In Vivo Reconstitution of Dopamine D_{2S} Receptor-Mediated G Protein Activation in Baculovirus-Infected Insect Cells: Preferred Coupling to G_{i1} versus G_{i2}[†]

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ABSTRACT: Agonist binding of the human D_{2S} receptor overexpressed in baculovirus-infected Sf9 insect cells was of low affinity and GppNHp-insensitive, yet, dopaminergic agonists were able to partly inhibit forskolin-stimulated cAMP accumulation. In order to prove full functionality of the receptor, we used an "*in vivo*" reconstitution system, which is based on coinfection of Sf9 cells with the appropriate receptor and G protein encoding baculoviruses. In cells coexpressing the D_{2S} receptor and either G_{i1} or G_{i2}, the dopaminergic agonist apomorphine effectively stimulated [^{35}S]GTPγS binding and GTPase activity. Agonist-stimulated [^{35}S]GTPγS binding was dependent on the ratio of G protein to receptor. Expression levels of receptor and G protein influenced each other reciprocally. G protein activation could be optimized by varying the multiplicity of infection of the receptor and G protein encoding baculoviruses. Coexpression of either G_{i1} or G_{i2} led to the appearance of GppNHp-sensitive high-affinity agonist binding. Detailed agonist competition binding analysis revealed that the percentage of high-affinity agonist binding sites was significantly higher in D_{2S} receptor-expressing cells coinfected with G_{i1} viruses than when coinfected with G_{i2} viruses. Moreover, the coexpressed G_i proteins seemed to modulate the affinity of agonists for the high-affinity form of the receptor. In cells coexpressing G_{i1}, agonist high affinity was 2−4-fold higher than in cells coexpressing G_{i2}. Na^+ increased the dissociation constant of apomorphine for the high-affinity site by 2−4-fold without affecting the percentage of high-affinity sites or the preference for G_{i1}. In some dopamine competition experiments with coinfected cells, displacement data were best fit assuming three noninteracting classes of sites in the absence and two independent classes of sites in the presence of GppNHp. Dopamine competition curves with cells highly overexpressing the D_{2S} receptor or with membranes from such cells were best fit assuming two independent classes of sites which were insensitive to GppNHp and might reflect abnormal compartimentalization and/or different states of aggregation.

The transduction of dopaminergic signals across cellular membranes is mediated by at least five different dopamine receptor subtypes that belong to the gene superfamily of GPCRs[1] (Civelli et al., 1993; Gingrich & Caron, 1993). The D_2 subtype was the first dopamine receptor to be cloned (Bunzow et al., 1988). It has been implicated in the development of psychiatric and neurological disorders, such as schizophrenia and Parkinson's disease (Lee et al., 1978; Seeman, 1987). At the molecular level, activation of D_2 dopamine receptors results in inhibition of adenylyl cyclase (Albert et al., 1990). In addition, these receptors have been shown to induce a variety of cell-type-specific signal transduction pathways such as stimulation of phosphatidylinositol turnover (Vallar et al., 1990), potentiation of arachidonic acid release (Kanterman et al., 1991), or regulation of K^+- and Ca^{2+}-channel activity (Vallar et al., 1990; Castellano et al., 1993). All these different signalling pathways involve the activation of the G_i/G_o family of guanine nucleotide binding proteins (G proteins).

Interestingly, the D_2 dopamine receptor exists in two protein isoforms, termed D_{2S} and D_{2L}, which are generated by differential splicing. Both isoforms are identical except for an insert of 29 amino acids in the putative third cytoplasmic loop of D_{2L} (Dal Toso et al., 1989; Grandy et al., 1989; Monsma et al., 1989). They coexist in all brain regions and tissues analyzed but at a highly variable ratio, with the longest isoform being expressed predominantly in all regions. They cannot be discriminated pharmacologically (Dal Toso et al., 1989; Giros et al., 1989; Grandy et al., 1989; Monsma et al., 1989). However, functional differences are beginning to emerge. The two isoforms have been shown to respond differently to desensitization in CHO cells (Zhang et al., 1994) and to display differential sensitivity to inhibitory modulation by protein kinase C (Liu et al., 1992). Furthermore, D_{2S} and D_{2L} have been demonstrated to couple differently to G proteins. Specifically, it seems that this 29 amino acid insert of D_{2L} confers interaction selectivity for $G_{i\alpha 2}$ (Liu et al., 1994; Guiramand et al., 1995).

We have overexpressed and characterized the D_{2S} dopamine receptor isoform in Sf9 insect cells (Grünewald et al., 1996). It is glycosylated and palmitoylated in insect cells and shows ligand binding characteristics identical to the native protein. In contrast to the results which have been

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[1] Abbreviations: GPCR, G protein-coupled seven-transmembrane receptor; G_s and G_{i1}, G_{i2}, stimulatory and inhibitory guanine nucleotide binding proteins of subtypes 1 and 2; G_α, $\alpha_{i(1or2)}$, G_β, β_1, G_γ, and γ_2, G protein α-, β-, and γ-subunits of denoted subtype; AppNHp, 5′-adenylyl imidodiphosphate; ATP, adenosine 5′-triphosphate; ATPγS, adenosine 5′-*O*-(3-thiotriphosphate); GTP, guanosine 5′-triphosphate; GTPγS, guanosine 5′-*O*-(3-thiotriphosphate); DTT, dithiothreitol; BSA, bovine serum albumin; moi, multiplicity of infection; PBST, phosphate-buffered saline containing 0.2% Tween 20; TBS, Tris-buffered saline; SBTI, soybean trypsin inhibitor; PTX, pertussis toxin; FSK, forskolin.

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reported for the human D_{2L} (Ng et al., 1994) and rat D_{2L} and D_{2S} isoforms (Boundy et al., 1996), we could not detect any high-affinity agonist binding indicating coupling to endogenous insect cell G proteins. Sf9 cells possess several G proteins including a G_s-like protein (Parker et al., 1991; Kleymann et al., 1993), G proteins belonging to the $G_{q/11}$ class (Kwatra et al., 1993), and a pertussis toxin-sensitive G_o-like protein (Ng et al., 1993; Mulheron et al., 1994). So far, however, inhibitory G_i proteins have not been found (Quehenberger et al., 1992; Kleymann et al., 1993; Mulheron et al., 1994). A common way to show full functionality of an overexpressed GPCR is the functional reconstitution of purified receptor with the appropriate purified G protein (Tota et al., 1987; Senogles et al., 1990). In order to avoid the time-consuming setting up of solubilization, purification, and reconstitution procedures for the receptor and the G proteins, we tested the possibility of coexpressing the G_i protein to be tested together with the D_{2S} receptor through coinfection of Sf9 cells with the receptor baculovirus and the viruses for the α-, β-, and γ-subunits of the G protein. This approach has successfully been used by Butkerait et al. (1995) to show G protein coupling of the 5-HT_{1A} receptor which displayed an uncoupled phenotype in infected Sf9 cells. We adapted G protein assays and agonist competition binding to this whole cell system which turned out to be very sensitive to the ratio of the multiplicity of infections used. We present a detailed comparison of the interaction between the D_{2S} receptor and two G_i subtypes, G_{i1} and G_{i2}, and show for the first time that the coexpressed G_i subtype modulates agonist high-affinity binding, revealing a preference of D_{2S} for G_{i1} over G_{i2}.

EXPERIMENTAL PROCEDURES

Materials. [^{3}H]Spiperone ([^{3}H]SPIRO; 700−900 GBq/mmol), [^{3}H]methylspiperone ([^{3}H]MS; 3108 GBq/mmol), [γ-^{32}P]GTP [1.11 TBq (30 Ci)/mmol], and [^{35}S]GTPγS (46−52 TBq/mmol) were purchased from DuPont NEN. *R*(−)-Apomorphine hydrochloride and *S*(+)-butaclamol hydrochloride were obtained from Research Biochemicals Inc. (Natick, MA). GTPγS was obtained from Boehringer Mannheim, ATP from Pharmacia, and GTP, GppNHp, AppNHp, and ATPγS from Sigma. Ethylene glycol monomethyl ether and the scintillation cocktail Rotiscint eco plus were from Roth (Karlsruhe, FRG). All other chemicals were reagent grade, obtained primarily from Sigma. Antipeptide G protein antibodies were purchased from Calbiochem. Anti-$G_{s\alpha}$ was directed against the C-terminal amino acids 385−394, anti-$G_{i\alpha}1$−2 was directed against the C-terminal amino acids 345−354, and anti-G_β was directed against the internal amino acids 127−139. The secondary goat anti-rabbit IgG antibody (alkaline phosphatase-labeled) was obtained from Biozol (Eching, FRG). Sf9 cells were purchased from ATCC (Rockville, MD). Cell culture medium ingredients were obtained from Sigma and Gibco BRL. Baculoviruses expressing G protein α_{i1}, α_s, β_1, and γ_2 subunits were a generous gift of Prof. M. Linder (Washington University School of Medicine, St. Louis, MO), and the baculovirus encoding G protein subunit α_{i2} was kindly provided by Prof. J. Garrison (University of Virginia Health Sciences Center, Charlottesville, VA). The baculovirus AcPolD2S encoding the D_{2S} receptor is described in the preceding paper (Grünewald et al., 1996).

Insect Cell Culture. Sf9 insect cells were propagated in TNM-FH medium supplemented with 5% fetal calf serum and 50 μg/mL gentamycin as monolayer or suspension culture at 27 °C as described by Summers and Smith (1987). Viruses were amplified, and virus titers were determined by end-point dilution as described in O'Reilly et al. (1992).

