brain, and free βγ dimers were chromatographically resolved from the α subunits (Casey *et al*., 1989).

Radioligand binding experiments. Equilibrium binding with the A_1 adenosine receptor agonist [^{125}I]HPIA and with the dopaminergic D_2 agonist [^{125}I]OH-PIPAT were carried out in a final volume of 40 μl containing 50 mM Tris·HCl, pH 8, 1 mM EDTA, 5 mM $MgCl_2$, 1 mM ascorbic acid, 8 μg/ml adenosine deaminase, 10 μg of membrane protein, and the concentrations of suramin and NF037 as indicated (ascorbic acid and adenosine deaminase are not required for determining binding to A_1 adenosine and D_2 dopamine receptors, respectively, but were present in all incubations to obtain identical incubation conditions). The binding reaction was carried out for 90 min at 25° and terminated by filtration over glassfiber filters using a cell harvester (Skatron, Lier, Norway). Nonspecific binding was determined in the presence of 1 μM XAC (for A_1 adenosine receptors) or 10 μM sulpiride (for D_2 dopamine receptors) and amounted to ~5–10% of total binding in the K_D concentration range. In experiments using membranes from clones with a high receptor expression level (1.5–3.9 pmol/mg of membrane protein) or low radioligand concentrations, the amount of membrane protein added and the assay volume was adjusted (up to 250 μl) to avoid depletion of the radioligand (bound <10% of total). Specific binding of agonist or antagonist radioligands ([^{3}H]DPCPX and [^{125}I]epideprid for A_1 adenosine and D_2 dopamine receptors, respectively) was not detectable in membranes prepared from untransfected HEK 293 cells. Radioli-

gand binding to membranes from transfected cells was displaced by unlabeled receptor ligands with the appropriate pharmacological specificity, and specific binding for both agonists and antagonist radioligands was saturable; B_{max} values for antagonist binding were ~1.2-fold higher than those for agonist radioligands, indicating that the majority of the receptors were capable of interacting with G proteins endogenous to the HEK 293 membranes (not shown).

Receptor-mediated [^{35}S]GTPγS binding. Receptor-promoted binding of [^{35}S]GTPγS was determined essentially as described previously (Nanoff *et al.*, 1995). In brief, membranes from HEK 293 cells (~10 μg) were suspended in 40 μl of buffer containing 25 mM HEPES·NaOH, pH 7.5, 1.5 mM $MgCl_2$, 150 mM NaCl, 1 mM EDTA, 0.01 mM GDP, and the concentrations of dopamine, CPA, and suramin analogues indicated in the respective figures. After a preincubation of 10 min at 25°, the assay was initiated by adding 10 μl of buffer containing [^{35}S]GTPγS to yield a final concentration of 1 nM (specific activity, 2000 cpm/fmol). The assay was terminated after 10 min by adding 0.5 ml of ice-cold stop buffer containing 10 mM Tris·HCl, pH 8.0, 100 mM NaCl, 20 mM $MgCl_2$ and 0.1 mM GTP. Bound and free nucleotides were separated by filtration over glass-fiber filters.

Determination of adenylyl cyclase activity. Adenylyl cyclase activity in HEK 293 membranes expressing the recombinant receptors was assayed in 0.1 ml containing 50 mM HEPES·NaOH, pH 8.0, 0.05 mM [α-^{32}P]ATP (~200 cpm/pmol), 5 mM $MgCl_2$, 0.1 mM rolipram, 10 mM creatine phosphate, membrane protein (25 μg), 1 mg/ml creatin kinase, 8 μg/ml adenosine desaminase, and 1% bovine serum albumin. Inhibitory regulation of adenylyl cyclase by the D_2 dopamine and A_1 adenosine receptor agonists was determined in the presence of 1 μM prostaglandin E_1 and 10 nM GTPγS. The reaction was carried out for 20 min at 25°; cAMP was separated from ATP by sequential chromatography on Dowex and Alumina (Johnson and Salomon, 1991).

Pertussis toxin treatment and reconstitution of HEK 293 cell membranes with $rG_i\alpha$-1. HEK 293 cells expressing the rat A_1 adenosine, human A_1 adenosine, or human D_2 dopamine receptors were incubated with 100 ng/ml pertussis toxin for 24 hr, and membranes were prepared as described. To insert exogenously added G protein into the membranes, the stable reconstitution protocol (Freissmuth *et al.*, 1991a) was adapted as follows: PTX-treated membranes were incubated with 4.5 ng pf $rG_{i\alpha\text{-}1}$/μg membrane protein in HME containing 1% octylglucoside. After 1 hr on ice, membranes were diluted 1:10 in detergent-free buffer and centrifuged at 38,000 × *g* for 12 min. Pellets were resuspended in HME and stored in aliquots at a concentration of ~10 mg/ml at −80°. The amount of $rG_{i\alpha\text{-}1}$ incorporated into the membranes was determined by immunoblotting. To assess the potency of $rG_{i\alpha\text{-}1}$ to restore high affinity agonist binding, $rG_{i\alpha\text{-}1}$ was combined with a 4-fold molar excess of purified βγ dimers in 1% octylglucoside (or 10 mM CHAPS); appropriate dilutions were added to the membranes to give 0.5% octylglucoside (or 5 mM CHAPS) and preincubated on ice for 15 min. Subsequently, radioligand binding assays were carried out after diluting the detergent 2-fold.

Immunoblots. Membrane proteins (~25 μg/lane) were separated on SDS-polyacrylamide gels (10% acrylamide, 0.13% bisacrylamide) and transferred to nitrocellulose membranes that were probed with AS7, an antiserum recognizing $G_{i\alpha\text{-}1}$ and $G_{i\alpha\text{-}2}$ (McClue *et al.*, 1992) or with the $G_{i\alpha\text{-}1}$-specific antiserum I1C (Selzer *et al.*, 1993). The immunostained bands were visualized by enhanced chemoluminescence using an anti-rabbit IgG antibody conjugated to horseradish peroxidase (Amersham, Arlington Heights, IL). Purified recombinant G protein α subunits were used as standards. To verify that comparable amounts of membrane proteins had been applied in individual lanes, blots also were probed with a rabbit antiserum directed against the G protein β subunit (Hohenegger *et al.*, 1995).

Results

Uncoupling of A_1 adenosine and D_2 dopamine receptors after heterologous expression. High affinity binding of agonists to G protein-coupled receptors depends on the formation of a ternary complex of agonist, receptor, and G protein (Hepler and Gilman, 1992).After stable expression of the human D_2 dopamine and the rat and human A_1 adenosine receptors in HEK 293 cells, the coupling of the receptors with G proteins in the membrane was assessed by using agonist radioligands. Suramin and NF037 did not block binding of appropriate antagonist radioligands to the receptors (not shown; see Beindl *et al.*, 1996) but inhibited equilibrium binding of the A_1-selective agonist [^{125}I]HPIA and the D_2-dopaminergic agonist [^{125}I]OH-PIPAT (Fig. 1); suramin (■ in Fig. 1) was >10-fold more potent than NF037 (● in Fig. 1) in suppressing ternary complex formation of the rat (Fig. 1A) and human (Fig. 1B) A_1 adenosine receptor. In contrast, the compounds were equipotent in inhibiting binding of the D_2 dopamine receptor agonist [^{125}I]OH-PIPAT (Fig. 1C). The same difference was seen if the rat A_1 adenosine receptor and human D_2 dopamine receptor were transiently expressed in a cell line of nonhuman origin, namely, COS-7 cells (not shown). In addition, the apparent affinity of suramin and of NF037 was highest for human D_2 receptor/G protein complexes and lowest for human A_1 receptor/G protein complexes (Table 1).

