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Biochemical Pharmacology 65 (2003) 1139–1150
Biochemical Pharmacology
www.elsevier.com/locate/biochempharm

Interaction of the D_{2short} dopamine receptor with G proteins: analysis of receptor/G protein selectivity

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Received 28 August 2002; accepted 20 December 2002

Abstract

The human D_{2short} (D_{2S}) dopamine receptor has been expressed together with the G proteins Gi2 and Go in insect cells using the baculovirus system. Levels of receptor were determined using [^{3}H]spiperone binding. Levels of G protein heterotrimer were determined using quantitative Western blot and using [^{35}S]GTPγS saturation binding experiments. Levels of the receptor and G protein and the receptor/G protein ratio were similar in the two preparations. Stimulation of [^{35}S]GTPγS binding by a range of agonists occurred with higher relative efficacy and in some cases higher potency in the preparation expressing Go, indicating that interaction of the D_{2S} receptor is more efficient with this G protein. The effects of various G protein-selective agents on 10,11-dihydroxy-*N*-*n*-propylnorapomorphine ([^{3}H]NPA) binding were used to examine the receptor/G protein complex in the two preparations. Suramin inhibited [^{3}H]NPA binding with slightly higher potency in the Gi2 preparation, whereas GppNHp inhibited [^{3}H]NPA binding with greater potency (~6-fold) in the Go preparation. This may imply that the G protein is more readily activated in the D_{2S}/Go preparation. [^{3}H]Spiperone binding occurred with an increased B_{max} in the presence of suramin in the Go preparation but not in the Gi2 preparation, suggesting a higher affinity interaction between the free receptor and this G protein. It is concluded that the higher efficiency activation of Go by the D_{2S} receptor may be a function of higher affinity receptor/G protein interaction as well as a greater ability to activate the G protein.

Keywords: D_2 dopamine receptor; G proteins; Selectivity; Agonist binding; [^{35}S]GTPγS binding

1. Introduction

The interaction of the receptor and G protein is a critical event in the mechanism of agonist action at G protein coupled-receptors (GPCRs). Agonist activation of GPCRs is often described using the ternary complex or extended ternary complex models [1,2]. In the extended ternary complex model, the receptor is proposed to exist in two forms, the ground state R and a partially activated state R*. R* is able to couple more efficiently to the G protein to form the fully active species R*G. Agonists (A) stabilise the R* and R*G forms of the receptor leading to the formation of the ternary complex AR*G. In the activated forms of the receptor (R*G and AR*G), the exchange of GTP for GDP occurs on the α subunit and once GTP is bound, both the α and the βγ subunits are released to alter the activity of effectors. The specific determinants of agonist efficacy and potency are, however, still unknown.

Some receptors have been shown to interact with multiple G proteins [3]. It has been proposed that, when a receptor interacts with more than one G protein, different agonists may have the ability to induce differentially activated forms of a receptor which have the ability to selectively activate one G protein over another. This concept has been termed agonist-induced receptor trafficking [4]. Evidence for this phenomenon has been demonstrated in a number of systems, including the 5-HT_{2C} receptor [5] and the Drosophila octopamine/tyramine receptor [6]. The introduction of compounds that disrupt the interaction of receptors and G proteins [3] may allow for selective disruption of signalling cascades.

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Abbreviations: ECL, enhanced chemiluminescence; GPCR, G protein-coupled receptor; K_l and K_h, lower and higher affinity dissociation constants; moi, multiplicity of infection; NPA, 10,11-dihydroxy-*N*-*n*-propylnorapomorphine; 3-PPP, 3-(3-hydroxyphenyl)-*N*-propylpiperidine.

0006-2952/03/$ – see front matter.
doi:10.1016/S0006-2952(03)00040-6

Receptors for the neurotransmitter, dopamine, constitute a family of GPCRs that can be divided into D_1-like (D_1, D_5) and D_2-like (D_2, D_3, D_4) receptor subtypes [7]. The D_2 dopamine receptor is an important site of action for the antipsychotic and anti-Parkinsonian drugs and has been shown to interact with different members of the Gi/o protein family to influence different signalling events. Signalling of the D_2 receptor through Gi2 leads to the inhibition of adenylyl cyclase [8,9], whereas signalling through Go leads to the inhibition of calcium channels [10]. A clear definition of the selectivity of the D_2 receptor for one G protein over another has, however, not yet emerged. The D_2 dopamine receptor also exists in short and long isoforms (D_{2S} and D_{2long} (D_{2L})) generated by alternative splicing [11,12]. There are also indications that the two isoforms may have different functions [13] and couple differentially to G proteins [14,15], but the precise selectivity has not been defined.

We studied the interaction of rat D_{2L} with the G proteins Go and Gi2 by expression in insect cells using the baculovirus system and showed a stronger interaction between the D_{2L} receptor and Go [16]. The baculovirus expression system is an ideal system for the study of interaction of D_2 receptors and G proteins as it does not contain endogenous dopamine receptors and there is minimal interaction between the receptor and the endogenous G proteins of the insect cells [17,18]. In both our previous [16] and the present study, we determined the receptor/G protein ratio in the membranes following infection as this may affect the potency and efficacy of agonists and inverse agonists [19–21].

In the present study, the human dopamine D_{2S} receptor has been co-expressed with G proteins Go and Gi2 in insect cells in order to examine the specificity of receptor/G protein interaction for this isoform. The potency and efficacy of a range of dopaminergic agonists has been measured using the stimulation of [^{35}S]GTPγS binding. Our data suggest that the human dopamine D_{2S} receptor couples preferentially to the G protein Go over the G protein Gi2.

2. Materials and methods

2.1. Materials

[*phenyl*-4-^{3}H]Spiperone (0.5–1.1 TBq/mmol) and [^{35}S]GTPγS (37–55 TBq/mmol) were obtained from Amersham Biosciences and [^{3}H]NPA (1.9–3.0 TBq/mmol) was obtained from Perkin-Elmer Life Sciences. All other chemicals were from Sigma. Oligonucleotides were synthesised and desalted by Invitrogen. Baculoviruses expressing the human G protein subunits, αo, αi1, αi2, αi3, β1 and γ2, were generously donated by Dr. T. Kozasa (University of Chicago). Purified human G protein subunits, αo, αi2 and βγ dimers [16], were generously donated by Dr. S. Graber (West Virginia University).

2.2. Construction of baculovirus expressing HIV-tagged D_{2S} receptor

Human D_{2S} DNA (generously supplied by Dr. Mark Cockett, Wyeth, Taplow, UK) was amplified using PCR supermix (GibcoBRL) following the manufacturer's instructions with primers designed to add an *Nde*I site to the 5′ end and an *Eco*RI site to the 3′ end. The PCR product was then subcloned into the pcTOPO2.1 vector (Invitrogen) to produce the plasmid TOPOD2S. Synthetic oligonucleotides encoding a tag derived from the gp120 protein of HIV (Ser-Thr-Ser-Ile-Arg-Gly-Lys-Val-Gln) and a 3′ *Nde*I site were annealed, and digested with *Nde*I. The receptor bearing the tag sequence exhibited identical properties in ligand binding and signalling assays as the native receptor (data not shown). TOPOD2S was digested with *Eco*RI and *Nde*I and the D_{2S} DNA fragment and the tag were ligated. The ligation mixture was subjected to PCR to selectively amplify tagged receptor, with one primer specific to the 3′ end of the D_2 DNA and the other primer specific to the 5′ end of the tag. The second primer also added a *Xho*I site and a start codon to the 5′ end of the tag. This PCR product was digested with *Xho*I and *Eco*RI and ligated into the plasmid pBlueBac4.5 (Invitrogen) to produce the plasmid pBBHD2S which was sequenced to ensure the integrity of the insert. The plasmid pBBHD2S was then co-transfected with Bac-N-Blue™ DNA (Invitrogen) and underwent recombination to produce recombinant virus. Recombination of the virus was confirmed by PCR using a virus-specific primer and a D_2 DNA-specific primer. All viruses were purified using plaque purification and amplified [22].