Coexpression of the D_{2S} Receptor and G Protein Subunits in Sf9 Cells. Typically, 5×10^6 cells were seeded in a 6 cm petri dish and simultaneously infected with the AcPolD2S baculovirus and a 1:1:1 mixture of the baculoviruses expressing the G-protein subunits α, β_1, and γ_2 at multiplicities of infection indicated in the text. Cells were collected by centrifugation (1000*g*, 10 min) usually 70 h after infection. Cell permeability was routinely checked by trypan blue uptake (0.4% trypan blue solution, Sigma). After a short wash in cold 50 mM Tris-HCl, pH 7.4, 100 mM NaCl (TN-buffer), the cells were resuspended to a density of 1×10^7 cells/mL in TN/PI-buffer [TN-buffer, supplemented with the protease inhibitors leupeptin (5 μg/mL), SBTI (5 μg/mL), and benzamidine (10 μg/mL)] for GTPγS assays or in 50 mM Tris-HCl, pH 7.4, 0.5 mM EDTA, 0.1 mM DTT, 2.5 mM $MgCl_2$, 0.1% ascorbate, 5 μg/mL leupeptin, 5 μg/mL SBTI, and 10 μg/mL benzamidine (buffer S) for agonist competition assays. The cells were kept on ice and used immediately for further experiments.

Immunoblot Analysis. Uninfected or infected Sf9 cells (10^7 cells/mL) were lysed in Laemmli sample buffer, heated for 5 min at 95 °C, and centrifuged at 12000*g* for 5 min. Protein in the supernatant was resolved by SDS−PAGE (12%, 10 μL $\sim$ 75 000 cells/lane) (Laemmli, 1970) and electrophoretically transferred to Immobilon-P poly(vinylidene difluoride) membranes (Millipore Corp., Bedford, MA). Immobilon-P membranes were probed with the appropriate anti-G protein antibody, diluted 1:1000 in blocking solution (3% BSA in PBST), as described previously (Grünewald et al., 1996).

Membrane Preparation. Membranes of AcPolD2S-infected Sf9 cells were prepared according to Ng et al. (1994) either in buffer S or in buffer N. Buffer N described therein consisted of 50 mM Tris-HCl, 5 mM EDTA, 1.5 mM $CaCl_2$, 5 mM $MgCl_2$, 5 mM KCl, 0.1% ascorbic acid, 5 μg/mL leupeptin, 5 μg/mL SBTI, and 10 μg/mL benzamidine, pH 7.4. Bovine striatal membranes were prepared as described in Kuno et al. (1983).

Receptor Binding Assays. For agonist competition binding, cells were diluted into buffer S with or without 100 mM NaCl to 40−70 pM [^{3}H]spiperone binding sites in a total volume of 1 mL and incubated for 2 h at 25 °C with increasing concentrations of dopamine (10^{-10}−10^{-2} M) or of *R*(−)-apomorphine (10^{-12}−$10^{-3.5}$ M) in the presence of about 600 pM [^{3}H]methylspiperone. The incubation was terminated by the addition of 3.5 mL of cold buffer T (50 mM Tris-HCl, pH 7.4) and vaccuum filtration over Whatman GF/F filters presoaked in 0.3% poly(ethylenimine) (Sigma) using a Brandel cell harvester. The filters were washed twice with 3.5 mL of cold buffer T and the filter disks counted for tritium using a Canberra-Packard Tri-Carb scintillation counter. [^{3}H]Methylspiperone saturation binding and total [^{3}H]spiperone binding sites were measured as described previously (Grünewald et al., 1996).

[^{35}S]GTPγS Binding Assays. Total [^{35}S]GTPγS binding was performed on whole cells (about 50 000 cells/assay) in buffer L [50 mM Tris-HCl, pH 7.4, 100 mM NaCl, 0.5 mM

EDTA, 0.1 mM DTT, 10 mM $MgCl_2$, 1 mM AppNHp, 1 mM ATPγS, 1 mM ATP, 0.1% Lubrol PX, and 1 μM GTPγS ($\approx$10 000 dpm/pmol)] in a total volume of 0.1 mL for 1 h at 30 °C. The assay was terminated by adding 3 mL of cold washing buffer (50 mM Tris-HCl, pH 7.4, 100 mM NaCl, and 25 mM $MgCl_2$), and bound [^{35}S]GTPγS was separated from free nucleotide by filtration through nitrocellulose filters (NC 45, Schleicher & Schuell). Subsequently, the filters were washed 3 times with 3 mL of cold washing buffer, placed in scintillation vials, and dissolved in 1 mL of ethylene glycol monomethyl ether. After 30 min, 3.5 mL of scintillation cocktail (Rotiscint eco plus) was added, and radioactivity was measured by liquid scintillation counting (Northup et al., 1982). Background binding was determined in the presence of 1 mM GTP. Agonist-sensitive [^{35}S]GTPγS binding was performed on whole cells in buffer G [50 mM Tris-HCl, pH 7.4, 100 mM NaCl, 0.5 mM EDTA, 0.1 mM DTT, 2.5 mM $MgCl_2$, 1 mM App(NH)p, 1 mM ATP, and 0.1% ascorbate] containing 100 nM GTPγS ($\approx$50 000 dpm/pmol) for 0−90 min at 25 °C (Senogles et al., 1990).

GTPase Assays. GTPase activity was determined as described by Tota et al. (1987) with the following alterations. A total of 10^7 cells/mL in TN-buffer or buffer S were diluted 1:9 into buffer G supplemented with 10 mM phosphocreatine and 50 units/mL creatine phosphokinase. The agonist *R*(−)-apomorphine or the antagonist *S*(+)-butaclamol was added to a concentration of 100 μM and 1 μM, respectively, and allowed to equilibrate for 5 min at 30 °C. The GTPase reaction was initiated by adding [γ-^{32}P]GTP to a concentration of 250 nM. After incubating for the desired amount of time, 50 μL aliquots were quenched by the addition to 250 μL of an ice-cold suspension of activated charcoal (5% w/v) in 20 mM phosphoric acid (pH 2.3). After 10 min on ice, the suspension was centrifuged at 12000*g* for 10 min at 4 °C. Free [^{32}P]P_i in 100 μL of the clear supernatant was measured by liquid scintillation counting. Background hydrolysis was determined in the presence of 100 μM GTP and subtracted from all determinations.

Measurement of Intracellular cAMP. Measurement of intracellular cAMP was performed on Sf9 cells 40−50 h either after infection with the AcPolD2S baculovirus alone at mois between 2 and 10 or after coinfection with the three G protein subunit viruses each at a moi of 4 in addition to the AcPolD2S baculovirus at a moi of 2. Infected cells were centrifuged at 1000*g*, resuspended to 2.5×10^6 cells/mL in Grace medium supplemented with 100 μM isobutylmethylxanthine (IBMX), and incubated for 30 min at 27 °C; 200 μL of these cells was added to tubes containing either no drugs (basal activity), 10 μM forskolin, or 10 μM forskolin plus 10 μM dopamine or, in addition, plus 10 μM (+)-butaclamol. After 20 min at 27 °C, the cells were placed on ice and lysed in 167 mM HCl. The lysate was incubated on ice for 1 h and, after neutralization with NaOH, was centrifuged at 15000*g* to remove cell debris. cAMP was measured in the supernatant using the commercially available cAMP ^{3}H assay system from Amersham.

Data Analysis. Saturation and competition binding data were analyzed by nonlinear least-squares curve-fitting using the LIGAND program (Munson & Rodbard, 1980) and graphically displayed with the Macintosh program KaleidaGraph. One-, two-, and three-site curve-fitting was tested in all experiments. Mostly, the two-site model gave adequate fits. In some cases, curve-fitting to a model assuming three subclasses of noninteracting agonist binding sites that bound antagonist with the same dissociation constant significantly improved the fit ($p \leq 0.001$, *F*-test). In single or coinfected Sf9 cells, the dissociation constant for [^{3}H]methylspiperone was stable ($K_D = 111 \pm 15$ pM), so that the K_D and total concentration of the radioactive ligand were supplied from independent determinations and held constant. When using membranes, saturation and competition binding data were analyzed simultaneously.

[^{35}S]GTPγS binding rates were generally analyzed according to apparent first-order kinetics. Apparent first-order rate constants, k_{app}, were obtained by nonlinear least-squares analysis (using KaleidaGraph) of the first-order rate equation: $(G_{eq} - G_t)/G_{eq} = \exp(-k_{app}t)$, where G_{eq} is the total amount of GTPγS bound at long times (after the plateau was reached) and G_t is the amount of GTPγS bound at individual shorter times.

Statistical comparisons were done using the Student's unpaired *t* test with InStat by GraphPad Software.

RESULTS

Simultaneous Expression of the D_{2S} Receptor and G Proteins in Coinfected Sf9 Insect Cells. For expression of the D_{2S} receptor in Sf9 cells, the baculovirus AcPolD2S was used, which contains a fusion of the first 12 codons of the viral polyhedrin gene to the 5′-end of the D_{2S} receptor gene [see Grünewald et al. (1996)]. Heterologous expression of the D_{2S} receptor and the appropriate G protein subunits after coinfection of Sf9 insect cells was confirmed by radioligand binding and immunoblot analysis (Figure 1). In cells infected only with the D_{2S} receptor baculovirus AcPolD2S, expression levels were higher than in coinfected cells [as measured by [^{3}H]spiperone binding, $\approx 1 \times 10^6$ receptors/cell, moi = 6−10, versus $(0.3-0.9) \times 10^6$ receptors/cell, moi of G protein viruses $\approx$ 10 with varying moi (1.25−40) of baculovirus AcPolD2S]. Expression of recombinant G proteins in coinfected Sf9 insect cells was shown by immunoblot analysis with commercially available antipeptide G protein antibodies (Figure 1A). The antibodies clearly recognized the recombinant $G_{\alpha s}$, $G_{\alpha i1}$, $G_{\alpha i2}$, and G_β subunits produced in coinfected cells, whereas in cells infected with the recombinant AcPolD2S baculovirus only or in noninfected cells no G protein subunits could be detected. This is in agreement with results of Graber and co-workers, who also did not detect any G proteins on Western Blots of uninfected Sf9 cell extracts (Graber et al., 1992). In contrast, other groups have reported the immunological detection of several endogenous insect cell G proteins, such as a PTX-sensitive Go-like (Ng et al., 1993; Mulheron et al., 1994), a Gs-like (Parker et al., 1991; Kleymann et al., 1993), and a Gq-like (Kwatra et al., 1993) protein. Successful immunological detection obviously depends on the sensitivity of the assay method used.