The agonist-liganded receptor catalyzes the GDP/GTP exchange reaction of the G protein; agonist-stimulated binding of [^{35}S]GTPγS therefore can be used as an alternative readout to assess receptor/G protein coupling. The A_1-selective agonist CPA stimulated [^{35}S]GTPγS binding with EC_{50} values of 4.1 ± 2.4 and 161 ± 57 nM (not shown) in membranes harboring the human and the rat A_1 adenosine receptor, respectively; after a 10-min incubation period, the receptor-promoted binding was ~2.5-fold higher than the basal binding (Fig. 2, A–C). In membranes expressing the D_2 dopamine receptor at low levels (used to generate the data shown in Fig. 1C), the dopamine-induced increment in [^{35}S]GTPγS binding was too low (~1.2-fold) to obtain a reliable signal-to-noise ratio for assessing the inhibitory effect of suramin and NF037. Hence, membranes from a cell clone that expressed the D_2 dopamine receptor at high levels (3.9 pmol/mg) were used where dopamine-stimulated basal [^{35}S]GTPγS binding ~2-fold (see Fig. 2D) with an EC_{50} value of 0.12 ± 0.02 μM. Fig. 2A summarizes experiments carried out with membranes harboring the rat A_1 adenosine receptor. The basal rate of [^{35}S]GTPγS binding was determined in the presence of receptor antagonists (1 μM XAC or 5 μM sulpiride) to eliminate nucleotide exchange catalyzed by the unliganded receptor. Suramin and NF037 decreased basal [^{35}S]GTPγS binding by ~50% (Fig. 2A, *open symbols*); these findings are consistent with the ability of the compounds to directly block the release of GDP from G protein α subunits (Freissmuth *et al.*, 1996). In contrast, suramin (IC_{50} = 1.5 ± 0.3 μM) was more potent than NF037 (IC_{50} = 15.9 ± 2.2 μM) in blocking [^{35}S]GTPγS binding promoted by the activated rat A_1 adenosine receptor (Fig. 2B). Similarly, the apparent affinity of suramin was higher than that of NF037 when inhibition of [^{35}S]GTPγS binding promoted by the agonist-liganded human A_1 adenosine receptor (Fig. 2C) was determined. In contrast, the two compounds were equipotent in inhibiting

the D_2 dopamine receptor-stimulated guanine nucleotide exchange reaction. Higher concentrations of suramin and NF037 are required to inhibit receptor-promoted [^{35}S]GTPγS binding than high affinity agonist binding (compare Figs. 1 and 2). This discrepancy is presumably due, in part, to the different assay conditions; that is, the catalytic turnover of the agonist-liganded receptor in the presence of a mixture of GTPγS and GDP (Fig. 2) versus stoichiometric interaction to form a ternary complex in the absence of guanine nucleotides (Fig. 1). Importantly, differences in receptor occupancy by the agonists contribute to the rightward shift of the inhibition curves (see also below); agonist radioligands were present at concentrations close to their K_D values, whereas CPA and dopamine were used at saturating concentrations (300 nM and 1 μM, respectively) to promote [^{35}S]GTPγS binding. If the human A_1 adenosine receptor was activated with 10 nM CPA, the IC_{50} value of suramin was 2.89 ± 0.77 μM (not shown), whereas it amounted to 8.78 ± 1.81 μM at 300 nM CPA (Fig. 2C).

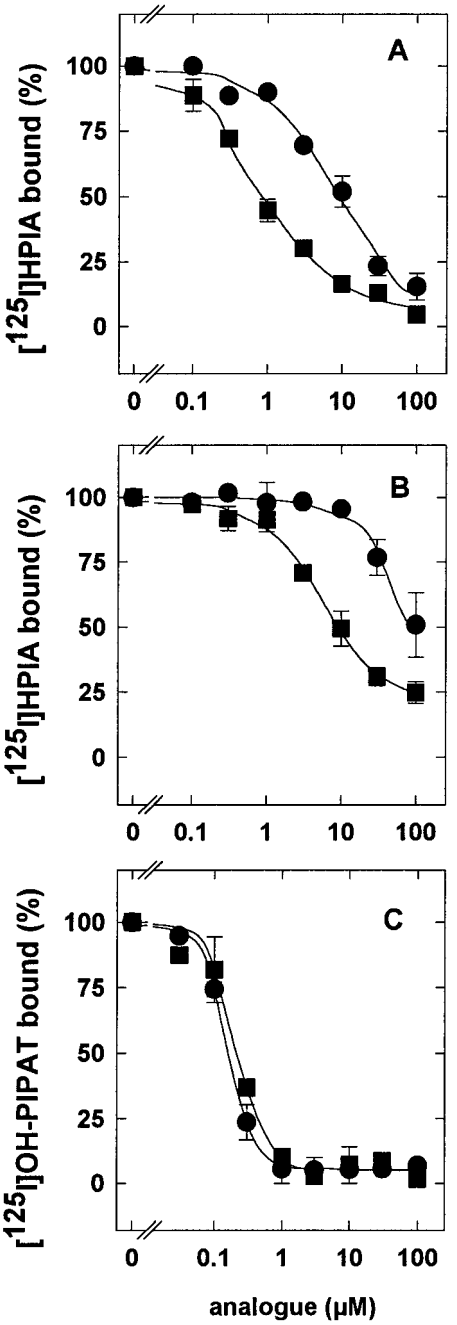

Fig. 1. Inhibition of agonist radioligand binding to rat A_1 (A), human A_1 adenosine (B), and human D_2 dopamine receptors (C) stably expressed in HEK 293 membranes by suramin and NF037. The binding reaction was carried out in 40 μl containing membranes (6–12 μg of protein), and [^{125}I]HPIA (final concentration, 1.5 nM, A and B) or [^{125}I]7-OH-PIPAT (0.7 nM, C) and increasing amounts of suramin (■) and NF037 (●) for 90 min at 25° as outlined in Experimental Procedures. The membranes used in C were from the cell clone expressing D_2 dopamine receptors at 0.3 pmol/mg. Nonspecific binding was determined in the presence of 1 μM XAC or 10 μM sulpiride. Specific binding in the absence of any analogue (1.5–2 fmol ligand bound) was set 100%. Data are mean ± standard error values from three separate experiments carried out in duplicate.