2.3. Cell culture

Suspension cultures of *Spodoptera frugiperda* (*Sf*9) cells were grown in TC-100 medium containing 10% foetal bovine serum and 0.1% pluronic F68® (GibcoBRL). Cells were seeded in suspension at a density of between 0.3×10^6 and 0.6×10^6 cells/mL, and allowed to grow until they reached a density of 1×10^6 cells/mL (log phase). The cells were then infected with a given moi of virus and harvested 48 hr after infection.

2.4. Membrane preparation

Cells were collected by centrifugation for 10 min at 1700 *g*. For every 50 mL of cell suspension used, 5 mL of ice-cold buffer (20 mM HEPES, 6 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, pH 7.4) was used to resuspend the cell pellet, which was transferred to a Dounce glass:glass homogeniser. The cells were homogenised at 4° by 40 strokes of the Dounce homogeniser. The homogenate was centrifuged for 10 min at 1700 *g*, the supernatant was collected and centrifuged at 48,000 *g* (4°) for 1 hr. The resulting pellet was resuspended in buffer and aliquots of

250 μL were stored at −80°. The method of Lowry *et al.* [23] was used to determine the protein content of membrane preparations, using BSA as standard.

2.5. Determination of G protein level using quantitative Western blot

Before analysis, proteins (*Sf*9 membrane or pure G protein subunits) were denatured by the addition of two times electrophoresis loading buffer (100 mM Tris–HCl, 200 mM dithiothreitol, 4% sodium dodecyl sulphate, 0.2% bromophenol blue, 20% glycerol) and heated at 90° for 5 min. *Sf*9 membrane proteins (20–40 μg) and G protein standards were separated by sodium dodecyl sulphate–polyacrylamide gel electrophoresis on 12% acrylamide gels. Samples were then transferred to nitrocellulose membranes using the Biorad semi-dry transfer system. Nitrocellulose membranes were incubated for 1 hr with 5% dried milk (w/v) in buffer (137 mM NaCl, 3 mM KCl, 25 mM Tris–HCl, 0.1% Tween). Blots were then incubated overnight at 4° with single primary antibodies (MAB3073 anti-αo, 1 μg/mL (Chemicon); C-10 anti-α1–3, 1 μg/mL (Santa Cruz); C-16 anti-β1, 0.4 μg/mL (Santa Cruz); A-16 anti-γ2, 0.4 μg/mL (Santa Cruz)) in buffer containing 5% dried milk (w/v). Blots were washed five times with buffer (10 min each) and then incubated with secondary antibody (anti-mouse (αo)/anti-rabbit (αi1–3, β1, γ2) immunoglobin horseradish peroxidase conjugate (1:5000; Sigma) for 1 hr. Blots were then washed three times (10 min each) with buffer, before exposure to equal volumes of enhanced chemiluminescence (ECL) detection reagents 1 and 2 (Amersham Biosciences). Blots were then wrapped in clingfilm and exposed to Hybond-ECL X-ray film for between 30 s and 2 min. Densitometry was performed using a GS710 Calibrated Imaging Densitometer (Biorad) and data analysed using GraphPad PRISM. Determinations of levels of G protein subunits were always performed using ECL exposures that ensured a linear dependence of band density on protein amount.

2.6. Saturation binding assays with [^{3}H]spiperone and [^{3}H]NPA

Cell membranes (25 μg protein) were incubated in triplicate with [^{3}H]spiperone at concentrations ranging from 0.001 to 2 nM, or [^{3}H]NPA at concentrations ranging from 0.0025 to 5 nM, in a total volume of 1 mL buffer (20 mM HEPES, 6 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, pH 7.4). Non-specific binding was determined by the inclusion of 3 μM (+)-butaclamol. The reaction was initiated by the addition of membranes and the tubes were incubated at 25° for 3 hr. The reaction was terminated by rapid filtration using a Brandel cell harvester (Semat) with four 3 mL washes of ice-cold PBS (140 mM NaCl, 2.7 mM KCl, 1.5 mM KH_2PO_4, 8 mM Na_2HPO_4, pH 7.4). The filters were soaked in 2 mL scintillation fluid (Optiphase Hisafe 3, Wallac) overnight before the amount of radioactivity present was determined by liquid scintillation counting.

2.7. Competition binding experiments vs. [^{3}H]spiperone and [^{3}H]NPA

Cell membranes (25 μg protein) were incubated with 0.3 nM [^{3}H]spiperone and various concentrations of competing ligand in triplicate, in a final volume of 1 mL buffer (20 mM HEPES, 6 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, pH 7.4). Non-specific binding was determined by the inclusion of 3 μM (+)-butaclamol. The reactions were initiated, incubated and terminated as described earlier. The effect of guanine nucleotides on agonist binding was assessed by the addition of 100 mM NaCl and 100 μM GTP to the buffer. Competition experiments vs. [^{3}H]NPA (0.35 nM unless otherwise stated) were carried out essentially as for [^{3}H]spiperone, but 100 mM NaCl was always included in the buffer. For both radioligands specific binding was ∼90% of total binding in these competition experiments.

2.8. Agonist stimulation of [^{35}S]GTPγS binding

Cell membrane (5 μg) was incubated in 80 μL buffer (20 mM HEPES, 100 mM NaCl, 6 mM $MgCl_2$, pH 7.4) containing 1 μM GDP and various concentrations of agonist. All experiments were performed in duplicate. The reaction was initiated by the addition of cell membranes and incubated at 30° for 30 min. A 20 μL volume of [^{35}S]GTPγS (0.2 nM final concentration) was then added and the incubation continued for a further 30 min. The reaction was terminated by rapid filtration with a Brandel cell harvester (Semat) and four 1.5 mL washes with ice-cold PBS. Radioactivity was determined as described under saturation analyses earlier.

2.9. Saturation analysis of [^{35}S]GTPγS binding

The number of G proteins in infected *Sf*9 cells was determined by [^{35}S]GTPγS isotopic dilution saturation binding. This method is based on the idea that if insect cells are infected at the same time, one flask with viruses encoding D_{2S}, αx, β1, γ2 and one with viruses encoding D_{2S}, β1, γ2, the increase in [^{35}S]GTPγS binding in membranes prepared from the first culture compared to the second is due to expression of the α subunit. Cell membranes (25 μg) from the two cultures were prepared and incubated for 30 min at 30°, in triplicate in a final volume of 1 mL of buffer (20 mM HEPES, 100 mM NaCl, 6 mM $MgCl_2$, pH 7.4) containing 0.1 nM [^{35}S]GTPγS. Binding of [^{35}S]GTPγS was inhibited with unlabelled GTPγS at concentrations ranging from 0.05 to 50 nM. Reactions were initiated by the addition of membranes. Termination and determination of radioactivity were as for [^{3}H]spiperone saturation binding experiments.

The number of G proteins was calculated by the following equation:

$$B_{\text{total}} = \frac{[[^{35}\text{S}]\text{GTP}\gamma\text{S}_{\text{bound}}][\text{GTP}\gamma\text{S}_{\text{total}}]}{[[^{35}\text{S}]\text{GTP}\gamma\text{S}_{\text{added}}]}$$

$[[^{35}\text{S}]\text{GTP}\gamma\text{S}_{\text{bound}}]$ is the $[^{35}\text{S}]$GTPγS binding determined as the difference in binding between the two preparations detailed earlier, $[\text{GTP}\gamma\text{S}_{\text{total}}]$ is the total concentration of GTPγS (radioactive plus non-radioactive) and $[[^{35}\text{S}]\text{GTP}\gamma\text{S}_{\text{added}}]$ is the concentration of $[^{35}\text{S}]$GTPγS added to assays. Saturation curves were then generated to determine the apparent $B_{\max}$ of functional G protein in the preparations containing α subunits.