It was difficult to accurately assess the amount of G protein produced in the infected cells due to the presence of nucleotide binding proteins other than trimeric G proteins. This might be one of the reasons for the high background [^{35}S]GTPγS binding of 50−70% that we found in our initial GTPγS binding assays when using GTP instead of GTPγS as competitor. The problem could be overcome by including 1 mM ATPγS (in addition to 1 mM ATP and 1 mM

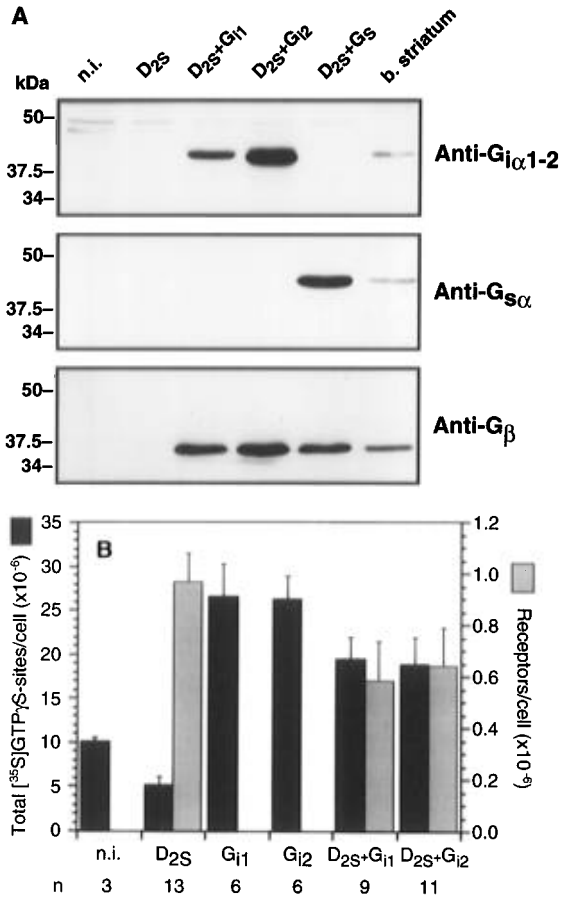

FIGURE 1: Coexpression of G proteins in coinfected Sf9 cells. (A) Aliquots from lysed Sf9 cells and bovine striatal membranes were subjected to immunoblot analysis. Sf9 cells were either not infected (n.i.), infected with AcPolD2S (moi ≈ 10) (D_{2S}), or coinfected with AcPolD2S (moi ≈ 6) together with β_1, γ_2, and either the α_{i1} (D_{2S}+G_{i1}) or the α_{i2} (D_{2S}+G_{i2}) or the α_s (D_{2S}+G_s) baculoviruses (moi ≈ 10). Cells were harvested 70 h after infection. (B) Total [^{35}S]GTPγS sites were determined in noninfected, single, and coinfected Sf9 cells as described under Experimental Procedures. Infections were as described above. G_{i1} and G_{i2} designate cells infected with the three G protein baculoviruses β_1, γ_2, and either α_{i1} or α_{i2}, each at a moi of 10, but without AcPolD2S. Data represent the mean ± SD of *n* independent determinations.

AppNHp) in the GTPγS binding buffer, keeping nonspecific binding down to 20−40% (depending on G protein expression levels), irrespective of whether GTP or GTPγS was used as competitor. Thus, total GTPγS binding sites could be determined reproducibly in uninfected as well as in infected insect cells (Figure 1B). Interestingly, Sf9 cells infected with the D_{2S} receptor baculovirus AcPolD2S only, with the β_1 and γ_2 baculoviruses only, or with a combination of these displayed a constant number of about 5 million [^{35}S]GTPγS binding sites per cell [(5.26 ± 0.87) × 10^6 sites/cell, n = 13], as long as infection was complete, versus 10 million GTPγS sites per cell [(10.2 ± 0.4) × 10^6 sites/cell, n = 3] in uninfected cells. A 5-fold increased number of GTPγS binding sites could be determined when Sf9 insect cells were simultaneously infected at a moi of ≈10 with the β_1, γ_2, and α_{i1} baculoviruses [(26.5 ± 3.7) × 10^6 sites/cell] or the β_1, γ_2, and α_{i2} baculoviruses [(26.3 ± 2.6) × 10^6 sites/cell] (Figure 1B). It is already known that baculovirus infection stops Sf9 host cell protein biosynthesis (Carstens et al., 1979), and, therefore, we assume that the remaining 5 × 10^6 GTPγS binding sites in infected cells not expressing recombinant G_α subunits can be attributed to other nucleotide binding proteins like transcription factors. So, for the determination of the amount of G_α expressed in infected Sf9 cells, these background GTPγS sites were subtracted from the total [^{35}S]-GTPγS sites measured. G_{i1} and G_{i2} could be expressed equally well with and without receptor coexpression (Figures 1 and 2).

Effect of Different Expression Levels of D_{2S} Receptor and G Protein on Receptor−G Protein Interactions. It is well-known for the D_2 (Senogles et al., 1990) as well as for other receptors that G protein coupling is dependent on the ratio of G protein to receptor. Expression levels of both receptor and G protein depended on the moi used as well as on the moi ratio of the viruses for the receptor and the G protein (Figure 2). In order to establish the optimal conditions for G protein−receptor coupling, Sf9 cells were coinfected with the recombinant AcPolD2S baculovirus at various mois, whereas the mois for the G protein viruses were kept constant (moi ≈ 10). Three days after infection, the expression levels for the receptor and the G_α proteins were determined (Figure 2A,D), and G protein coupling was measured as agonist-sensitive [^{35}S]GTPγS binding (Figure 2B,E). With increasing G_i to receptor moi ratio, both G_{i1} and G_{i2} coinfections showed decreasing receptor expression levels and simultaneously increasing G_{i1} or G_{i2} protein levels (Figure 2A,D). The ratio of G protein to receptor expressed in coinfected Sf9 cells can be varied more or less linearly by changing the moi ratio of the appropriate baculoviruses used for coinfection (Figure 2C,F). There are only minor, not significant, differences in expression levels and ratios in dependence of the G_α baculovirus used for coinfections. At all moi ratios tested, the agonist apomorphine significantly increased GTPγS binding above basal levels, whereas in cells infected with the AcPolD2S baculovirus only (i.e., moi ratio G_i:R = 0 in Figure 2B,E) or in cells infected with the three G_{i1} or G_{i2} subunit viruses only, apomorphine had no effect on [^{35}S]GTPγS binding (data not shown). Maximal stimulation was about 2-fold for G_{i1} and G_{i2}, respectively, and was achieved with cells that had been infected with G_i protein subunit viruses each at a moi of 10 and with the AcPolD2S virus at a moi between 5 and 7. Under these conditions, the coinfected cells expressed about (1.7−2.1) × 10^7 GTPγS sites versus (5−7) × 10^5 receptors per cell, resulting in an optimal ratio of G protein to receptor (after subtracting the background GTPγS sites) between 17 and 25 for both G_{i1} and G_{i2}. It seems, however, that in the case of G_{i1} a 2-fold agonist stimulation of GTPγS binding can already be achieved at G protein to receptor ratios as low as 10, indicating a more effective interaction with G_{i1} as compared to G_{i2}.

Similar results were obtained when Sf9 cells were coinfected at lower mois, i.e., at a moi of 3 for each of the G_{i1} or G_{i2} subunit viruses and at increasing mois from 1 to 12 for the AcPolD2S baculovirus. G protein and receptor expression levels influenced each other reciprocally. Apomorphine-stimulated GTPγS binding again was maximal at a ratio of the moi of the G protein viruses to the moi of the receptor virus between 1 and 2 (data not shown).

The $G_{i\alpha}$ subunits were always expressed together with β_1 and γ_2 with all three baculoviruses used at the same moi. This was to ensure the production of comparable amounts of α- and βγ-subunits necessary for the assembly of the functional G protein heterotrimer. Expression of $G_{i\alpha2}$