Stable reconstitution of high affinity agonist binding to membranes from pertussis toxin-treated cells by $rG_{i\alpha\text{-}1}$. The different affinity of suramin and NF037 for ternary complexes formed by the A_1 adenosine and D_2 dopamine receptor may have been due to an interaction of the receptors with distinct G proteins. This possibility is substantiated by the following observations: both A_1 adenosine and D_2 dopamine receptors physiologically recruit adenylyl cyclase inhibition as one of the signaling pathways to elicit a biological response, and a marked (>50%) inhibition of prostaglandin E_1-stimulated cAMP formation was observed for both receptor types in intact transfected HEK 293 cells (Roka F and Nanoff C, unpublished observations). However, attenuation of adenylyl cyclase activity was observed only on activation of the D_2 dopamine receptor in HEK 293 membranes, whereas A_1 adenosine receptors were inactive (data not shown). We therefore determined the expression of $G_{i\alpha}$ in the transfected cells. Two forms of $G_{i\alpha}$ were detected in HEK 293 membranes (Fig. 3A). The top band commigrates with purified $rG_{i\alpha\text{-}1}$ and is recognized by I1C, an antiserum specific for $G_{i\alpha\text{-}1}$ (see Fig. 3B). The bottom band is detected only by the antiserum that reacts with $G_{i\alpha\text{-}1}$ and $G_{i\alpha\text{-}2}$ but not by antiserum I1C and commigrates with $rG_{i\alpha\text{-}2}$. The levels of $G_{i\alpha\text{-}2}$ and of the G protein β subunits (migrating as a β35/β36 doublet) were comparable in all cell lines. In contrast, there was some variability in the level of $G_{i\alpha\text{-}1}$; the membranes from the cell line that expressed the A_1 adenosine receptor (Fig. 3A, *lanes 4* and *5*) had more $G_{i\alpha\text{-}1}$ than the control cells (Fig. 3A, *lane 2*), whereas those harboring the D_2 dopamine receptor contained less $G_{i\alpha\text{-}1}$ (Fig. 3A, *lane 3*). However, this is most likely not related to an effect of receptor expression *per se*; other cell clones that expressed more D_2 receptors than that shown in Fig. 2A had $G_{i\alpha\text{-}1}$ levels comparable to those of untransfected control cells (not shown). This suggests that the variation in the amount of $G_{i\alpha\text{-}1}$ may be due to clonal selection.

To force the receptors to interact with a defined G protein α subunit, we disrupted the coupling of the receptors to the endogenous $G_{i\alpha}$ subunits by pretreating the cells with pertussis toxin and subsequently incorporating exogenously added $rG_{i\alpha\text{-}1}$ into the membrane. ADP-ribosylation of $G_{i\alpha}$ subunits, which occurs at a cysteine residue at position −4 from the carboxyl terminus, retards the migration of the proteins, which can be detected by gel electrophoresis under appropriate conditions (Linder *et al.*, 1990); $G_{i\alpha\text{-}1}$, which was visualized in pertussis toxin-treated membranes (Fig. 3B), was shifted to a slightly lower mobility. Pertussis toxin-treated membranes were incubated with $rG_{i\alpha\text{-}1}$ in the presence of detergent followed by a dilution far below the critical micellar concentration of the detergent and its removal by

centrifugation; this stable reconstitution resulted in incorporation of substantial amounts of the protein into the membranes (Fig. 3B, lanes labeled *Rec*). As expected, pertussis toxin treatment eliminated the high affinity binding of agonist radioligands (□ in Fig. 4). In membranes stably reconstituted with exogenous $rG_{i\alpha\text{-}1}$, high affinity agonist binding to the uncoupled receptors was restored. As shown in Fig. 4 (compare ● and ○), the saturation isotherms showed varying reconstitution efficiencies for the different receptors; however, the affinities for the agonist radioligands were similar in native and reconstituted membranes. K_D values (three experiments) were 0.7 ± 0.3 and 0.7 ± 0.2 nM for binding of [^{125}I]OH-PIPAT to the D_2 dopamine receptor in control and reconstituted membranes, and 1.8 ± 0.3 and 2.2 ± 0.4 nM and 0.7 ± 0.1 and 0.7 ± 0.1 nM for binding of [^{125}I]HPIA to the rat and human A_1 adenosine receptor in control and reconstituted membranes, respectively.

Uncoupling of A_1 adenosine and D_2 dopamine receptors after stable reconstitution. The reconstituted membranes in which the receptors were forced to interact with identical G protein α subunits were used to evaluate the ability of suramin and NF037 to inhibit receptor agonist binding (Fig. 5). The selectivity of the two compounds toward the individual receptor/G protein tandems was essentially unchanged (compare Figs. 1 and 5). In addition, the IC_{50} estimates obtained for inhibition of [^{125}I]OH-PIPAT binding to the D_2 dopamine and of [^{125}I]HPIA binding to the human A_1 adenosine receptor were identical for suramin and NF037 in native and reconstituted membranes (Table 1). Only after reconstitution of the rat A_1 adenosine receptors with exogenous $rG_{i\alpha\text{-}1}$ complement was the inhibitory potency of both compounds moderately shifted to higher IC_{50} values.

From the data summarized in Table 1, it is clear that suramin (and NF037) displayed the highest affinity for D_2 dopamine receptor/G protein complexes regardless of whether it was assessed in native or in reconstituted membranes; in addition, suramin and NF037 were more potent inhibitors of rat A_1 adenosine receptor/G protein tandems than those formed by the human homologue. If the site of action of suramin and NF037 is at the receptor/G protein interface, the ability to dissociate agonist binding relies on a competition between the receptor and the suramin analogue for binding to the G protein docking site. In this case, one would predict that the ability of the suramin analogues to discriminate among specific receptor/G protein tandems should be inversely correlated with the affinity of the receptors for the G protein. We therefore have assessed the ability of receptors to interact with $rG_{i\alpha\text{-}1}$ by restoring high affinity agonist binding to pertussis toxin-treated membranes. Membranes were reconstituted with increasing concentrations of $rG_{i\alpha\text{-}1}$. Because $\beta\gamma$ dimers are required for efficient interaction of the α subunit with the receptor (Freissmuth *et al.*, 1991b), the association of $rG_{i\alpha\text{-}1}$ with $\beta\gamma$ dimers endogenous to the membrane may be limiting for estimating the affinity of the α subunit for the receptor. This confounding effect, however, was eliminated by combining $rG_{i\alpha\text{-}1}$ with a 4-fold molar excess of purified $\beta\gamma$ dimers to reform the oligomer ($rG_{i\alpha\text{-}1}.\beta\gamma$) before the incubation. After detergent dilution, agonist radioligand binding was measured at a fixed concentration (see Experimental Procedures). Fig. 6 shows a concentration-dependent restoration of agonist binding to the human (○) and rat A_1 adenosine receptor (●) and the D_2 dopamine receptor (▽). [^{125}I]HPIA binding to membranes carrying either the human or the rat A_1 adenosine receptor was restored to ≥75% of the values obtained in untreated control membranes; at the highest concentrations of $G_{i\alpha\text{-}1}$ added (300 nM), the reconstitution efficiency amounted to only ~40% for the D_2 dopamine receptor as evaluated by [^{125}I]OH-PIPAT binding. The EC_{50} values for $G_{i\alpha\text{-}1}$ in the presence of $\beta\gamma$ dimers were estimated to be 5.9 ± 1.7, 44.4 ± 9.1, and >400 nM in restoring agonist binding to the human and rat A_1 adenosine and the D_2 dopamine receptor, respectively. This is the inverse of the rank order of the selectivity that suramin and NF037 displayed in uncoupling the individual receptor/G protein tandems. The same difference in affinity between human and rat A_1 adenosine receptor was also observed if CHAPS was used as the detergent (instead of octylglucoside) to dilute the G protein subunits; however, agonist (and antagonist) binding to D_2 dopamine receptors was greatly reduced if the membranes were exposed to CHAPS.