2.10. Data analysis

Data were analysed by non-linear regression using PRISM (GraphPAD Software Inc.). Ligand binding data (saturation analyses and competition analyses with dopamine or NPA) were analysed using one- or two-binding site models and the best fit to the data determined using a partial *F*-test. For experiments on the effects of guanine nucleotides or suramin on [^{3}H]NPA binding, data were analysed using one-binding site models or a model with a variable Hill coefficient and the best fit determined using a partial *F*-test. K_i values of compounds (or corrected IC_{50} values (IC_{50}corr), where the Hill coefficient of the competition curve was less than 1) for inhibition of [^{3}H]spiperone or [^{3}H]NPA binding were determined using the method of Cheng and Prusoff [24]. For assays with [^{3}H]NPA, the higher affinity dissociation constant of NPA was used in this calculation, as at the concentration of [^{3}H]NPA used (0.35 nM) the majority of binding will be to the higher affinity state. Statistical significance determined using either Student's two-tailed *t*-tests or one-sample *t*-tests or ANOVA followed by Tukey's *post hoc* test as indicated.

3. Results

3.1. Quantification of receptors and G proteins

Human D_{2S} receptors were co-expressed with G protein αi2 or αo subunits and β1 and γ2 subunits, at moi of R:α:β:γ of 3:6:6:6. D_{2S} was also co-expressed with β1 and γ2 subunits but without α subunit as a negative control for α subunit expression in studies on saturation analyses of GTPγS binding (see the following description and Section 2).

Saturation analysis of [^{3}H]spiperone binding to D_{2S} receptors gave a K_d of 89 pM ($pK_d = 10.05 \pm 0.12$, mean ± SEM (N = 3)). This dissociation constant was unaffected by the co-expression of G protein subunits. Receptor levels, as measured by saturation binding analyses with [^{3}H]spiperone in the two preparations used for co-expression studies, were comparable at approximately 1.5 pmol/mg. G protein levels were estimated by isotopic dilution GTPγS saturation studies and the level of Go was not significantly different to the level of Gi2 (Student's *t*-test, $P > 0.05$) (Table 1, Fig. 1).

Table 1
Expression levels of D_{2S} receptors and the G protein subunits (αo, αi2, β1 and γ2)

	$D_{2S}\beta1\gamma2$	$D_{2S}\alpha o\beta1\gamma2$	$D_{2S}\alpha i2\beta1\gamma2$	N
$B_{\max}$ D_{2S} (pmol/mg)	1.38 ± 0.09	1.51 ± 0.13	1.42 ± 0.09	3
$B_{\max}$ GTPγS (pmol/mg)	–	10.33 ± 1.68	9.11 ± 1.36	4
α (pmol/mg)	–	408 ± 120	263 ± 161	6
β1 (pmol/mg)	n.d.	163 ± 33	168 ± 43	6
γ2 (pmol/mg)	n.d.	21 ± 5	40 ± 20	6

D_{2S} expression levels were measured using saturation binding with [^{3}H]spiperone and G protein levels were measured using isotopic dilution GTPγS saturation studies ($B_{\max}$ GTPγS) and quantitative Western blot (α, β1, γ2) as described in the Section 2. Values are expressed as mean ± SEM (N experiments). n.d.: not determined.

G protein levels were also estimated by quantitative Western blotting to confirm this result (Fig. 2). Using this method it was shown that, although α and β1 expression levels were very high (in excess of 100 pmol/mg), expression of γ2 subunits was limiting in both the Go and the Gi2 preparations (Table 1). Nevertheless, no significant difference in the level of the G protein subunits α, β or γ was observed between the two preparations (Student's *t*-test, $P > 0.05$). The antibodies used were unable to detect endogenous insect cell G protein subunits under the conditions used (data not shown).

Based on these data and following the method described by Cordeaux *et al.* [16], a ratio of 6–7 (GTPγS saturation

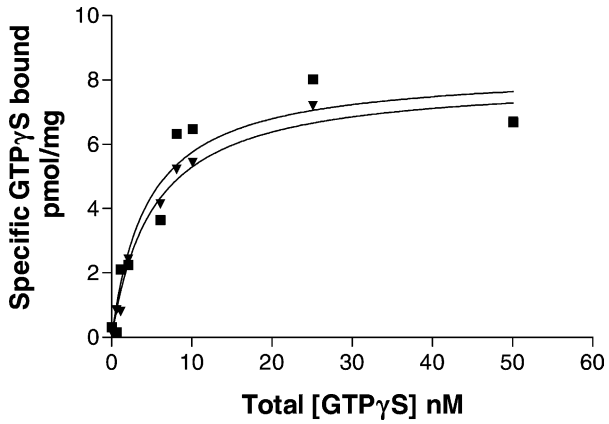

Fig. 1. Isotopic dilution saturation analysis of G protein expression in membranes from cells expressing $D_{2S}\alpha o\beta1\gamma2$ or $D_{2S}\alpha i2\beta1\gamma2$. Competition of non-radioactive GTPγS vs. 100 pM [^{35}S]GTPγS binding was determined as described in the Section 2 in membranes expressing $D_{2S}\alpha o\beta1\gamma2$ (▼) or $D_{2S}\alpha i2\beta1\gamma2$ (■); membranes from cells infected with $D_{2S}\beta1\gamma2$ were used as a control to determine the binding of GTPγS to endogenous insect cell GTPγS binding sites. Data for GTPγS binding in the $D_{2S}\alpha o\beta1\gamma2$ and $D_{2S}\alpha i2\beta1\gamma2$ preparations were corrected for GTPγS binding in the $D_{2S}\beta1\gamma2$ preparation and presented as a saturation curve. Graphs are representative of four independent experiments replicated as in Table 1.

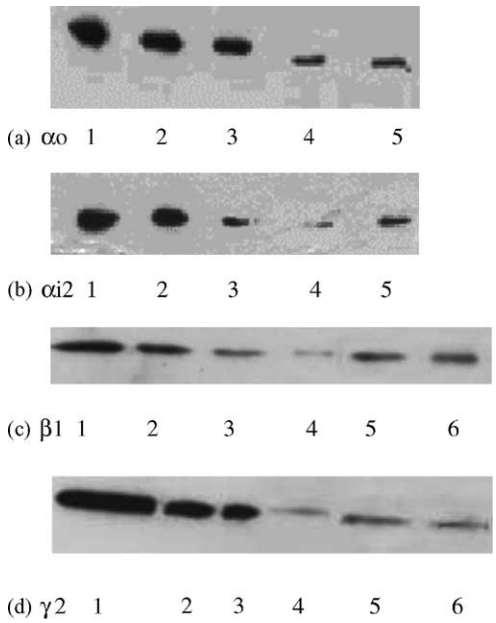

Fig. 2. Quantitative Western blot for G protein subunit levels in membranes from *Sf*9 cells expressing D_{2S} and G proteins. (a) Detection of αo: lanes 1–4 contain 1, 0.5, 0.25 and 0.1 μg pure αo subunit, lane 5 contains 20 μg of membrane protein from preparation expressing D_{2S}αoβ1γ2. (b) Detection of αi2: lanes 1–4 contain 0.5, 0.25, 0.1 and 0.075 μg pure αi2 subunit, lane 5 contains 20 μg of membrane protein from preparation expressing D_{2S}αi2β1γ2. (c) Detection of β1, lanes 1–4 contain 300, 200, 100, 50 and 25 ng of pure βγ subunit, lanes 5 and 6 contain 20 μg membranes from αo and αi2 preparations, respectively. (d) Detection of γ1, lanes 1–4 contain 150, 50, 25 and 10 ng of pure βγ subunit, lanes 5 and 6 contain 40 μg membranes from αo and αi2 preparations, respectively.

analyses) or 14–28 (quantitative Western blot) G proteins to every receptor was observed in both the Go and Gi2 preparations. The ratios in the two preparations were not significantly different. The G/R ratio is higher when estimated by quantitative Western blot, but we presume this reflects a proportion of inactive G protein. The data obtained using GTPγS saturation analyses reflect active heterotrimeric G protein and thus are likely to be more accurate.