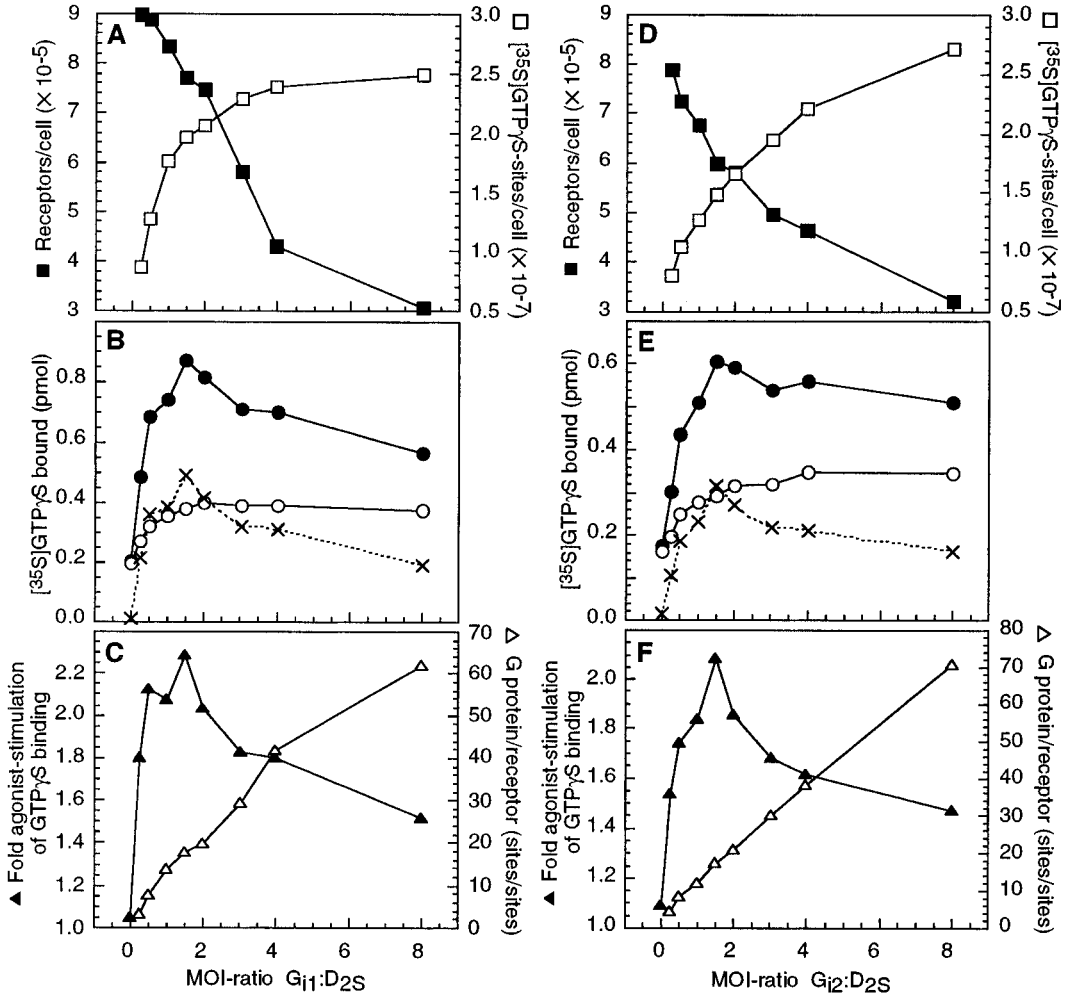

FIGURE 2: Agonist-stimulated [^{35}S]GTPγS binding in coinfected Sf9 insect cells expressing different levels of D_{2S} receptor and G_i protein. A total of 5×10^6 cells were coinfected with β_1, γ_2, and either α_{i1} (A−C) or α_{i2} (D−F) subunit viruses at constant moi (moi $\approx$ 10 for each virus) and with the D_{2S} receptor virus AcPolD2S at various mois (mois of 0, 1.25, 2.5, 3.3, 5, 6.7, 10, 20, and 40). Three days after infection, receptor binding and coupling assays were performed. (A and D) Receptor and G_i protein levels were determined by [^{3}H]-spiperone and [^{35}S]GTPγS binding, respectively. (B and E) The cells were assayed for [^{35}S]GTPγS binding in the presence of the agonist apomorphine (100 μM) (●) or in the presence of the antagonist *S*(+)-butaclamol (1 μM) (○) for 10 min. The difference between these two curves (dotted line, ×) represents the specific agonist-stimulated [^{35}S]GTPγS binding. (C) and (F) show the G protein to receptor ratio estimated from the expression levels in (A) and (D) as well as the fold agonist stimulation of GTPγS binding as calculated from (B) and (E), respectively, in dependence of the ratio in the multiplicity of infection of the G_i subunit viruses and AcPolD2S. The results from one typical experiment out of two for G_{i1} and out of five for G_{i2} are shown.

together with the D_{2S} receptor, but without β_1 and γ_2, resulted in only about 40% of the GTPγS sites detected in cells that were coinfected with all three G protein subunit viruses, and agonist-stimulated GTPγS binding was barely detectable (data not shown).

D_{2S} Receptor-Mediated Effects on [^{35}S]GTPγS Binding and GTPase Activity of G_i Subtypes. Using the established approach of the *in vivo* reconstitution described above, coupling of the D_{2S} receptor to G_{i1} and G_{i2}, respectively, was examined in more detail. The time course of [^{35}S]GTPγS binding to cells coinfected with the AcPolD2S virus and the G_{i1} or G_{i2} viruses in comparison to cells coinfected with either G_{i1} or G_{i2} viruses only was measured (results are summarized in Table 1). According to the results presented above (Figure 2), the mois of the viruses were chosen to give optimal agonist-stimulated [^{35}S]GTPγS binding. Only in cells expressing both receptor and the respective G protein was the extent of [^{35}S]GTPγS binding increased in the presence of agonist. Table 1 shows the rate of [^{35}S]GTPγS binding of the G_i subtypes derived from the first-order rate equation. The basal rate of [^{35}S]GTPγS binding for G_{i1} and G_{i2} was the same in cells expressing either G_i only or G_i and the D_{2S} receptor simultaneously. Even so, these experiments have been done with whole cells and not with purified G proteins; the apparent basal association rates of 0.06−0.07 min^{-1} are in very good agreement with those published for pure recombinant α_i-subunits produced in Sf9 insect cells (Graber et al., 1992) as well as with those purified from mammalian tissues (Senogles et al., 1990). The agonist apomorphine significantly increased the rate of binding of [^{35}S]GTPγS to G_{i1} or G_{i2} almost 2-fold when the D_{2S} receptor was coexpressed in the cells (Table 1). In order to compare the extent of agonist-stimulated [^{35}S]GTPγS binding in different experiments, [^{35}S]GTPγS bound at various times was normalized to the total [^{35}S]GTPγS binding sites present in the assay. In cells coexpressing the D_{2S} receptor and either G_{i1} or G_{i2}, about 40% of the total [^{35}S]GTPγS sites present were occupied under basal conditions, defined here as being in the presence of the antagonist *S*(+)-butaclamol. Apomorphine significantly stimulated [^{35}S]GTPγS binding to 52% for G_{i1} and 62% for G_{i2}, respectively (Table 1). This difference is not statistically significant (Student's *t*-test, *p*

Table 1: [^{35}S]GTPγS Association in Coinfected Sf9 Cells[a]

condition	G_{eq}/G_T	k_{app} (min^{-1})
G_{i1}	0.398 ± 0.024	0.072 ± 0.004
D_{2S}+G_{i1}, Apo	0.524 ± 0.037	0.113 ± 0.005
D_{2S}+G_{i1}, But	0.414 ± 0.029	0.062 ± 0.005
G_{i2}	0.610 ± 0.037	0.065 ± 0.005
D_{2S}+G_{i2}, Apo	0.622 ± 0.027	0.116 ± 0.007
D_{2S}+G_{i2}, But	0.456 ± 0.028	0.072 ± 0.005

[a] A total of 5×10^6 cells in a 6 cm petri dish were infected with G protein subunit β_1, γ_2, and either α_{i1} or α_{i2} viruses each at a moi of 10 with (D_{2S}+G_{i1} or D_{2S}+G_{i2}) or without (G_{i1} or G_{i2}) the AcPolD2S baculovirus at a moi of 6. Seventy hours after infection, total [^{35}S]GTPγS binding and time-dependent [^{35}S]GTPγS binding in the presence of either 100 μM *R*(−)-apomorphine (Apo) or 1 μM *S*(+)-butaclamol (But) were measured as described under Experimental Procedures. [^{35}S]GTPγS bound at time t (G_t) was normalized to the total [^{35}S]GTPγS sites present (G_T). G_{eq} designates the GTPγS bound after the plateau was reached. The values shown for G_{eq}/G_T and k_{app} were determined by nonlinear least-squares fitting to the first-order rate equation and represent the means ± SE of five independent experiments performed in duplicate.

> 0.05). Taking into account that under the experimental conditions 25−30% of the total [^{35}S]GTPγS sites measured were estimated not to belong to recombinant α_i (Figure 1B), agonist-stimulated GTPγS binding typically represented 70−80% of the estimated total G_i present. There is an unexpected difference in the extent of GTPγS binding in cells infected with either the three G_{i1} or G_{i2} subunit viruses (but without the AcPolD2S virus). In the case of G_{i1}, 40% of the total [^{35}S]GTPγS sites were occupied, which correspond to the basal conditions in coinfection experiments with the D_{2S} receptor AcPolD2S virus. In the case of G_{i2}, however, the extent of [^{35}S]GTPγS binding (61%) (but not the apparent first-order rate) equals that of agonist-stimulated GTPγS binding in AcPolD2S-coinfected cells (62%). Similar behavior was found when cells were infected with lower mois (data not shown). The reason for this difference between G_{i1} and G_{i2} is not clear at the moment.

Under identical conditions, the agonist-stimulated GTPase activity was comparable in D_{2S} receptor expressing cells coinfected with either G_{i1} or G_{i2}. In control cells either infected with AcPolD2S only or coinfected with G_s, GTPase activity was low and insensitive to agonist stimulation (Figure 3).

Recently, the use of permeable cell systems has been suggested for studying receptor−G protein−effector coupling (Rasenick et al., 1993). In the GTPγS binding and GTPase experiments described above, typically about 75−85% of the cells were trypan blue-positive. Additional permeabilization of the cells with saponin (0.025% or 0.05% saponin in TBS, pH 7.4) prior to the assays, resulting in nearly 100% permeabilized cells, had no significant effect on GTPγS binding and GTPase activity (data not shown).