Effect of receptor occupancy on the apparent affinity of suramin and NF037. Suramin and NF037 do not compete for binding of antagonists to the A_1 adenosine and D_2 dopamine receptors, nor do they inhibit the binding of agonists in the absence of a productive interaction between receptor and G protein (Beindl *et al.*, 1996). If the receptors were allowed to couple to G proteins, suramin inhibited agonist binding in a quasicompetitive manner (i.e., the IC_{50} values increased at higher concentrations of the agonist radioligand; Beindl *et al.*, 1996; see also below). This phenomenon may result from a competition of the agonist-liganded receptor with suramin for binding to the G protein or, alternatively, from the direct action of suramin on the receptor to prevent the agonist-promoted transition of the receptor to the active conformation R*. In this case, variations in the membrane concentration of the receptor should not affect the IC_{50} values of suramin analogues. This was tested by using membranes from the three clones of HEK 293 cells expressing different D_2 dopamine receptor densities (0.3, 1.3, and 3.9 pmol/mg). The IC_{50} value of suramin and NF037 was determined in the presence of the agonist radioligand [^{125}I]OH-PIPAT at a concentration close to the K_D value (0.5 nM). As shown in Fig. 7A for NF037, the IC_{50} value was shifted to the

TABLE 1

Inhibition of receptor agonist binding: affinity estimates for suramin and NF037

IC_{50} mean ± standard deviation values were calculated by fitting the data shown in Figs. 1 and 4 to a four-parameter logistic equation [$B = B_o*(I^{nH}/(I^{nH\cdot} + IC_{50}{}^{nH}) + C$, where B is the radioligand bound, B_o is the radioligand bound in the absence of inhibitor, I is the concentration of the inhibitor, n_H is the slope of the curve, and C is the term estimating residual binding insensitive to the effect of suramin analogues].

	Human A_1 receptor		Rat A_1 receptor		Human D_2 receptor	
	Control	Reconstituted	Control	Reconstituted	Control	Reconstituted
Suramin IC_{50} (μM)	5.2 ± 1.0	5.4 ± 1.2	0.57 ± 0.1	1.66 ± 0.5	0.21 ± 0.01	0.23 ± 0.07
NF037 IC_{50} (μM)	92 ± 18	112 ± 20	8.5 ± 3.2	23.9 ± 11	0.16 ± 0.01	0.18 ± 0.06

right with increasing expression levels of the receptors. The same was true for suramin and the decrease in the apparent affinity of suramin and of NF037 was related in a linear manner to the amount of bound agonist (Fig. 7B, *solid symbols*). Control experiments were carried out with the D_2-dopaminergic antagonists sulpiride and haloperidol; as expected, receptor density did not affect IC_{50} values of the receptor antagonists (data not shown).

If the clone expressing intermediate levels of D_2 dopamine receptors was incubated with increasing concentrations of [^{125}I]OH-PIPAT, the IC_{50} estimates of suramin and of NF037 varied with the concentration of the radioligand (Fig. 7B, *open symbols*). Again, in the plot of IC_{50} versus receptor occupancy, the affinity estimates fall onto a straight line. The slope of the regression line is comparable within experimental error with that calculated for the IC_{50} values that were observed by varying receptor density (Fig. 7B, *solid symbols*). Hence, the number of agonist-liganded receptor present was responsible for the rightward shift of the inhibition curves (Fig. 7A) and the increase in the IC_{50} estimates (Fig. 7B). The dependency of IC_{50} estimates on the activator concentration is determined by the Cheng-Prusoff relation [$K_i = IC_{50}/(1 + A/K_{DA})$]; on rearranging, the equation yields $IC_{50} = K_i/K_{DA}{}^*A + K_i$, stating that the IC_{50} value of an inhibitor depends in a linear manner on the concentration of the activator A and is determined by both the dissociation constant K_{DA} of the activator and that of the inhibitor K_i. Thus, the y-axis intercept yields an estimate of the K_i (~0.16 μM; see Fig. 7B) and the slope is given by the ratio of K_i/K_{DA}. The slope of the regression line in Fig. 7B is ~0.2; thus, the K_{DA} estimate for the activator (A) is ~0.8 μM. Obviously, because this number is calculated by a division with two derived parameters, it is inherently imprecise. However, this K_{DA} estimate (~0.8 μM) is 3 orders of magnitude higher than the K_D value for [^{125}I]OH-PIPAT binding to the D_2 dopamine receptor (~0.7 nM; see Fig. 4); in contrast, the K_{DA} estimate for the activator is consistent with the affinity estimated for the interaction between agonist-liganded D_2 dopamine receptors and exogenously added $rG_{i\alpha\text{-}1}$ (Fig. 6). Taken together, these findings imply that the activator (A) for which suramin and NF037 compete is not the agonist [^{125}I]OH-PIPAT but the agonist-liganded receptor.