3.2. Stimulation of [^{35}S]GTPγS binding by agonists

Agonist stimulation of [^{35}S]GTPγS binding was used as a measure of receptor-mediated G protein activation. Dopamine was unable to stimulate [^{35}S]GTPγS binding in membranes expressing only receptor and β1γ2 subunits. On co-expression with α subunits, basal [^{35}S]GTPγS binding increased in both preparations although basal binding increased to a greater extent in the Go preparation compared to the Gi2 preparation. Also clear stimulation of [^{35}S]GTPγS binding by dopamine was observed only when α subunits were co-expressed. The ability of saturating concentrations of dopamine to stimulate [^{35}S]GTPγS binding in the Go and Gi2 preparations, in terms of fmol [^{35}S]GTPγS bound per mg of protein was not significantly different (Fig. 3) although the percent stimulation of [^{35}S]GTPγS binding over basal was different in the two preparations (D_{2S}/Go 42 ± 2% and D_{2S}/Gi2 108 ± 7%, mean ± SEM (N = 4)). The potency of dopamine to stimulate [^{35}S]GTPγS binding was, however, higher in the Go preparation relative to the Gi2 preparation (Student's *t*-test, $P < 0.05$) (Table 2). This suggests that dopamine, acting through the D_{2S} receptor, displays a preference for the G protein Go over the G protein Gi2.

The ability of various dopamine agonists to activate the G proteins Go and Gi2 was measured using [^{35}S]GTPγS binding. Agonists exhibited a higher relative efficacy (compared to the maximal dopamine response) in the Go preparation compared with the Gi2 preparation (Table 2). It should be noted that, as the maximal stimulation for dopamine in terms of fmol/mg in each preparation was similar, the relative efficacy of the other agonists may be compared directly between the preparations. The agonists *m*-tyramine and 3-(3-hydroxyphenyl)-*N*-propylpiperidine ((+)-3-PPP), similarly to dopamine, also exhibited higher potencies in the Go preparation, but none of the other agonists tested showed significant differences in potency (Table 2, Fig. 3). Subtle variations in the order of magnitude of efficacy and potency of agonists were

Table 2
Potency and relative efficacy of dopamine agonists at D_{2S} receptors co-expressed with either αo or αi2 and β1 and γ2 G protein subunits

	Potency pEC_{50} (EC_{50}, nM)		Relative efficacy (percent maximal dopamine response)	
	D_{2S}αoβ1γ2	D_{2S}αi2β1γ2	D_{2S}αoβ1γ2	D_{2S}αi2β1γ2
Dopamine (N = 5)	6.91 ± 0.14 (123)	6.15 ± 0.15* (708)	100	100
m-Tyramine (N = 6)	5.86 ± 0.23 (1380)	5.29 ± 0.11* (5130)	93 ± 4	59 ± 9*
p-Tyramine (N = 4)	4.99 ± 0.28 (10230)	4.29 ± 0.20 (51290)	65 ± 4	32 ± 6*
(+)-3-PPP (N = 4)	6.27 ± 0.12 (540)	5.56 ± 0.23* (2750)	102 ± 6	60 ± 6*
(−)-3-PPP (N = 5)	6.86 ± 0.23 (245)	6.40 ± 0.30 (400)	54 ± 7	27 ± 10*
Quinpirole (N = 5)	6.18 ± 0.26 (660)	6.00 ± 0.20 (1000)	95 ± 9	84 ± 5
NPA (N = 3)	9.16 ± 0.20 (0.69)	8.89 ± 0.17 (1.29)	134 ± 8	106 ± 6*

Potency values were determined from the stimulation of [^{35}S]GTPγS binding as described in the Section 2 and are expressed as mean ± SEM (N experiments). Relative efficacy values are the percentage of the maximal dopamine stimulation observed in that preparation and are expressed as mean ± SEM (N experiments).

* Values at αo are significantly different from those at αi2 (Student's *t*-test, $P < 0.05$).

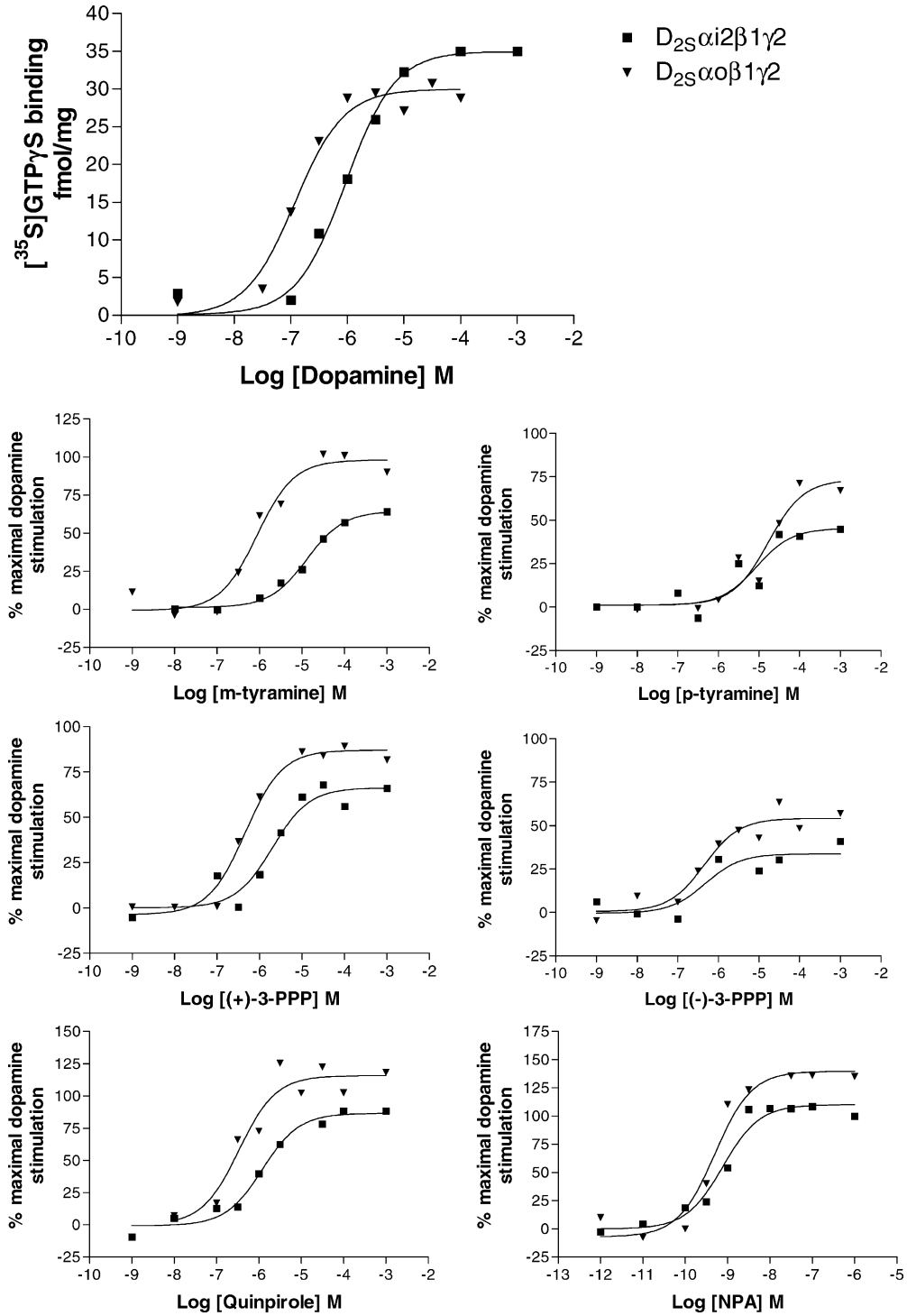

Fig. 3. Stimulation of [^{35}S]GTPγS binding by dopamine agonists in membranes expressing D_{2S}αoβ1γ2 (▼) or D_{2S}αi2β1γ2 (■). Dopamine stimulation is expressed as fmol [^{35}S]GTPγS bound per mg of protein; all other data are expressed as percent maximal dopamine stimulation. All graphs are representative of three to six independent experiments replicated as in Table 2.