Appearance of High and Low Agonist Affinity Sites in Coinfected Sf9 Cells. The receptor−G protein coupling experiments as probed on the G protein side were done with saturating concentrations of agonist. Under such conditions, no significant difference in coupling of the D_{2S} receptor to either G_{i1} or G_{i2} could be detected. To further characterize the receptor to G protein interactions "in vivo", agonist competition experiments were performed in single infected (AcPolD2S only) and coinfected (AcPolD2S + G_{i1} or G_{i2}) Sf9 cells (Figure 4, Table 2). In AcPolD2S-infected cells, the agonists dopamine and apomorphine bound to a single

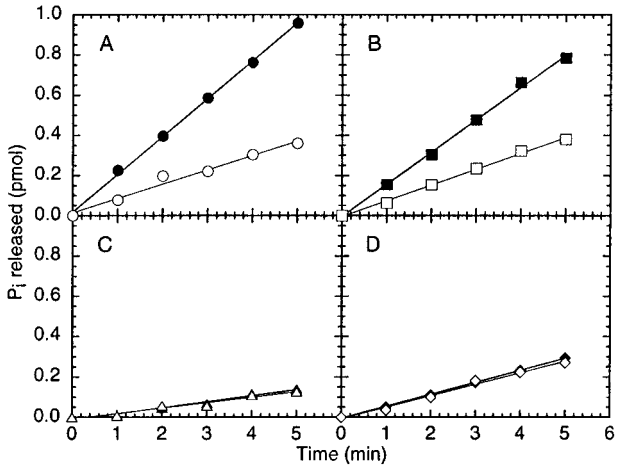

FIGURE 3: Comparison of GTPase activity promoted by the D_{2S} receptor. Sf9 cells were infected as described in the legend to Figure 1. Seventy hours postinfection, GTPase activity was measured in the presence of agonist (closed symbols) or antagonist (open symbols). (A) D_{2S}+G_{i1}. (B) D_{2S}+G_{i2}. (C) D_{2S}+G_s. (D) D_{2S}.

class of low-affinity GppNHp-insensitive sites (Figure 4A, but see Figure 5 and Table 3). In contrast, the human D_{2L} receptor isoform has been reported to display a low- and a high-affinity dopamine binding site in membranes from infected Sf9 cells, indicating coupling to an endogenous G protein (Ng et al., 1994). Furthermore, Boundy et al. (1996) reported recently the detection of the high-affinity state of both rat D_2 receptor isoforms in Sf9 cells after short infection times, resulting in low receptor expression levels. Both authors used different assay buffers in their experiments. Therefore, we prepared membranes from AcPolD2S-infected Sf9 cells and repeated agonist competition binding according to the protocols given by Ng et al. (1994). Again, only the low-affinity dopamine binding site could be detected (Table 2). The low-affinity dissociation constant, K_L, of dopamine obtained in buffer N with membranes containing D_{2S} receptors (12.3 ± 0.9 μM) was virtually identical to the K_L determined in buffer S either with or without NaCl (11.5 ± 0.6 μM and 11.7 ± 0.8 μM, respectively) and corresponds well with the K_L found for the D_{2L} receptor in Sf9 cells [15.4 ± 0.058 μM (Ng et al., 1994)]. We also examined the inhibition of [^{3}H]methylspiperone binding by dopamine in AcPolD2S-infected Sf9 cells at 18−22 h after infection. Expression levels were very low, ranging from 40 000 to 150 000 receptors/cell (0.12−0.5 pmol/mg of total cell protein). Contrary to Boundy et al. (1996), we could not detect any high-affinity agonist binding. The low-affinity dissociation constant of dopamine (K_L = 8.8 ± 1.4, n = 4) was similar to that obtained at higher expression levels. In cells coinfected with either G_{i1} or G_{i2}, however, agonist interactions were best fit to a model predicting two subclasses of sites (or three sites, see Figure 5 and Table 3). The agonist-dependent high-affinity state of the receptor was sensitive to GppNHp as shown by the rightward shift of the displacement curves. Addition of GppNHp completely converted the high-affinity to the low-affinity state (Figure 4). These results again clearly indicate that the D_{2S} receptor heterologously produced in Sf9 cells was able to interact with both the coexpressed G_{i1} and G_{i2} proteins. Yet, a detailed analysis of the competition binding data revealed a significant difference in the agonist binding properties of the D_{2S} receptor coexpressed with G_{i1} versus G_{i2}. When coexpressed

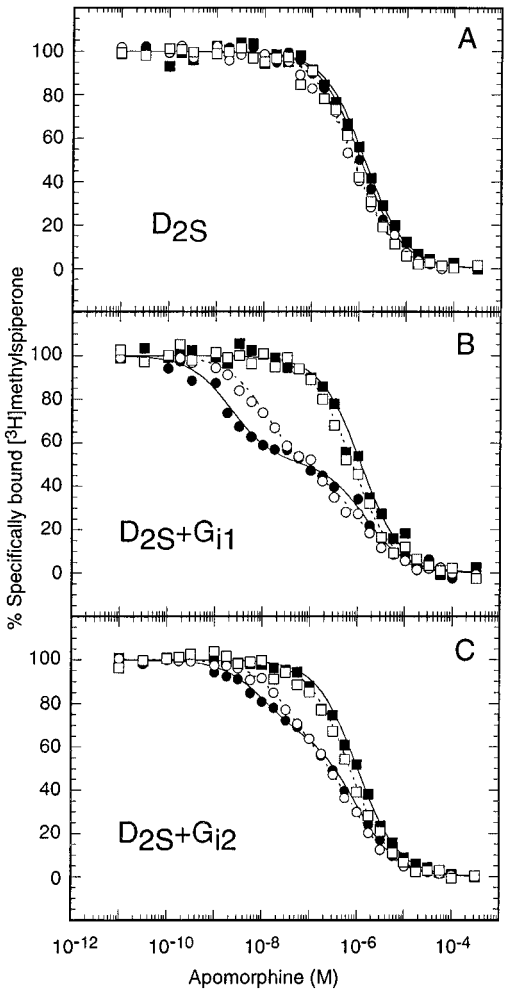

FIGURE 4: Apomorphine competition for radioligand binding at the D_{2S} receptor heterologously coexpressed with G_i proteins in Sf9 insect cells. Sf9 cells were infected with the AcPolD2S baculovirus alone (moi = 1.5) (A) or coinfected with the AcPolD2S (moi = 1.5), the β_1, the γ_2, and either the α_{i1} (B) or the α_{i2} (C) viruses (moi = 3 for each of the G protein subunit viruses). Seventy hours postinfection, cells were harvested, and total [^{35}S]GTPγS and [^{3}H]spiperone binding sites were determined. Apomorphine competition binding was performed in buffer S with (open symbols) or without (closed symbols) 100 mM NaCl in the absence (●, ○) or presence (■, □) of 150 μM GppNHp. The percent inhibition of specific [^{3}H]methylspiperone binding (K_D = 111 pM) is plotted. The data shown are means of duplicates from one representative experiment that was repeated several times (Table 2). The solid and dotted lines represent the theoretical inhibition curves using the fitted binding parameters obtained by LIGAND for a one-site [with GppNHp, $K_L = K_L(G)$] or two-site (without GppNHp) agonist competition model. Expression levels and fitted parameters for this experiment are as follows: (A) [^{35}S]GTPγS sites/cell were not determined, 1.23×10^6 [^{3}H]spiperone sites/cell, −NaCl: $K_L = 120 \pm 6$ nM, $K_L(G) = 145 \pm 9$ nM; +NaCl: $K_L = 86 \pm 5$ nM, $K_L(G) = 83 \pm 5$ nM. (B) 3.03×10^7 [^{35}S]GTPγS sites/cell, 5.90×10^5 [^{3}H]spiperone sites/cell, −NaCl: $K_H = 0.23 \pm 0.09$ nM, $R_H = 51 \pm 3\%$, $K_L = 158 \pm 28$ nM, $K_L(G) = 127 \pm 15$ nM; +NaCl: $K_H = 1.33 \pm 0.34$ nM, $R_H = 55 \pm 4\%$, $K_L = 136 \pm 20$ nM, $K_L(G) = 123 \pm 15$ nM. (C) 2.53×10^7 [^{35}S]GTPγS sites/cell, 5.35×10^5 [^{3}H]spiperone sites/cell, −NaCl: $K_H = 1.06 \pm 0.36$ nM, $R_H = 34 \pm 3\%$, $K_L = 104 \pm 8$ nM, $K_L(G) = 110 \pm 4$ nM; +NaCl: $K_H = 3.85 \pm 1.40$ nM, $R_H = 43 \pm 5\%$, $K_L = 104 \pm 14$ nM, $K_L(G) = 76 \pm 5$ nM.

with G_{i1}, the D_{2S} receptor displayed a greater proportion of high-affinity agonist binding sites, which, in addition, showed a higher affinity for the agonist than when coexpressed with G_{i2} (all differences are statistically significant with $p < 0.005$, $p < 0.01$, or $p < 0.05$; see Table 2). Thus, dopamine had a 2-fold and apomorphine a 4-fold increased affinity for the G protein-coupled state when coupling was to G_{i1} as compared to G_{i2}. The dopamine dissociation constants for the high- and low-affinity states, K_H and K_L, in AcPolD2S- and G_{i1}-coinfected cells match those published for the D_{2L} receptor in Sf9 cells. However, when compared to the D_{2S} receptor expressed in mammalian cells [LTk$^-$ (Castro & Strange, 1993); CHO-K1 (Hayes et al., 1992)], K_H was 2−3 times and K_L 5−6 times higher, with about the same fraction of high-affinity binding sites. For apomorphine, K_H, K_L, and R_H measured in buffer S with D_{2S} and G_{i1} coexpressing Sf9 cells correspond well with those reported for the D_{2S} receptor in LTk$^-$ cells (Falardeau, 1994).

Figure 4 and Table 2 demonstrate that binding of apomorphine to the D_{2S} receptor was sensitive to sodium. Inclusion of 100 mM NaCl in the binding buffer led to a 4-fold increase of the dissociation constant K_H in the presence of G_{i1} and to a 2-fold increase in the presence of G_{i2}. Low-affinity binding was not affected, and preferential coupling to G_{i1} was preserved. Dopamine binding was not significantly modulated by NaCl.

For performance of these competition binding experiments, a higher ratio of GTPγS sites to receptor sites was favorable. Optimal mois turned out to be about 1.5 for the AcPolD2S virus and 3 for each of the three G protein subunit viruses, resulting in an estimated G protein to receptor ratio of 41 ± 4 and 37 ± 6 for G_{i1} and G_{i2}, respectively. A similar observation has been reported for muscarinic receptors heterologously expressed in Sf9 cells. Here, a higher G protein to receptor ratio was necessary to observe G protein-induced high-affinity agonist binding than to observe receptor-mediated GTPγS binding and GTPase activity (Richardson & Hosey, 1992).