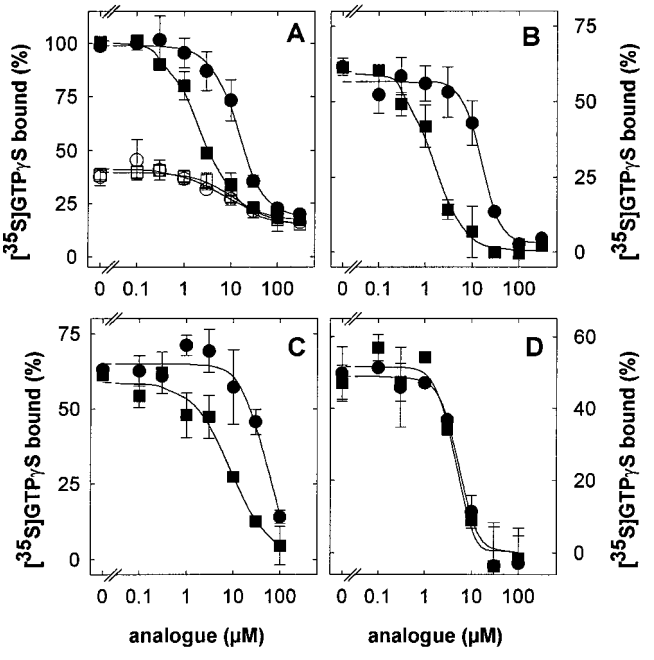

Fig. 2. Inhibition of [^{35}S]GTPγS binding to HEK 293 membranes expressing the rat (A and B) and human A_1 adenosine receptor (C) and the human D_2 dopamine receptor (D) by suramin and NF037. A and B, [^{35}S]GTPγS binding to HEK 293 membranes (10 μg) expressing the rat A_1 adenosine receptor was determined in the presence of 0.3 μM CPA (■, ●) or of 1 μM XAC (□, ○) and of increasing concentrations of suramin (□, ■) and NF037 (○, ●). The binding reaction was initiated by the addition of 1 nM [^{35}S]GTPγS and carried out for 10 min at 25° as outlined in Experimental Procedures. B, Basal binding of [^{35}S]GTPγS was subtracted from CPA-stimulated binding. C, [^{35}S]GTPγS binding was determined as in A using membranes expressing the human A_1 adenosine receptor. Shown is the agonist-stimulated binding (i.e., the difference between total binding in the presence of CPA and basal binding in the presence of XAC). D, [^{35}S]GTPγS binding was determined as in A using membranes expressing the human D_2 dopamine receptor at a level of 3.9 pmol/mg, dopamine (1 μM) as the agonist, and sulpiride (10 μM) as the antagonist. Shown is the agonist-stimulated binding (i.e., the difference between total binding in the presence of dopamine and basal binding in the presence of sulpiride). Total [^{35}S]GTPγS binding in the presence of CPA (human A_1, 167 ± 33 fmol/mg; rat A_1, 168 ± 12 fmol/mg) or dopamine (153 ± 13 fmol/mg) was set at 100%. Data are mean ± standard error from three experiments carried out in duplicate.

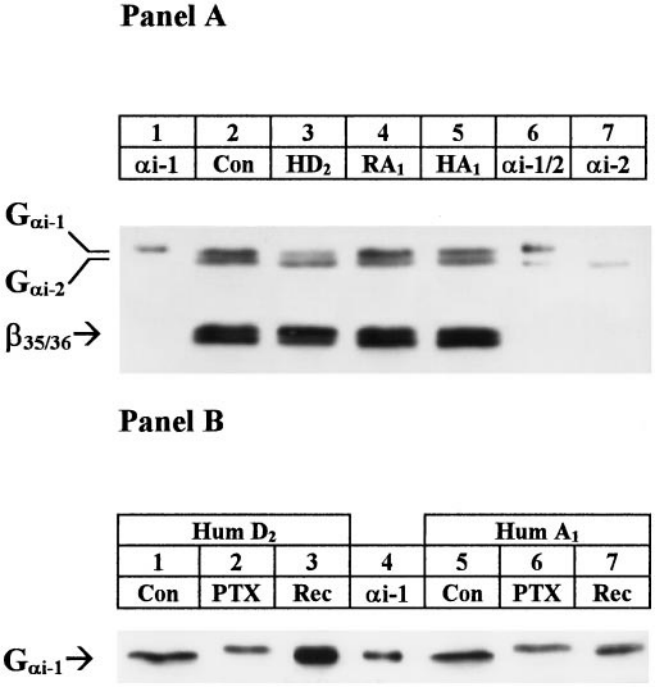

Fig. 3. Immunodetection of $G_{i\alpha\alpha}$ subunits in native HEK 293 cell membranes (A) and after pertussis toxin treatment and stable reconstitution (B). A, Membrane proteins (25 μg/lane) from untransfected control cells (*lane 2*), HEK 293 cells expressing the human D_2 dopamine receptors (*lane 3*), as well as the rat (*lane 4*) and human A_1 adenosine receptor (*lane 5*) were resolved on a 10% SDS-polyacrylamide gel and transferred to nitrocellulose; the blot was immunostained with AS7 (an antiserum recognizing $G_{i\alpha\text{-}1}$ and $G_{i\alpha\text{-}2}$). *Lane 1*, $rG_{i\alpha\text{-}1}$ (10 ng). *Lane 7*, $rG_{i\alpha\text{-}2}$ (5 ng). *Lane 6*, combination of $rG_{i\alpha\text{-}1}$ (10 ng) and $rG_{i\alpha\text{-}2}$ (5 ng). To rule out differences in the amount of protein loaded in each lane, the blot was also probed with a G protein β-subunit antiserum (*arrow*). B, Confluent cultures of HEK 293 cells expressing the recombinant receptors were treated with vehicle (*Con*) or 100 ng/ml pertussis toxin (*PTX*) for 24 hr; membranes prepared from the latter cells were also stably reconstituted with recombinant $rG_{i\alpha\text{-}1}$ (*Rec*) as outlined in Experimental Procedures. Membrane proteins (~25 μg) from HEK 293 cells expressing the D_2 dopamine receptor (*lane 1–3*) and the human A_1 adenosine receptor (*lane 5–7*) were separated on a 10% polyacrylamide gel and transferred to nitrocellulose; the blot was analyzed using the $G_{i\alpha\text{-}1}$-specific antiserum I1C. *Lane 4*,immunostaining of 5 ng $G_{i\alpha\text{-}1}$. Two additional experiments gave comparable results.

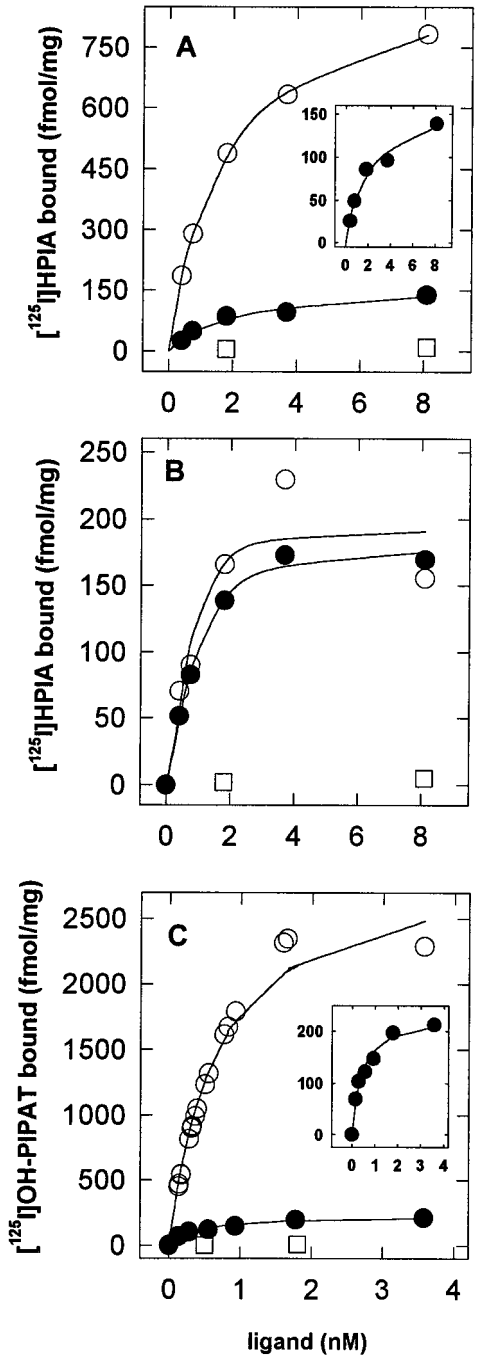

Fig. 4. Agonist radioligand binding to the rat (A) and human A_1 adenosine (B) and human D_2 dopamine receptors (C) stably expressed in HEK 293 cell membranes. Membranes were prepared from control (○) and pertussis toxin-treated (□) HEK 293 cells expressing the recombinant rat (A) and human (B) A_1 adenosine receptors and the human D_2 dopamine receptors (C). Membranes prepared from pertussis toxin-treated cells were also stably reconstituted with $G_{i\alpha\text{-}1}$ (4.5 ng/mg; ●). Saturation isotherms were generated with the agonist radioligands [^{125}I]HPIA for rat (A) and human A_1 adenosine receptors (B) or [^{125}I]OH-PIPAT for the human D_2 dopamine receptor (C). The binding reaction was carried out in 40 μl containing membranes (2–5 μg) and the indicated concentrations of the radioligands for 90 min at 25°. Nonspecific binding was determined in the presence of 1 μM XAC (A, B) or 10 μM sulpiride (C) and amounted to ~5% of total binding. *Insets* (A and C), saturation isotherms of the stably reconstituted membranes (●) with the *y*-axis range (fmol/mg of radioligand bound) scaled down. Data are mean values of duplicate determination; two additional experiments gave similar results.