observed between the two different preparations. In membranes co-expressing Go, the order of potency was NPA ⩾ dopamine ∼ (−)-3-PPP > (+)-3-PPP ∼ quinpirole > *m*-tyramine > *p*-tyramine. In membranes co-expressing Gi2, the order of potency was NPA ⩾ (−)-3-PPP ∼ dopamine ∼ quinpirole > (+)-3-PPP > *m*-tyramine > *cp*-tyramine. In membranes co-expressing Go, the order of efficacy was NPA > (+)-3-PPP ∼ dopamine ∼ quinpirole ∼ *m*-tyramine > *p*-tyramine > (−)-3-PPP. In membranes co-expressing Gi2, the order of efficacy was NPA ∼ dopamine > quinpirole > (+)-3-PPP ∼ *m*-tyramine > *p*-tyramine ∼ (−)-3-PPP.

Table 3
Binding of dopamine and NPA in membranes expressing D_{2S} receptors and G protein subunits

	Dopamine			NPA		
	$D_{2S}\beta1\gamma2$	$D_{2S}\alpha o\beta1\gamma2$	$D_{2S}\alpha i2\beta1\gamma2$	$D_{2S}\beta1\gamma2$	$D_{2S}\alpha o\beta1\gamma2$	$D_{2S}\alpha i2\beta1\gamma2$
$pK_h \pm$ SEM (K_h, nM)	7.42 ± 0.18 (38)	7.67 ± 0.28 (21)	7.58 ± 0.45 (26)	10.08 ± 0.16 (0.083)	10.50 ± 0.27 (0.032)	10.28 ± 0.30 (0.052)
pK_l + SEM (K_l, nM)	4.83 ± 0.07 (14800)	4.99 ± 0.21 (10200)	4.84 ± 0.28 (14500)	7.59 ± 0.09 (25.7)	8.10 ± 0.10 (7.94)	8.10 ± 0.06 (7.94)
Percent Rh	22 ± 3	47 ± 3*	35 ± 4	19 ± 4	36 ± 3*	34 ± 4*
$pK_{i\ GTP} \pm$ SEM ($K_{i\ GTP}$, nM)	4.88 ± 0.06 (13200)	4.92 ± 0.06 (12000)	4.87 ± 0.06 (13500)	7.40 ± 0.07 (39.8)	7.83 ± 0.22 (14.8)	7.86 ± 0.20 (13.8)

The binding of dopamine and NPA was determined in competition analyses vs. [^{3}H]spiperone binding as described in the Section 2. Values are expressed as mean ± SEM (N = 4).

* Significantly different from membranes not co-expressing α subunits (ANOVA followed by Tukey's *post hoc* test, $P < 0.05$).

3.3. Measurement of high affinity agonist binding

The affinity of agonists for the dopamine D_{2S} receptor was assessed through competition experiments vs. [^{3}H]spiperone. Competition experiments with dopamine and NPA, in the absence of sodium ions and GTP, were best described by a two-binding site model with higher and lower affinity states (K_h and K_l) (Table 3). The percentage of receptors exhibiting higher affinity for dopamine was increased by the co-expression of G protein subunits although this was only significant in the preparations containing Go (ANOVA followed by Tukey's *post hoc* test, $P < 0.05$). The percentage of receptors exhibiting higher affinity for NPA was significantly increased by the co-expression of either G protein (ANOVA followed by Tukey's *post hoc* test, $P < 0.05$). Competition experiments were fitted best by a one-binding site model when GTP and sodium ions were included. The dissociation constant for the lower affinity site for dopamine or NPA (K_l) was similar to the dissociation constant measured in the presence of sodium ions and GTP ($K_{i\ GTP}$) (Table 3). NPA exhibited affinities for the D_{2S} receptor approximately three orders of magnitude greater than dopamine in all membrane preparations.

The higher affinity site was examined further using the binding of the agonist [^{3}H]NPA. Saturation analyses with [^{3}H]NPA were fitted well by a one-binding site model in about three quarters of the experiments. Where a one-binding site model described the data, however, the K_d value of [^{3}H]NPA was approximately 3-fold higher than the K_h value obtained in competition experiments. Also, the proportion of sites labelled with higher affinity by the agonist, as inferred from the relative B_{max} values for [^{3}H]NPA and [^{3}H]spiperone, was higher than that inferred from competition studies. These data suggest that [^{3}H]NPA may have been labelling both the higher and lower affinity states of the receptor in these saturation analyses. In support of this idea, if the saturation data are analysed using a two-binding site model, with the higher affinity dissociation constant fixed to the value obtained in competition studies, an excellent description of all of the saturation data is obtained (Fig. 4).

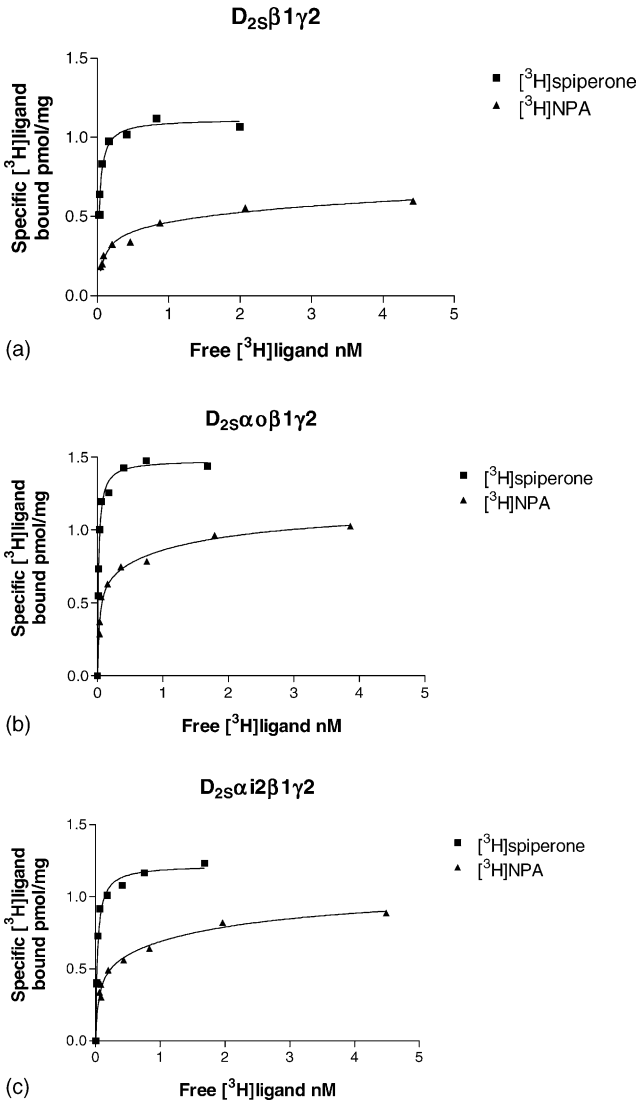

Fig. 4. Saturation analysis of [^{3}H]spiperone and [^{3}H]NPA binding to (a) D_{2S} receptors co-expressed with β1γ2 or (b) D_{2S} receptors co-expressed with αoβ1γ2 or (c) D_{2S} receptors co-expressed with αi2β1γ2. Graphs are representative of three independent experiments. Data were fitted best to one-binding site ([^{3}H]spiperone) or two-binding site ([^{3}H]NPA) models. For [^{3}H]NPA, the data were fitted using a two-binding site model with K_h given the value derived from competition experiments (Table 3). The lower affinity sites were characterised by the following binding parameters ($pK_l \pm$ SEM (K_l, nM) (four experiments)): $D_{2S}\beta1\gamma2$, 8.58±0.15 (2.6); $D_{2S}\alpha o\beta1\gamma2$, 8.63±0.16 (2.3); $D_{2S}\alpha i2\beta1\gamma2$, 8.63 ± 0.09 (2.3).