In some of the dopamine competition experiments, displacement data were best fit assuming three noninteracting classes of sites in the absence and two independent classes of sites in the presence of GppNHp ($p \leq 0.001$, F test). Representative displacement plots for coupling to G_{i1} and G_{i2}, respectively, are shown in Figure 5. Theoretical two- (−GppNHp) and one-site (+GppNHp) fits as calculated by LIGAND are shown by dotted lines for comparison. The dissociation constants are summarized in Table 3. In the presence of GppNHp, the proportion of high-affinity agonist sites in either G_{i1}- or G_{i2}-coinfected cells was quantitatively converted to low-affinity sites, whereas the fraction of sites displaying intermediate affinity remained unchanged. The values for the dissociation constants K_H and K_M correspond well with K_H and K_L reported for the high- and low-affinity sites of the D_{2S} receptor in LTk$^-$ or CHO-K1 cells (Hayes et al., 1992; Falardeau, 1994), except that K_M belongs to a receptor state that does not seem to couple to G proteins. In membranes from AcPolD2S-infected cells, only the GppNHp-insensitive intermediate and low-affinity binding sites were found. Their proportions were the same as in GppNHp-treated coinfected cells. Two competition binding experiments out of four with membranes in buffer N, however, gave K_M and K_L values about 3−4-fold higher with inverse proportions. The same values and proportions were found when AcPolD2S-infected cells instead of membranes were used for the binding experiments. GppNHp had no influence (Table 3).

In the presence of G_{i1}, dopamine again bound with 2-fold higher affinity, and there was a greater proportion of high-

Table 2: Quantitative Analysis of Agonist Competition Curves for [^{3}H]Methylspiperone Binding to Single and Coinfected Sf9 Cells[a]

experimental condition		competing agonist: dopamine				competing agonist: apomorphine			
expression	assay buffer	K_H (nM)	K_L (μM)	% R_H	n	K_H (nM)	K_L (nM)	% R_H	n
D_{2S}+G_{i1}	S	20 ± 5[b]	8.8 ± 2.1	56 ± 2[b]	4	0.44 ± 0.11[c,e]	148 ± 22	50 ± 2[b]	5
	S+GppNHp		6.6 ± 0.1		4		108 ± 11		3
	S+NaCl	29 ± 5[d]	8.5 ± 1.6	56 ± 1[b]	4	1.82 ± 0.42[c]	155 ± 36	58 ± 4[d]	4
	S+NaCl+GppNHp		6.9 ± 0.3		4		99 ± 10		4
D_{2S}+G_{i2}	S	48 ± 4	7.1 ± 0.5	41 ± 3	4	1.65 ± 0.35[e]	112 ± 4	34 ± 3	3
	S+GppNHp		7.6 ± 0.5		4		126 ± 12		3
	S+NaCl	63 ±10	9.8 ± 1.9	46 ± 1	4	3.59 ± 0.14	109 ± 7	39 ± 3	4
	S+NaCl+GppNHp		7.8 ± 0.7		4		133 ± 35		3
D_{2S}	S		11.5 ± 0.6		4		109−120		2
	S+GppNHp		13.3 ± 1.2		3		113−145		2
	S+NaCl		11.7 ± 0.8		3		86−115		2
	S+NaCl+GppNHp		12.4 ± 1.0		3		83−175		2
membranes	N		12.3 ± 0.9		4				
D_{2L} in Sf9 [f]	N	25.1 ± 4.6	15.4 ± 0.06	28	3				
	N+GppNHp		14.0 ± 0.18		3				
D_{2S} in LTk$^-$ [g]	−NaCl	7.7 ± 4	0.75 ± 0.5	58	4	0.75 ± 0.31	41.7 ± 3.7	48	5
D_{2S} in LTk$^-$ [h]		6.2 ± 3.3	1.40 ± 0.13	52 ± 5	3	0.77 ± 0.32	134 ± 32	55 ± 6	3
D_{2S} in CHO[i]	+120 mM NaCl	9.43 ± 5.33	1.44 ± 0.84	65 ± 10	3				

[a] Sf9 cells were infected with the AcPolD2S (moi = 1.5), the β_1, the γ_2, and either the α_{i1} (D_{2S}+G_{i1}) or the α_{i2} (D_{2S}+G_{i2}) baculoviruses (moi = 3 for each of the G protein subunit viruses) or with the AcPolD2S virus alone (moi 1.5−6) (D_{2S}). Seventy hours postinfection, cells were harvested. Total [^{35}S]GTPγS and [^{3}H]spiperone binding sites were determined, and agonist competition binding was performed in buffer S ±100 mM NaCl with or without 150 μM GppNHp. Membranes of AcPolD2S-infected Sf9 cells were prepared as in Ng et al. (1994) and were used for dopamine competition in buffer N in control experiments. Average [^{35}S]GTPγS sites/cell and [^{3}H]spiperone binding sites/cell were (2.86 ± 0.19) × 10^7 and (5.94 ± 0.83) × 10^5 for D_{2S}+G_{i1} and (2.68 ± 0.25) × 10^7 and (6.56 ± 0.59) × 10^5 for D_{2S}+G_{i2}, resulting in an average estimated G protein to receptor ratio of 41 ± 4 for D_{2S}+G_{i1} and 37 ± 6 for D_{2S}+G_{i2}, respectively. Dissociation constants for high (K_H) and low (K_L) affinity sites and the proportion of high-affinity sites (% R_H) were statistically determined by LIGAND analysis and are reported as the mean and standard error of several independent determinations (n). Statistical comparisons of the results were made using the Student's unpaired t test (InStat, GraphPad Software). [b] $p < 0.005$, [c] $p < 0.01$, [d] $p < 0.03$ compared to the K_H or % R_H value, respectively, obtained in the same assay buffer with D_{2S}+G_{i2} expression. [e] $p < 0.01$ compared to the K_H value obtained in the presence of 100 mM NaCl in the same infection. [f] Ng et al. (1994). [g] Castro & Strange (1993). [h] Falardeau (1994). [i] Hayes et al. (1992).

Table 3: Dopamine Dissociation Constants for the Human D_{2S} Receptor Coexpressed with G_i Proteins in Sf9 Cells According to a Model Predicting Three Independent Subclasses of Agonist Sites[a]

expression	assay buffer	K_H (nM)	% R_H	K_M (μM)	% R_M	K_L (μM)	% R_L	n
D_{2S}+G_{i1}	S	4.5 ± 0.8[b]	39 ± 4[c]	0.51 ± 0.21	30 ± 2	20.4 ± 9.5	31 ± 3	3
	S+GppNHp			0.37 ± 0.17	28 ± 1	13.7 ± 2.5	72 ± 1	3
D_{2S}+G_{i2}	S	10.4 ± 0.7	25 ± 3	0.85 ± 0.18	42 ± 4	27.1 ± 5.8	33 ± 2	3
	S+GppNHp			0.91 ± 0.13	38 ± 7	20.0 ± 4.3	62 ± 7	3
D_{2S} (membranes)	S			0.91 ± 0.38	28 ± 4	39.6 ± 12.5	72 ± 4	3
	S+GppNHp			1.90	24	15	76	1
	N			1.10−1.63	22−34	14−22	66−78	2
	N			3.5−4.4	64−71	83−111	29−36	2
	N+GppNHp			5.9 ± 1.4	70 ± 10	105 ± 37	30 ± 10	3
D_{2S} (cells)	S			4.6 ± 0.8	72 ± 5	58 ± 13	28 ± 5	3
	S+GppNHp			6.0 ± 1.4	71 ± 11	90 ± 27	29 ± 11	3

[a] Sf9 cells were infected with the AcPolD2S, the β_1, the γ_2, and either the α_{i1} (D_{2S}+G_{i1}) or the α_{i2} (D_{2S}+G_{i2}) baculoviruses (moi ≈ 3 for each of the viruses) or with the AcPolD2S virus alone (moi ≈ 14) (D_{2S}). Seventy hours postinfection, cells were harvested. Total [^{35}S]GTPγS and [^{3}H]spiperone binding sites were determined, and dopamine competition binding was performed in buffer S with or without 150 μM GppNHp as described under Experimental Procedures. In some cases, membranes were prepared from AcPolD2S-infected cells as described by Ng et al. (1994) prior to assay. Average [^{35}S]GTPγS sites/cell and [^{3}H]spiperone binding sites/cell were (2.67 ± 0.45) × 10^7 and (6.94 ± 2.18) × 10^5 for D_{2S}+G_{i1} and (2.42 ± 0.06) × 10^7 and (6.76 ± 0.79) × 10^5 for D_{2S}+G_{i2}, resulting in an average estimated G protein to receptor ratio of 32 ± 3 for D_{2S}+G_{i1} and 28 ± 2 for D_{2S}+G_{i2}, respectively. Dissociation constants K_H, K_M, and K_L and their proportion were statistically determined by LIGAND analysis and are reported as the mean and standard error of three independent determinations except for the few cases where the results of one or two experiments are shown. For coinfected cells, the three-site model in the presence of GppNHp or for single-infected cells the two-site model significantly improved the curve fit ($p \leq 0.001$, F-test). Statistical comparisons of the results were made using the Student's unpaired t test (InStat, GraphPad Software). [b] $p < 0.005$, [c] $p < 0.05$ compared to K_H and % R_H values, respectively, obtained in the D_{2S}+G_{i2} infection.

affinity dopamine binding sites than with coexpression of G_{i2}. Interestingly, the fraction of low-affinity binding sites was the same in both G_{i1}- and G_{i2}-coinfected cells, so that the increased fraction of high-affinity sites in G_{i1}- versus G_{i2}-coexpressing cells was at the cost of sites displaying intermediate affinity and not, as one might have expected, at the cost of low-affinity sites.