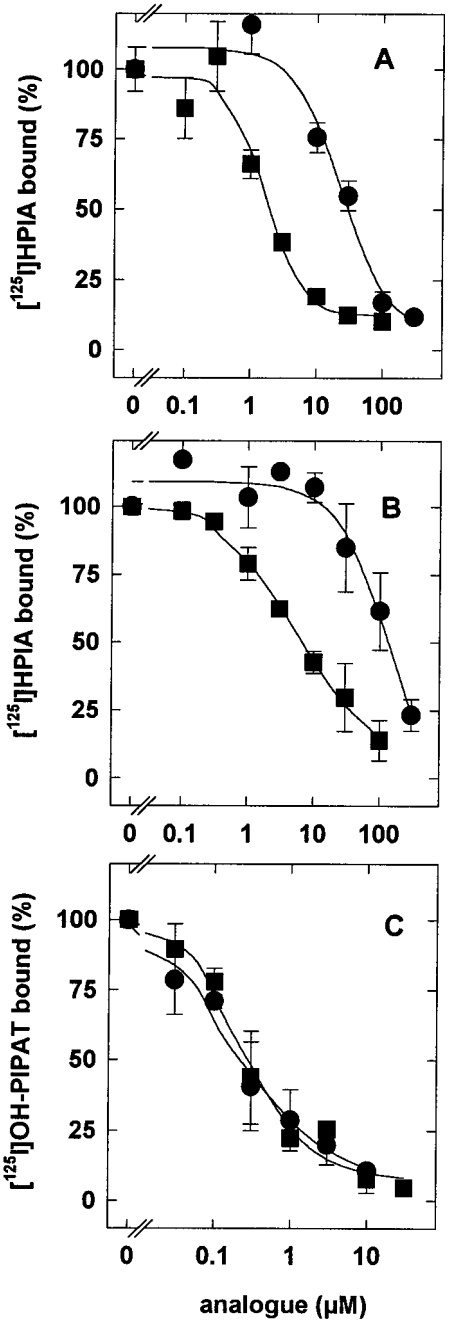

Fig. 5. Suramin- and NF037-mediated inhibition of agonist radioligand binding to membranes prepared from pertussis toxin-treated rat A_1 (A), human A_1 adenosine (B), and human D_2 dopamine receptors (C) in HEK 293 membranes that had been reconstituted with $rG_{i\alpha\text{-}1}$. Membranes prepared from pertussis toxin-treated cells were stably reconstituted with $rG_{i\alpha\text{-}1}$, and the binding reaction was carried out in 40 μl containing membrane protein (~10 μg), [^{125}I]HPIA (final concentration, 1.5 nM in A and B), or [^{125}I]OH-PIPAT (0.7 nM, C) and increasing amounts of suramin (■) and NF037 (●) for 90 min at 25° as outlined in Experimental Procedures. The reconstituted membranes used in C were from the cell clone expressing D_2 dopamine receptors at 3.9 pmol/mg. Specific binding in the absence of any analogue (~1 fmol ligand bound) was set 100%. Data are mean ± standard error from three independent experiments carried out in duplicate.

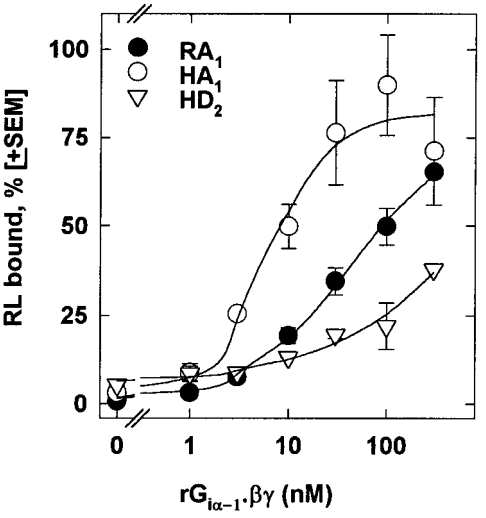

Fig. 6. Reconstitution of high affinity agonist binding to A_1 adenosine and D_2 dopamine receptors in pertussis toxin-treated HEK 293 cell membranes by $rG_{\alpha i\text{-}1}$. Membranes (~10 μg protein) prepared from pertussis toxin-treated cells expressing the rat A_1 (●), the human A_1 adenosine (○), or the human D_2 dopamine receptors (▽) were preincubated with increasing concentrations of a combination of $rG_{i\alpha\text{-}1}$ and $\beta\gamma$ (molar ratio = 1:4) in the presence of 0.5% octylglucoside. The binding reaction included radioligand (~1.9 nM [^{125}I]HPIA or ~0.6 nM [^{125}I]PIPAT), $rG_{i\alpha\text{-}1}$ at the indicated concentrations, and octylglucoside at a final concentration of 0.25%. Nonspecific binding was assayed in the presence of 1 μM XAC or 10 μM sulpiride. In the same experiment, specific binding was determined in untreated HEK 293 membranes and was set at 100%. These values were 158 ± 41, 447 ± 5, and 576 ± 87 fmol/mg for human and rat A_1 adenosine and the human D_2 dopamine receptor, respectively. Data are mean ± standard error from three experiments.

In the control experiments, in which haloperidol and sulpiride were allowed to compete with [^{125}I]OH-PIPAT at radioligand concentrations covering the range 0.3–3 nM, the intercepts yielded K_i estimates of ~0.6 and ~5 nM for haloperidol and sulpiride, respectively (data not shown); the K_{DA} of [^{125}I]OH-PIPAT was estimated from these experiments to be in the range of 0.55–1.0 nM (i.e., consistent with the K_D determined in saturation binding experiments; see Fig. 4).

In an analogous experiment, the occupancy of the human A_1 adenosine receptor in HEK 293 membranes was varied by using [^{125}I]HPIA concentrations covering the range of 0.15–7.5 nM. The IC_{50} value of suramin increased in a linear manner with receptor occupancy (Fig. 7C, ●). For the purpose of comparison, the data obtained by varying occupancy of the D_2 dopamine receptors with increasing [^{125}I]OH-PIPAT have been replotted as a fraction of B_{max} (Fig. 7C, ■). It is evident that the slope of the regression line determined for uncoupling the A_1 adenosine receptor (~18) was considerably steeper than that determined for inhibition of [^{125}I]OH-PIPAT binding, whereas the y-axis intercepts are similar within experimental error. Division of the y-axis intercept (0.18 μM) by the slope (~18) yielded a value of ~10 nM for the K_{DA} of the activator. This is in reasonable agreement with the affinity of the agonist-liganded human A_1 adenosine receptor for $rG_{i\alpha\text{-}1}$ determined in the reconstitution experiment (see Fig. 6).