Table 4
Inhibition of [^{3}H]NPA binding by GppNHp and GDP in membranes expressing D_{2S} receptors and G protein subunits

	GppNHp			GDP		
	$D_{2S}\beta1\gamma2$	$D_{2S}\alpha o\beta1\gamma2$	$D_{2S}\alpha i2\beta1\gamma2$	$D_{2S}\beta1\gamma2$	$D_{2S}\alpha o\beta1\gamma2$	$D_{2S}\alpha i2\beta1\gamma2$
pIC_{50}corr (IC_{50}corr, nM)	n.d.	9.31 ± 0.03 (0.49)	8.55 ± 0.13[a] (2.82)	7.05 ± 0.12 (89)	7.71 ± 0.07[b] (19)	7.91 ± 0.04[b] (12)
Percent [^{3}H]NPA residual binding	38 ± 3[c]	21 ± 6	19 ± 3[b]	36 ± 5	33 ± 3	27 ± 1
*n*H	0.22 ± 0.06	0.43 ± 0.10	0.51 ± 0.08	1	1	1

Inhibition of [^{3}H]NPA binding by GppNHp and GDP was determined as described in the Section 2 and inhibition curves were analysed using PRISM to provide values for IC_{50} and Hill coefficient (*n*H) and the percent residual [^{3}H]NPA binding at high concentrations of inhibitor. Inhibition data for GppNHp were fitted best by a model assuming a Hill coefficient less than 1, whereas data for GDP were fitted best by a model with a Hill coefficient of 1. Values are expressed as mean ± SEM (three experiments for $D_{2S}\beta1\gamma2$ and four experiments for $D_{2S}\alpha o\beta1\gamma2$ and $D_{2S}\alpha i2\beta1\gamma2$). n.d.: not determined.

[a] Significantly different from membranes co-expressing αo subunits (Student's *t* test, $P < 0.05$).

[b] Significantly different from membranes not co-expressing α subunits (ANOVA followed by Tukey's *post hoc* test, $P < 0.05$).

[c] At 100 μM GppNHp.

3.4. Inhibition of [^{3}H]NPA binding by guanine nucleotides

The guanine nucleotides GDP and GppNHp were used to inhibit [^{3}H]NPA binding (Table 4, Fig. 5). GDP inhibited 60–70% of the specific [^{3}H]NPA binding and the inhibition was described well by a one-binding site model. We have assumed that the component of specific [^{3}H]NPA binding inhibited by GDP corresponds to the higher affinity component seen in saturation analyses. In support of this, the residual binding was higher in the preparation expressing only exogenous βγ subunits, where there is less coupled receptor (see earlier description). The potency of GDP to inhibit [^{3}H]NPA binding was lowest for membranes lacking exogenous α subunits, and slightly higher for those co-expressing D_{2S} and Gi2 compared with membranes co-expressing D_{2S} and Go.

GppNHp also inhibited 60–70% of specific [^{3}H]NPA binding in the preparations expressing exogenous α subunits and the inhibition was best fitted by inhibition curves with Hill coefficients of ∼0.5. GppNHp exhibited higher potency for inhibiting [^{3}H]NPA binding in membranes containing D_{2S} and Go as compared to D_{2S} and Gi2. GppNHp exhibited very low potency for inhibiting [^{3}H]NPA binding in membranes lacking exogenous α subunits, and due to the low Hill coefficient a corrected IC_{50} value could not be determined.

Overall, in membranes co-expressing α subunits, [^{3}H]NPA binding was inhibited with higher potency by GppNHp than GDP and the ratio of potencies for the two guanine nucleotides was greatest in the Go preparation (∼4-fold and 40-fold in the Gi2 and Go preparations, respectively). In inhibition experiments with both guanine nucleotides and with suramin (see the following description), there was usually less residual [^{3}H]NPA binding in membranes that had been infected with α subunits compared to membranes lacking exogenous α subunits. This is consistent with the greater formation of the coupled state in membranes expressing exogenous α subunits.

3.5. Inhibition of high affinity agonist binding with suramin

The G protein antagonist suramin inhibited 70–80% of specific [^{3}H]NPA binding and suramin inhibition curves fitted a one-binding site model well (Fig. 6a) in all the membrane preparations. Suramin exhibited higher potency at inhibiting [^{3}H]NPA binding in membranes containing D_{2S} and Gi2 compared to D_{2S} and Go (ANOVA followed by Tukey's *post hoc* test, $P < 0.05$) (Table 5). Suramin inhibition curves were obtained in the two preparations expressing exogenous G proteins for different concentrations of [^{3}H]NPA corresponding to different receptor occupancies. The IC_{50} values for inhibition were higher

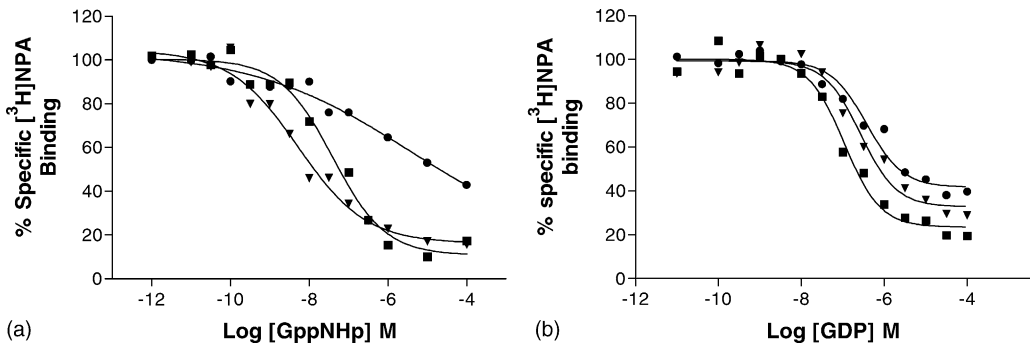

Fig. 5. Inhibition of [^{3}H]NPA binding by GppNHp (a) or GDP (b), in membranes containing D_{2S} receptors and either $\beta1\gamma2$ (●), $\alpha o\beta1\gamma2$ (▼) or $\alpha i2\beta1\gamma2$ (■) G protein subunits. Graphs are representative of three to four independent experiments replicated as in Table 4.