Effect of Dopamine on Forskolin-Stimulated cAMP Accumulation in AcPolD2S-Infected Sf9 Cells. In order to find out whether the overexpressed D_{2S} receptor is able at all to interact to a certain extent with endogenous G proteins, as shown by Boundy et al. (1996), we examined whether dopamine is able to inhibit forskolin-stimulated cAMP accumulation in AcPolD2S-infected Sf9 cells. In cells expressing more than 1 × 10^6 receptors/cell, dopamine inhibited forskolin-stimulated cAMP accumulation by about 40%. This effect could be blocked by the dopaminergic antagonist (+)-butaclamol (Figure 6). In uninfected cells,

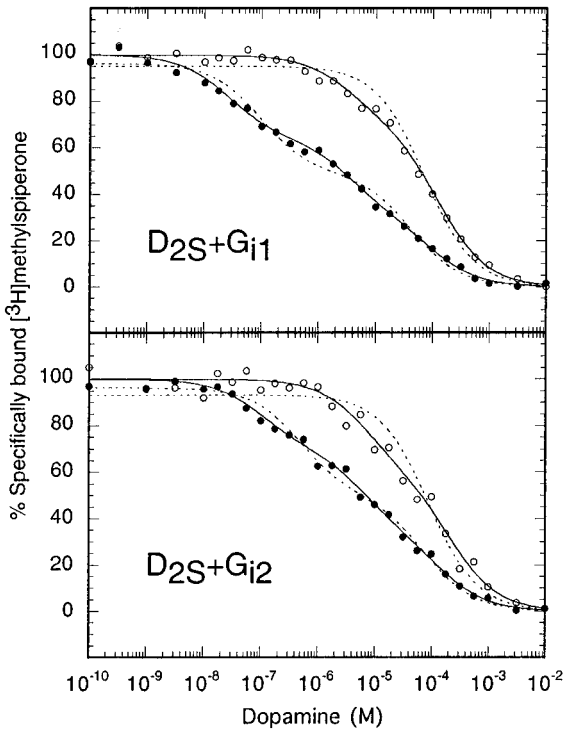

FIGURE 5: A model of two independent classes of sites is not always satisfactory for describing dopamine competition binding to the D_{2S} receptor coexpressed with G_i proteins in Sf9 insect cells. Sf9 cells were coinfected with the AcPolD2S, the β_1, the γ_2, and either the α_{i1} (A) or the α_{i2} (B) baculoviruses at a moi of 3 for each. Binding experiments were performed as described. Data are the means of duplicates from one out of three independent experiments and were fit using LIGAND assuming three (closed symbols, without GppNHp) or two (open symbols, with 150 μM GppNHp) noninteracting classes of agonist sites that bound antagonist with the same dissociation constant. Fits are shown by the solid curve. For comparison, the fits assuming two independent agonist sites, or one site in the presence of GppNHp, are shown by the dotted curves. Expression levels and fitted parameters are as follows: (A) 2.20×10^7 [^{35}S]GTPγS sites/cell, 4.76×10^5 [^{3}H]spiperone sites/cell, $K_H = 2.77 \pm 1.12$ nM, $R_H = 36 \pm 4\%$, $K_M = 0.39 \pm 0.20$ μM, $R_M = 33 \pm 5\%$, $K_L = 10.1 \pm 2.7$ μM, $R_L = 31 \pm 4\%$, $K_M(G) = 0.35 \pm 0.16$ μM, $R_M(G) = 28 \pm 4\%$, $K_L(G) = 13.4 \pm 1.3$ μM, $R_L(G) = 72 \pm 4\%$. (B) 2.43×10^7 [^{35}S]GTPγS sites/cell, 5.96×10^5 [^{3}H]spiperone sites/cell, $K_H = 11.5 \pm 6.5$ nM, $R_H = 30 \pm 6\%$, $K_M = 0.72 \pm 0.46$ μM, $R_M = 36 \pm 6\%$, $K_L = 15.8 \pm 4.8$ μM, $R_L = 34 \pm 7\%$, $K_M(G) = 0.74 \pm 0.47$ μM, $R_M(G) = 39 \pm 9\%$, $K_L(G) = 23.1 \pm 6.4$ μM, $R_L(G) = 61 \pm 9\%$.

dopamine had no effect on intracellular cAMP levels (data not shown). In cells expressing only $(2-4) \times 10^5$ receptors/cell, inhibition of forskolin-stimulated cAMP accumulation by dopamine was only about 20%. The same degree of inhibition was reached in cells that were coinfected with G_{i1} or G_{i2} subunit baculoviruses and therefore expressed similarily low receptor levels.

DISCUSSION

The human D_{2S} receptor overexpressed in baculovirus-infected insect cells is glycosylated and palmitoylated and shows ligand binding characterisitics similar to the native protein (Grünewald et al., 1996). Yet, coupling to G proteins endogenous to Sf9 insect cells was not evident with either agonist competition studies or G protein activation assays. The receptor exhibited a K_D for the agonist apomorphine similar to that of the low-affinity form of brain D_2 receptors (Sibley et al., 1982) or of D_{2S} receptors heterologously

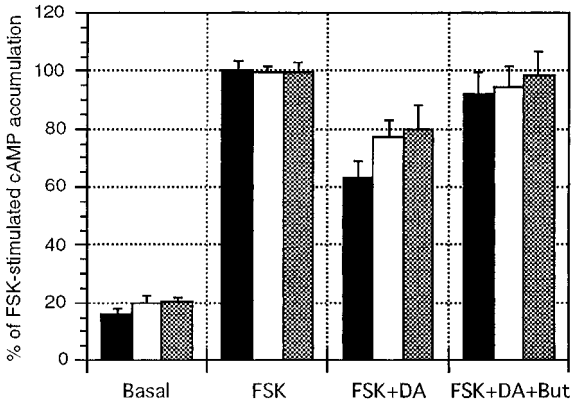

FIGURE 6: Dopamine inhibits forskolin-stimulated cAMP accumulation in Sf9 cells expressing the human D_{2S} receptor. cAMP accumulation was measured in Sf9 cells expressing the D_{2S} receptor either alone (black) or together with G_{i1} (white) or G_{i2} (grey) as outlined under Experimental Procedures. Stimulation was with 10 μM forskolin (FSK) in the presence of either 10 μM dopamine (FSK+DA) or 10 μM dopamine plus 10 μM (+)-butaclamol (FSK+DA+But). For better comparison of the effect of dopamine, values for each infection were normalized to maximal forskolin-stimulated cAMP levels. The results shown are the mean ± SD of three to five independent experiments performed in duplicate.

expressed in mammalian cell lines (Castro & Strange, 1993; Falardeau, 1994). Although competition binding using the natural agonist dopamine was more complicated, only low-affinity GppNHp-insensitive binding sites were detected. We originally thought that the lack of high-affinity agonist binding in Sf9 cells was due to the absence of appropriate endogenous G proteins. All the signaling events evoked by D_2 receptors including inhibition of adenylyl cyclase (Albert et al., 1990), activation of K^+ channels (Castellano et al., 1993), or modulation of intracellular calcium levels (Vallar & Meldolesi, 1989) are pertussis toxin-sensitive, indicating mediation by the $G_{i/o}$ family of G proteins. So far, however, G_i proteins could not be detected in Sf9 cells (Quehenberger et al., 1992; Mulheron et al., 1994). We hypothesized that the GppNHp-sensitive high-affinity dopamine binding of the human D_{2L} isoform reported by Ng et al. (1994) was due to the additional 29 amino acid insert in the putative third intracellular loop which in this artificial cellular environment allowed interaction with a pertussis toxin-insensitive G protein.

In a recent report, Boundy et al. (1996) showed that in Sf9 cells dopamine binding of both the rat D_{2S} and D_{2L} receptor isoforms was guanine nucleotide-sensitive but only at very low expression levels (below 1 pmol/mg). Similarly, receptor−G protein coupling of the 5-HT_{1A} receptor in Sf9 cells was dependent on receptor levels (Mulheron et al., 1994; Parker et al., 1994; Butkerait et al., 1995). So, with $(1-2) \times 10^6$ D_{2S} receptors per cell, corresponding to 10−19 pmol/mg of membrane protein, overexpression in Sf9 cells could have been too high so that endogenous G proteins were limiting. In this context, one should also keep in mind that the virus shuts off host gene expression (Carstens et al., 1979). This could lead to progressive uncoupling of a recombinant receptor with increasing infection time as has already been shown for the heterologously expressed murine gastrin-releasing peptide receptor (Kusui et al., 1995). However, dopamine competition binding performed on membranes of a stably transformed Sf9 cell line, thus not being affected by viral action, also was only of low affinity (unpublished results). The expression level of the D_{2S}

receptor in these membranes with 2.2 pmol/mg is in the range of that achieved by Ng et al. (1994) with the D_{2L} receptor (1.1 pmol/mg) or by Mills et al. (1993) with the D_4 receptor (5 pmol/mg) in Sf9 cells, both showing GTP-sensitive agonist binding.

On the other hand, dopamine was able to significantly inhibit forskolin-stimulated cAMP accumulation in D_{2S} receptor expressing Sf9 cells. So, the expressed D_{2S} receptor obviously is able to couple to some extent to endogenous G proteins. Since expression levels of more than 1×10^6 receptors/cell were necessary to achieve an inhibition of forskolin-stimulated cAMP accumulation of 40%, coupling to the endogenous G protein(s) does not seem to be very specific and could not be detected by agonist competition binding experiments using a tritiated radioligand.

Since full functionality of the overexpressed D_{2S} receptor, including the ability to efficiently couple to G proteins, is a prerequisite for any further pharmacological, biophysical, or structural studies, reconstitution of the receptor with its appropriate G proteins was necessary. The endogenous G protein population in baculovirus-infected Sf9 cells seems to be very low. Indeed, purification of recombinant G proteins from infected insect cells resulted in contamination by endogenous G proteins of less than 0.12% (Graber et al., 1992). The low content of endogenous G proteins and the ability to coinfect Sf9 cells with different baculoviruses simultaneously make them a suitable *in vivo* reconstitution system that allows one to examine the selectivity of interaction between different receptors and G proteins as well as to screen for a given receptor's possible coupling partners as demonstrated by Butkerait et al. (1995). This "in vivo" reconstitution system was established for the D_{2S} receptor and allowed us in a convenient way to examine G protein coupling without the laborious purification and reconstitution into lipid vesicles of the receptor and G proteins involved. In the coinfected cells, D_2 agonists effectively stimulated binding of GTPγS and significantly increased GTPase activity. The heterologously produced receptor displayed high-affinity agonist binding which was sensitive to Gpp-NHp, thus proving functional coupling of the receptor to the coexpressed G_i protein.