Discussion

The current results unequivocally demonstrate that suramin and its didemethylated analogue NF037 discriminate among receptor/G protein tandems formed by the A_1 adenosine and the D_2 dopamine receptor regardless of whether the inhibition of high affinity agonist binding or of agonist-stimulated binding of [^{35}S]GTPγS was determined. Suramin was more potent than NF037 in uncoupling A_1 adenosine receptors, whereas the two compounds were equipotent in preventing the interaction of the D_2 dopamine receptor with G proteins. These observations complement and extend previous work that was carried out on A_1 adenosine receptors in human brain cortex and D_2 dopamine receptors in rat striatum. Here, we used both the rat and human A_1 adenosine receptor and therefore rule out species differences as a trivial explanation for the distinct activity profiles of the two compounds. Because the receptors were stably expressed in the same cell line, an effect of cellular heterogeneity also can be ruled out. In agreement with this conclusion, the activity profile of the compounds was indistinguishable from the findings obtained in HEK 293 cells when the receptors were transiently expressed in COS-7 cells to obtain a nonhuman tissue readout system (not shown). Finally, the receptors may have been targeted to different subcellular compartments that possibly differed in composition of G protein subunits; when heterologously expressed in a cell line derived from a polarized epithelium, α_2-adrenergic and A_1 adenosine receptors are localized in the basolateral and apical membrane, respectively (Saunders *et al.*, 1996; Wozniak and Limbird, 1996). This potential source of error was eliminated by pretreating the cells with pertussis toxin and stably reconstituting high affinity agonist binding to the membranes with a defined G protein α subunit ($rG_{i\alpha\text{-}1}$). Thus, the higher affinity of NF037 for uncoupling the D_2 dopamine receptor (compared with its ability to uncouple A_1 adenosine receptors) is maintained even when the receptors are forced to interact with identical G protein α subunits. Uncoupling of the D_2 dopamine receptor/G protein complex by suramin analogues gave inhibition curves with varying slopes (Figs. 1C and 5C). On pertussis toxin treatment and reconstitution with $G_{i\alpha\text{-}1}$, the inhibition curves were shallower than in the control membranes. A steep slope (Hill coefficient ~ −2) suggests interference with a reaction different from the 1:1 mode of receptor/G protein coupling, such as through the formation of receptor dimers. Dimerization of G protein-coupled receptors might result in enhanced signaling efficacy as opposed to the monomeric form of receptor (Hebert *et al.*, 1996). On the basis of evidence obtained with other types of G protein-coupled receptors (Hebert *et al.*, 1996; Cvejic and Devi, 1997), it is attractive to speculate that the D_2 dopamine receptor in HEK 293 cells undergoes dimerization leading to steep inhibition curves with the suramin analogues. On pertussis toxin treatment of the membranes and reconstitution with $G_{i\alpha\text{-}1}$ shallow inhibition curves (Hill coefficient ~ −1) would suggest that the ability to dimerize is lost after manipulation of the membranes. Nevertheless, the slope of the inhibition curves but not the IC_{50} values was independent of the fractional receptor occupancy generated in the inhibition experiments (see Fig. 7A). Thus, although we have no direct evidence to explain the changes in slopes, we believe that this discrepancy does not interfere with our conclusions. Finally, we stress that experiments with purified α subunit and [^{3}H]suramin demonstrate a binding stoichiometry of 1:1 (Hill coefficient ~ 1.0; Hohenegger *et al.*, 1998).

If the pertussis toxin-treated membranes were stably re-

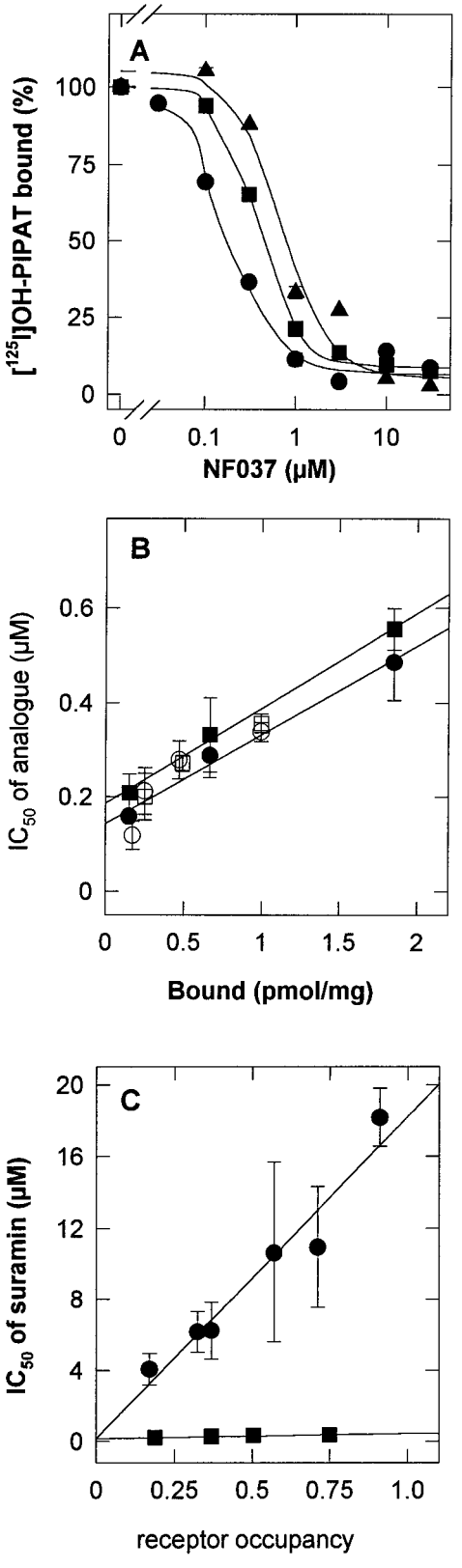

Fig. 7. Correlation between receptor agonist occupancy and the IC_{50} values for suramin and NF037 in uncoupling the human D_2 dopamine receptor. A, Inhibition of [^{125}I]OH-PIPAT binding was performed with membranes derived from three different HEK 293 cell clones expressing the D_2 dopamine receptor at various densities (● 0.3, ■ 1.3, ▲ 3.9 pmol/mg). Concentration-dependent inhibition of agonist binding by NF037 was determined at a concentration of 0.5 nM [^{125}I]OH-PIPAT with 1–10 μg of membrane protein such that the proportion of bound radioligand was <10% of the amount of radioligand added to the incubation volume. Inhibition curves were fitted by nonlinear least-squares regression analysis. B, The IC_{50} of NF037 (●) determined in Fig. 1A and those of suramin (■) determined in parallel were replotted as a function of [^{125}I]OH-PIPAT bound. In addition, inhibition binding experiments with NF037 (○) and suramin (□) were carried out at logarithmically spaced concentrations of radioligand (0.1–1 nM) on membranes prepared from the clone with intermediate expression levels (B_{max} = 1.3 pmol/mg) resulting in the indicated occupancy of the receptor by [^{125}I]OH-PIPAT. Each point represents the IC_{50} ± standard error of the estimate from three independent experiments. *Straight lines*, drawn by calculating the linear regression through all data points. C, The IC_{50} value of suramin was determined in inhibition experiments with various concentrations of [^{125}I]HPIA (0.15–7.5 nM) on HEK 293 membranes that carried the human A_1 adenosine receptor (●). These IC_{50} values were plotted as a function of relative receptor occupancy (i.e., B_{max} was set at 1.0). The IC_{50} values of suramin in inhibiting [^{125}I]OH-PIPAT binding to the membranes with intermediate expression levels (■; B_{max} = 1.3 pmol/mg) from B were replotted for the purpose of comparison and receptor occupancy was also expressed as a fraction of B_{max}.