Table 5
Inhibition of [^{3}H]NPA (0.35 nM) binding by suramin in membranes expressing D_{2S} receptors and G protein subunits

	$D_{2S}\beta1\gamma2$	$D_{2S}\alpha o\beta1\gamma2$	$D_{2S}\alpha i2\beta1\gamma2$
pIC_{50}corr (IC_{50}corr, nM)	6.61 ± 0.06 (245)	6.35 ± 0.09 (447)	6.86 ± 0.11* (138)
Percent [^{3}H]NPA residual binding	33 ± 5	20 ± 3	22 ± 2

Inhibition of [^{3}H]NPA binding by suramin was determined as described in the Section 2 and analysed using PRISM as in Table 4. Data were fitted best by a model with a Hill coefficient of 1. Values are expressed as mean ± SEM (N = 4).
* Significantly different to the corresponding values in membranes co-expressing αo (ANOVA followed by Tukey's *post hoc* test, $P < 0.05$).

for the Go preparation as compared to the Gi2 preparation but were virtually independent of [^{3}H]NPA concentration (Fig. 6).

3.6. Effect of suramin and GppNHp on [^{3}H]spiperone saturation analyses

In order to examine effects of receptor/G protein coupling on antagonist/inverse agonist binding, the effect of suramin (100 μM) and GppNHp (10 μM) on [^{3}H]spiperone binding was assessed using saturation analysis in the presence of sodium ions (100 mM). Sodium ions alone had no effect on the binding of [^{3}H]spiperone (data not shown). In the Go preparation suramin increased the B_{max} of [^{3}H]spiperone binding by 19% (one-sample *t*-test, $P < 0.05$) (Fig. 7). A small increase in B_{max} of 6% was observed in the Gi2 preparation, but this was not significant. GppNHp increased the B_{max} of [^{3}H]spiperone binding in the Go preparation by 11%, but this was not significant and no effect was observed in the Gi2 preparation.

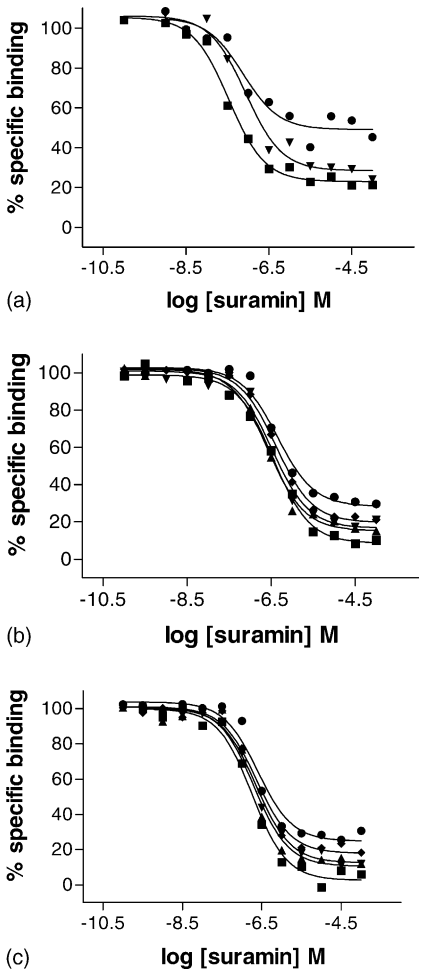

Fig. 6. (a) Inhibition of [^{3}H]NPA (0.35 nM) binding by suramin in membranes containing D_{2S} receptors and either β1γ2 (●), αoβ1γ2 (▼) or αi2β1γ2 (■) G protein subunits. Graphs are representative of four independent experiments replicated as in Table 5. (b) and (c) Effect of the [^{3}H]NPA concentration on suramin inhibition of [^{3}H]NPA binding to the D_{2S}αoβ1γ2 (b) or D_{2S}αi2β1γ2 (c) preparations. The concentration (nM) of [^{3}H]NPA was 0.05 (■), 0.09 (▲),0.19 (▼), 0.35 (◆), 0.7 (●).

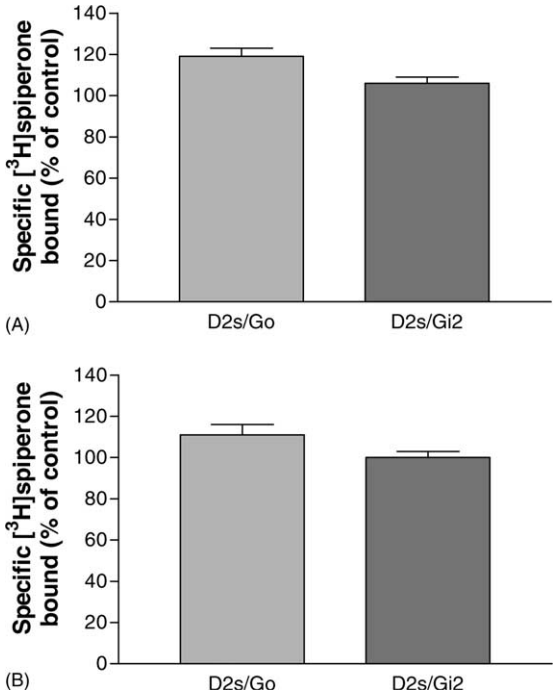

Fig. 7. Effect of suramin (100 μM) (A) or GppNHp (10 μM) (B) on [^{3}H]spiperone binding to D_{2S} receptors co-expressed with αoβ1γ2 or αi2β1γ2. [^{3}H]Spiperone saturation binding experiments were performed as described in the Section 2 and values for B_{max} are expressed as percent of control. Data are the mean±SEM of four independent experiments.

4. Discussion

In this report, we have examined the specificity of interaction of the D_{2S} dopamine receptor with Go and Gi2 proteins by co-expressing D_{2S} with either G protein subunits αo or αi2 and β1 and γ2 in the baculovirus expression system. β1γ2 subunits have previously been found to support the interaction between the D_2 receptor

and several G α isoforms [16–18]. The data in the present report show that agonists influence the efficiency of interaction of the D_2 receptor with the two G proteins.

4.1. Characterisation of preparations

The levels of receptor and G proteins expressed in the two preparations (D_{2S}/Go and D_{2S}/Gi2) were determined. D_{2S} receptor was quantified using radioligand binding and this showed that comparable levels of receptor were expressed in the preparations containing either Go or Gi2. G protein levels were determined in two ways. Total active G protein levels were determined in [^{35}S]GTPγS saturation binding studies. GTPγS binding was determined in membranes from cells infected with α subunit (D_{2S}αxβ1γ2) and corrected for binding in membranes from cells infected with viruses but without the α subunit virus (D_{2S}β1γ2). We used cells infected with D_{2S}β1γ2 as our negative control rather than uninfected cells as baculovirus infection has been shown to reduce host cell protein synthesis [25] and so could reduce the number of endogenous guanine nucleotide binding sites [18]. Using this method the G/R ratio was found to be ∼6 and similar in both preparations.

G protein levels were also assessed by quantitative Western blot. These studies revealed high levels of both G protein subunits α and β1. Levels of γ2 subunits were much lower and this may reduce active G protein heterotrimer formation [16]. The G/R ratio measured in this way is 14–28. These values are higher than those obtained using [^{35}S]GTPγS binding and may reflect detection of inactive protein in Western blots. Nevertheless, the G/R ratios in the two preparations are similar so that the two preparations may be compared. The receptor to G protein ratios found in this study are similar to both our recent work on the D_{2L} receptor, in which G protein levels were estimated by Western blot analysis and a G/R ratio of 20 was found [16] and the ratio of 17–25 [18] using [^{35}S]GTPγS binding.

4.2. Agonist stimulation of [^{35}S]GTPγS binding

The binding of [^{35}S]GTPγS stimulated by agonists has been used to determine agonist efficacy and potency in the two preparations. Dopamine was unable to stimulate [^{35}S]GTPγS binding in membranes from cells expressing only the receptor and G protein subunits β1γ2, but was able to stimulate binding in membranes from cells co-expressing α subunits. Dopamine exhibited higher potency in membranes co-expressing Go, although the total G protein activation (during the 30-min incubation) was equivalent in terms of fmol of [^{35}S]GTPγS bound per mg of protein. It is, therefore, concluded that dopamine acting *via* the D_{2S} receptor preferentially activates Go over Gi2. Agonist binding affinities in the preparations expressing Go and Gi2 were similar (Table 3) so that the greater ability of dopamine to activate G proteins in the Go preparation must be due to events beyond agonist binding.