In light of the fact that the D_{2L} isoform has been shown to preferentially couple to $G_{i\alpha2}$, the potential of this *in vivo* reconstitution system to compare in detail coupling of the D_{2S} isoform to $G_{i\alpha1}$ versus $G_{i\alpha2}$ was exploited. The α_i-subunits were always coexpressed with β_1 and γ_2, since α_i levels were much lower and G protein coupling was barely detectable when expressed alone with the D_{2S} receptor. The requirement for both α and $\beta\gamma$ for effective G protein coupling has also been observed for the stimulation of transducin by rhodopsin (Fung, 1983) or of G_o by muscarinic receptors (Florio & Sternweis, 1989) and fits the common picture of the G protein cycle (Neer, 1995). In addition, coexpressed $\beta\gamma$-subunits seem to stabilize α-subunits as was shown for recombinant $G_{q\alpha}$ and $G_{11\alpha}$ in Sf9 cells (Hepler et al., 1993). We could not detect significant differences in expression levels between G_{i1} and G_{i2} irrespective of receptor coexpression. Basal GTPγS association rates were the same for both G_{i1} and G_{i2} and equalled those published for purified native and recombinant G_{i1} or G_{i2} (Senogles et al., 1990; Graber et al., 1992).

In contrast to the results reported for the 5-HT_{1A} receptor (Butkerait et al., 1995), the coexpression of G proteins influenced receptor levels and vice versa. Varying the multiplicity of infection for the receptor and the three G protein viruses allowed us to obtain different G protein to receptor ratios. In liposome reconstitution studies with D_2 (Senogles et al., 1990) as well as other receptors (Tota et al., 1987; Florio & Sternweis, 1989), it has been shown that G protein coupling is dependent on the G protein to receptor ratio. So, agonist-stimulated GTPγS binding was optimized by varying the ratio of the multiplicity of infection for receptor and G protein viruses. Maximum fold agonist stimulation of GTPγS binding was the same for G_{i1} and G_{i2}. Both G_{i1} and G_{i2} showed similar basal and agonist-dependent GTPγS association rates, and GTPase activity could also be stimulated to the same extent.

The percentage of high-affinity agonist binding sites, however, is significantly higher in cells coinfected with the G_{i1} virus than after coinfection with the G_{i2} virus. Moreover, coexpressed G_i proteins seemed to modulate the affinity of agonists for the high-affinity form of the receptor. In cells coexpressing G_{i1}, agonist high affinities were 2−4-fold higher than in cells coexpressing G_{i2}. Low-affinity binding was not affected and was the same as in cells expressing the D_{2S} receptor only. This clearly demonstrates a preferential interaction of the D_{2S} isoform with G_{i1} than with G_{i2}. These results are consistent with results obtained in $G_{i\alpha2}$-deficient JEG3 cells (Montmayeur et al., 1993; Guiramand et al., 1995). In these cells, the D_{2S} isoform coupled more efficiently to endogenous G_i (presumably G_{i1} or G_{i3}) than the D_{2L} isoform, which required $G_{i\alpha2}$ to elicit the same inhibitory effect on stimulated adenylyl cyclase. In addition, the D_{2S} receptor had a 2-fold higher affinity for the agonist propylnorapomorphine than the D_{2L} receptor in these cells. Furthermore, in transfected GH4C1 cells expressing the D_{2L} but not the D_{2S} isoform, receptor-mediated inhibition of cAMP accumulation was impaired by coexpressing antisense to $G_{\alpha i2}$ (Liu et al., 1994). Using a different experimental approach, namely, eliminating endogenous G proteins by pertussis toxin treatment, Senogles (1994) reported preferred signaling of the D_{2S} isoform in GH4C1 cells through a cotransfected, pertussis toxin-insensitive $G_{i\alpha2}$ mutant and of the D_{2L} isoform through a cotransfected, pertussis toxin-insensitive $G_{i\alpha3}$ mutant. These results indicate that there are differences in signal transduction mediated by the two D_2 receptor isoforms.

However, measuring the second messenger response, e.g., changes in intracellular cAMP levels, in order to characterize the interactions between receptor and G protein, might result in ambiguous results since the signal depends on the interaction of the receptor and the G protein as well as on the interaction of the G protein and the effector. For instance, the different adenylate cyclase types present in the various cell systems used display different sensitivity to $G_{i\alpha}$-subunits (Kozasa & Gilman, 1995). So the elicited second messenger signal might be low not because of weak coupling of the receptor with the G protein but because of insensitivity of the adenylate cyclase present to the transfected $G_{i\alpha}$ subunit. In this context, it is interesting to note that both G_{i1} and G_{i2} did not increase the effect of dopamine on forskolin-stimulated cAMP accumulation in D_{2S} receptor-expressing Sf9 cells. This excludes dose−response curves of the inhibition of endogenous adenylate cyclase to further prove the preferential coupling of the D_{2S} receptor to mammalian G_{i1}.

A general characteristic of the G_i-coupled receptor group is their sensitivity to sodium ions which can affect both agonist and antagonist binding (Horstman et al., 1990; Jagadeesh et al., 1990; Neve, 1991). Several reports demonstrated that sodium decreased the proportion of D_2 receptors displaying high-affinity agonist binding without a change in the dissociation constants for the high- or low-affinity site (Grigoriadis & Seeman, 1985; Malmberg & Mohell, 1995). Often, NaCl must be present for GppNHp to fully convert high-affinity sites into low-affinity sites. On the other hand, Reader et al. (1990) demonstrated that sodium decreased agonist high and low affinity in dopamine/[^{3}H]-raclopride competition studies without a conversion of the two states. Also, Neve (1991) reported a decrease by sodium of agonist low affinity in an uncoupled system. In this study, sodium decreased high-affinity binding of apomorphine 2−4-fold but had no influence on the low-affinity dissociation constant K_L, on the proportions of high- and low-affinity, sites or on binding of dopamine. GppNHp was able to fully convert high-affinity sites into low-affinity sites in the absence or presence of NaCl. It seems that the measurable effects of sodium on agonist binding depend on the experimental system and on the agonist and radioactive antagonist (Na^+-dependent substituted benzamide versus Na^+-independent antagonists like spiperone) used. Whether in this baculovirus/insect cell "in vivo" reconstitution system Na^+ directly regulates ligand binding to overexpressed D_{2S} receptors without involvement of the G proteins present, or whether Na^+ exerts its effects primarily by decreasing the intrinsic affinity of the (eventually precoupled) receptor to the G protein (Tian & Deth, 1993), awaits further investigation.

Some dopamine binding data were not compatible with a simple two-state model (De Lean et al., 1980) but fit well to a model assuming three noninteracting classes of sites. Since GppNHp was able to quantitatively convert R_H to R_L in the dopamine competition curves, the highest dopamine affinity state is assigned to receptors complexed with G_i and, tentatively, the low-affinity state to free receptors. The appearance of three binding sites had no influence on the preferential coupling of the D_{2S} isoform to G_{i1} versus G_{i2}. The intermediate affinity state does not seem to be capable of interacting with G proteins and is undefined molecularly. This is somewhat surprising since the intermediate affinity corresponds to the low affinity found for dopamine in D_{2S} receptor expressing mammalian cell lines (Hayes et al., 1992; Castro & Strange, 1993). One would rather expect that GppNHp shifts the high-affinity sites to those of intermediate affinity. Given the unphysiologically high overexpression of the receptor and its predominant localization within the cytoplasm, i.e., presumably at intracellular membranes (Grünewald et al., 1996), possible explanations for these binding data include abnormal compartimentalization and state of aggregation.

The detection of three agonist binding sites has also been described for the porcine m2 muscarinic receptor highly overexpressed in CHO cells (Hirschberg et al., 1995; Vogel et al., 1995). Detailed kinetic analysis led to a model where the receptor could exist as a monomer or dimer in the G protein-coupled state as well as in the absence of ligands (Hirschberg et al., 1995). The existence of dimers is also being discussed for D_2 receptors (Seeman & Van Tol, 1994). In Sf9 cells expressing the D_{2S} receptor only, the dopamine dissociation constants were 3−4-fold higher but with inverse proportions than in G_i coexpressing cells. The same was found in some membrane preparations. In these cells, receptor expression levels were higher than in coinfected cells, and aggregation levels as well as compartimentalization might have been different. In addition, the coexpressed G_i protein might exert stabilizing effects on the overproduced receptor. In this context, it is interesting to note that the increased portion of high-affinity sites caused by G_{i1} compared to G_{i2} was at the cost of the undefined intermediate state and not at the cost of the low-affinity state into which GppNHp converted the high-affinity sites. Agonist binding at very low expression levels reproducibly yielded one, and not two, low-affinity agonist binding site. Preliminary agonist binding studies with D_{2S} receptor constructs containing the melittin signal sequence, which seems to be processed more completely and transported more easily to the plasma membrane (Grünewald et al., 1996), also revealed two low-affinity agonist binding sites at high expression levels. We therefore hypothesize that the second low-affinity binding site found in this Sf9 cell expression system is artifactual and due to aggregation of the receptor.

In summary, we have demonstrated that the D_{2S} receptor overexpressed in baculovirus-infected Sf9 cells is fully functional. We have also shown for the first time direct modulation of agonist high-affinity binding by specific G protein subtypes such that the D_{2S} receptor isoform prefers G_{i1} over G_{i2}. Given the fact that in all tissues studied the D_{2L} isoform is more abundant than D_{2S}, the latter will be imperative to cell function in those cells where the presence of a combination of specific G protein subtypes and effectors favors interaction with D_{2S}. The *in vivo* reconstitution system presented here should prove very valuable in further mapping the specificity of receptor−G protein interactions. It will be interesting to compare all members of the "D_2-like" family of dopamine receptors. Furthermore, this system offers the possibility to study agonist-modulated posttranslational modifications like phosphorylation or palmitoylation in the presence of different coexpressed G proteins.

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