constituted with $rG_{i\alpha\text{-}1}$, large differences in reconstitution efficiencies were observed, and the rank order was human A_1 > rat A_1 > human D_2 receptor; the amount of G protein incorporated into the membrane was clearly not limiting because the α subunit was present in vast excess over the receptor level. We therefore hypothesized that the differences in reconstitution reflected the rank order of affinity of the individual receptors for $rG_{i\alpha\text{-}1}$. If correct, it was likewise sensible to assume that the affinities of individual receptors for the same G protein is the major determinant for the potency of suramin in differentially inhibiting receptor/G protein coupling. The difference in G protein affinity was confirmed by titrating the ability of exogenously added $rG_{i\alpha\text{-}1}$ to reconstitute high affinity binding to pertussis toxin-treated membranes. We previously determined the affinity of the human A_1 adenosine receptor expressed in *E. coli* in reconstitution experiments with individual forms of recombinant G protein α subunits (Jockers *et al.*, 1994); the affinity currently observed for the interaction of the human A_1 adenosine receptor in pertussis toxin-treated HEK 293 membranes with $rG_{i\alpha\text{-}1}.\beta\gamma$ (~6 nM) was in reasonable agreement with that estimated in the earlier work (~15 nM). It was, on the other hand, somewhat surprising that the affinity of the D_2 dopamine receptor for $rG_{i\alpha\text{-}1}.\beta\gamma$ was so low.

The interaction between the D_2 dopamine receptor and $rG_{i\alpha\text{-}1}.\beta\gamma$ may have been impeded by the presence of octylglucoside. We therefore exploited the Cheng-Prusoff relation to independently estimate the affinity of the human D_2 dopamine receptor for its cognate G protein in HEK 293 membranes. This approach is valid if suramin competes with the agonist-liganded receptor for binding to the G protein α subunit. All available evidence supports this assumption: (1) suramin analogues bind directly to G protein α subunits (Freissmuth *et al.*, 1996), (2) they do not affect binding of antagonists or agonists to the receptor in the absence of receptor/G protein coupling (Beindl *et al.*, 1996), (3) the inhibition of receptor/G protein coupling can be overcome by raising the concentration of active receptor in the membrane (see Fig. 7), and (4) if the site of action of suramin is on the G protein, the regression lines in the plot of IC_{50} versus receptor occupancy are expected to yield similar *y*-axis intercepts; this was indeed observed. The affinity values estimated for the interaction between receptor and G protein were ~0.8 μM and ~10 nM for agonist-liganded D_2 dopamine receptor and the human A_1 adenosine receptor, respectively, and hence

consistent with the findings in the reconstitution experiments. Based on our experimental observations, we conclude that the affinity of the individual receptors for $rG_{i\alpha\text{-}1}$ is inversely correlated to the potency of suramin in uncoupling the receptors. This conclusion predicts that a selective action of suramin on receptor/G protein coupling can also result from the difference in affinity of individual agonist-liganded receptors for the same G protein. This may also explain the earlier observation that in membranes from NG108–15 cells, suramin inhibited activation of pertussis toxin-sensitive G proteins by δ-opioid agonists but not by serum factors (Huang *et al*., 1990).

The structural basis for the different activity profile of suramin analogues in uncoupling A_1 adenosine (suramin > NF037) and D_2 dopamine receptors (NF037 = suramin) is not known. The contact points by which receptors interact with their cognate G proteins are formed by those segments of the receptor that are juxtaposed to the transmembrane spans. These discontiguous segments cooperatively support binding of the receptor to the G protein oligomer (Ernst *et al*., 1995; Gomeza *et al*., 1996) and determine the G protein specificity of the receptor (Wong *et al*., 1994; Liu and Wess, 1996; for review, see Gudermann *et al*., 1996b). The amino acids DRY (ERY in rhodopsin) at the beginning of the second intracellular loop are invariant and are required for G protein activation (Scheer *et al*., 1996). Apart from this triplet, only very few amino acids are conserved within the intracellular loops; hence, a clearcut consensus sequence that would allow to predict the G protein specificity of a given receptor cannot be deduced. In addition, the ability of a receptor to activate multiple G proteins is specified by distinct portions within the intracellular loops; the α_{2A}-adrenergic receptor can couple to both $G_{s\alpha}$ and $G_{i\alpha}$. These two coupling modes, however, require distinct amino acid stretches in the second and third intracellular loops (Eason and Ligett, 1996). It is even more striking that different amino acids in the third intracellular loop are required to support coupling of the α_{1B}-adrenergic receptor to the closely related α subunits $G_{\alpha q}$, $G_{\alpha\text{-}14}$, and $G_{\alpha\text{-}16}$ (Wu *et al*., 1995). These findings predict that the surface the receptors cover on a given G protein α subunit varies in individual receptor/G protein tandems. This is indeed the case; if the five last amino acids in the carboxyl terminus are exchanged between $G_{s\alpha}$ and $G_{q\alpha}$, some, but not all, receptors are capable of recruiting this mutated α subunit in a manner similar to their cognate G protein (Conklin *et al*., 1996). Hence, the contact sites that are formed in individual receptor/G protein tandems must be different to account for this observation. It is attractive to speculate that in the A_1 receptor/$G_{i\alpha\text{-}1}$ tandem, the receptor covers a larger area of the G protein α subunit than in the D_2 receptor/$G_{i\alpha\text{-}1}$ tandem. This hypothesis would explain both the higher affinity of the A_1 receptor for $G_{i\alpha\text{-}1}$ and the lower relative potency of NF037 in disrupting the A_1 receptor/G complex; in this model, suramin, which has two additional methyl groups, competes more efficiently than NF037 with the A_1 adenosine receptor for binding to the G protein, whereas the difference in surface covered by the two compounds does not affect the formation of the D_2 receptor/G protein complex. Taken together, our data show that two factors contribute to the selectivity of inhibitors of receptor/G protein tandem formation, namely (1) differences in affinity of individual receptors for the G protein (which determines the apparent IC_{50} value of an inhibitor) and (2) differences in the contact site between individual receptors and the G protein (which gives rise to a distinct structure activity relation for inhibitors). Both aspects are relevant in the development of G protein inhibitors that may eventually be useful *in vivo*.

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