When a range of agonists was tested, each of these exhibited higher relative efficacy (in comparison to dopamine) in the preparation containing Go as compared to Gi2. *m*-Tyramine and (+)-3-PPP (in addition to dopamine) also exhibited higher potency in the preparation expressing Go, but none of the other agonists tested exhibited significantly different potencies for stimulating [^{35}S]GTPγS binding in the two preparations. Therefore, although this study is not an example of agonist trafficking as proposed by Kenakin [4], as there are no significant reversals in efficacy or potencies of agonists, it does show that the receptor/G protein combination can affect the response of the receptor to different agonists. This is similar to results found for the β_2-adrenoceptor [26], for which true agonist trafficking was seen at different classes of G protein, but only subtle differences in efficacy and potency were found within the same class of G protein. Interestingly, in the present study, the relative efficacies of quinpirole and (+)-3-PPP are approximately equal in the Go preparation, but significantly different in the Gi2 preparation. The relative efficacies of these two compounds have been found to reverse in certain tissues [27]. Furthermore, these results are similar to those reported for the rat D_{2L} receptor, co-expressed with Go and Gi2 [16].

4.3. Agonist binding studies

The binding of agonists to the receptors expressed with G protein subunits was examined in competition vs. [^{3}H]spiperone binding and using the binding of the radiolabelled agonist [^{3}H]NPA. Competition studies with dopamine or NPA vs. [^{3}H]spiperone binding gave data that could be described well by a two-binding site model. In the presence of GTP, however, the competition curves for the two agonists were described well by a one-binding site model. These data are consistent with interaction between the expressed receptors and G proteins. This behaviour was, however, seen even in the absence of exogenous G protein showing that the receptor can interact with the endogenous G proteins of the cells. In line with this, several studies have reported the presence of endogenous G proteins in insect cells [28–32]. This interaction does not lead to G protein activation in the present study in that no stimulation of [^{35}S]GTPγS binding could be observed in the preparation lacking exogenous G protein. In the preparations expressing exogenous G proteins, the affinities of the higher and lower affinity sites were similar but the proportion of higher affinity sites was greater, consistent with interaction between expressed receptor and G proteins.

4.4. Receptor/G protein interaction

In order to investigate the mechanism of receptor/G protein interaction and the apparently higher efficiency of D_{2S} to activate Go, we tested the effects of the guanine nucleotide GDP and the receptor/G protein antagonist

suramin on the binding of the agonist [^{3}H]NPA. [^{3}H]NPA binding stabilises formation of the ternary complex that is thought to be important for receptor activation and signalling. NPA exhibits greater efficacy for stimulation of [^{35}S]GTPγS binding in the Go preparation, so that studying [^{3}H]NPA binding may help understand this difference in efficacy. GDP inhibited [^{3}H]NPA binding with a similar potency in the two preparations. GDP is thought to affect high affinity agonist binding by reducing the affinity of the receptor for agonist by binding to the G protein in the ternary complex, destabilising it and leading to sequestration of α subunit. Hence, the NPA/D_2 receptor complex may have a similar affinity for the two G proteins. This may not be the case for other agonist/D_2 receptor combinations or for the unliganded receptor (see the following description).

Suramin inhibited [^{3}H]NPA binding more potently (3.2-fold) in the Gi2 preparation as compared with the Go preparation. When the effects of suramin on [^{3}H]NPA binding were determined at different [^{3}H]NPA concentrations, the IC_{50} for inhibition by suramin was largely unaffected. It has been proposed that suramin inhibits high affinity agonist binding by disrupting ternary complex formation by competing with the receptor for binding to the G protein [33]. A competitive interaction would suggest that the IC_{50} for suramin should be dependent on the concentration of [^{3}H]NPA. The present data are, therefore, more consistent with a model where the suramin binds to the G protein and allosterically prevents interaction with the receptor. From these data, therefore, we are unable to determine whether the greater potency of suramin to inhibit [^{3}H]NPA binding in the Gi2 preparation reflects differences in the affinity of suramin for the two G proteins or differences in receptor/G protein affinity. An alternative explanation for the observations comes from data on the effects of suramin on rhodopsin/transducin interaction where effects of suramin on membrane anchoring of transducin have been proposed [34].

We, therefore, tested the guanine nucleotide analogue GppNHp for its effects on [^{3}H]NPA binding. Inhibition occurred with greater potency (∼ 6-fold) in the Go preparation than the Gi2 preparation. The inhibition of [^{3}H]NPA binding by GppNHp was characterised by Hill coefficients less than 1. Overall GppNHp inhibited [^{3}H]NPA binding with greater potency than GDP in preparations co-expressing α subunits. Effects of GppNHp on high affinity agonist binding are thought to reflect the disruption of the ternary complex of agonist/receptor/G protein. This is not a simple competitive process, it depends on receptor activation, and this may account for the low Hill coefficient. The higher potency of GppNHp to act in the Go preparation may, therefore, be a reflection of multiple events, including the activation of the G protein by the receptor, which seems, therefore, to be greater for the Go preparation. GppNHp inhibition of [^{3}H]NPA binding was particularly poor in the preparation expressing D_2 receptor alone. In this preparation, [^{3}H]NPA binding most likely reflects formation of a ternary complex, including endogenous insect cell G proteins. From the studies on agonist stimulation of [^{35}S]GTPγS binding, it is clear that the receptor is unable to stimulate nucleotide exchange on the endogenous G proteins. Possibly, therefore, the poor inhibition of [^{3}H]NPA binding in this preparation by GppNHp is a reflection of the poor activation of G proteins. The greater ability of GppNHp to inhibit [^{3}H]NPA binding to the Go preparation may, therefore, reflect the greater efficiency of G protein activation in this complex.

The effects of inhibition of receptor/G protein coupling on the binding of [^{3}H]spiperone were also examined. Some studies have shown that the ligand spiperone has a higher affinity for the uncoupled form of the receptor than the coupled form [35,36], so that the binding of this ligand may be sensitive to the effects of inhibitors of receptor/G protein coupling. The binding of [^{3}H]spiperone was examined in the present study in the presence of GppNHp and suramin, both of which have been shown to prevent receptor/G protein interaction (see the earlier description). Saturation binding of [^{3}H]spiperone in the presence of these inhibitors gave a higher B_{max} than in their absence but only in the preparation expressing Go. This would be consistent with spiperone labelling, in the absence of inhibitors, a population of uncoupled receptors with high affinity. The affinity of spiperone for the coupled receptor would, however, be rather low. Inhibition of R/G interaction increases the population of uncoupled receptor, hence the increase in B_{max}. This effect is only seen in the Go preparation so that in this preparation there must be some precoupling of receptor and G protein in the absence of agonist. This suggests, in turn, that the affinity of the free receptor is higher for Go than for Gi2. Larger effects of suramin on binding of the antagonist [^{125}I]epidepride to the D_2 receptor expressed in rat striatum have been described [37], but this presumably reflects differences in the experimental systems.

In conclusion, therefore, the present data show that agonists acting at the D_{2S} dopamine receptor activate the G protein Go with higher efficacy and in some cases with higher potency than the G protein Gi2. The basis of this difference may lie in differences in receptor/G protein affinity and ability to activate the G protein.

Acknowledgments

We thank the University of Reading for financial support, Stephen Graber and Lori Flood for supplying pure G protein subunit and Sam Hoare for helpful comments on the manuscript.

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