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Agonist Regulation of D₂ Dopamine Receptor/G Protein Interaction

EVIDENCE FOR AGONIST SELECTION OF G PROTEIN SUBTYPE

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The D₂ dopamine receptor has been expressed in Sf21 insect cells together with the G proteins G₀ and G₁₂, using the baculovirus system. Expression levels of receptor and G protein (α, β, and γ subunits) in the two preparations were similar as shown by binding of [³H]siperone and quantitative Western blot, respectively. For several agonists, binding data were fitted best by a two-binding site model in either preparation, showing interaction of expressed receptor and G protein. For some agonists, binding to the higher affinity site was of higher affinity in D₂/G₀ than in the D₂/G₁₂ preparation. Some agonists exhibited binding data that were best fitted by a two-binding site model in D₂/G₀, and a one-binding site model in D₂/G₁₂. Therefore, receptor/G protein interaction seemed to be stronger in the D₂/G₀ preparation. Agonist stimulation of [³⁵S]GTPγS (guanosine 5′-3-O-(thio)triphosphate) binding in the two preparations also gave evidence for higher affinity D₂/G₀ interaction. In the D₂/G₀ preparation, agonist stimulation of [³⁵S]GTPγS binding occurred at higher potency for several agonists, and a higher stimulation (relative to dopamine) was achieved in D₂/G₀ compared with D₂/G₁₂. Some agonists were able to stimulate [³⁵S]GTPγS binding in the D₂/G₀ preparation but not in D₂/G₁₂. The extent of D₂ receptor selectivity for G₀ over G₁₂ is therefore dependent on the agonist used, and thus agonists may stabilize different conformations of the receptor with different abilities to couple to and activate G proteins.

There is considerable interest in understanding the action mechanisms of agonists at receptors (1–3). Agonists must bind to receptors, and this may be characterized in terms of an affinity of agonist binding. Agonists must also activate the receptor and associated signaling systems, and this property is often referred to as efficacy. Efficacy is exhibited in terms of the maximal effect induced by the agonist and also in the EC₅₀ of the agonist in activating the signaling system, which is often lower than the concentration of agonist which achieves half-maximal occupancy of the receptor.

For G protein-coupled receptors, an influential model of agonist action is the ternary complex model and its recent extensions (4–6). In this model the receptor exists in an inactive ground state, which may isomerize to a partially activated state (R*)¹ that is able to couple more efficiently to the G protein to form the coupled active species (R*G). The formation of R*G may occur spontaneously, but in the presence of an agonist both R* and R*G are stabilized, and the ternary complex (AR*G) is formed. Guanine nucleotide exchange (GDP/GTP) occurs in both the binary complex (R*G) and the ternary complex (AR*G). The binary and ternary complexes dissociate releasing αGTP and βγ subunits of the G protein which can alter effector activity. The agonist may also influence ternary complex breakdown (7, 8) so that there are several places at which agonism is determined.

There is, however, evidence that some receptors may interact with more than one G protein so that influences on different signaling pathways can occur. If a receptor can interact with more than one G protein this may influence the potency of agonist action and the pattern of agonist effects, i.e. the pharmacological profile of the response observed through the different G proteins. For the 5HT1A serotonin receptor, it was shown that the receptor interacts preferentially with Gᵢ, Gₒ, and Gₑ subtypes of G protein (9) and that the nature of the G protein subtype influenced the agonist selectivity of the response (10). This question was addressed more explicitly for the α₂-adrenergic receptor (11). Expression of Gₒ, together with the endogenous G proteins of NIH 3T3 cells, altered the agonist selectivity of the receptor; the partial agonists, oxymetazoline and clonidine, exhibited increased efficacy. The possibility that the pharmacological profile of the response depends on the nature of the G protein has been termed “agonist trafficking” (12).

The D₂ dopamine receptor has been shown to interact with different G proteins to influence different signaling events (13, 14). In one study, interaction with Gₒ has been shown to lead to inhibition of calcium channels, whereas interaction with Gₑ subtypes has been shown to lead to inhibition of adenyl cyclase (15). Also, the two splice variants of the D₂ receptor (D₂short and D₂long) have been reported to interact with different G proteins (13), although a clear definition of the selectivity pattern has not emerged as yet. Furthermore, the relative efficacies of quinpirole and (+)-3-PPP are reversed when tested on the D₂ receptor in the striatum and the pituitary gland (16), suggesting agonist trafficking, possibly via different G proteins.

To investigate these phenomena we have expressed the D₂

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¹ The abbreviations used are: R*, receptor in partially activated state; R*G, binary complex of G protein coupled to activated receptor; AR*G, ternary complex of agonist and R*G; GTPγS, guanosine 5′-3-O-(thio)triphosphate; CHO, Chinese hamster ovary; NMDG, N-methyl D-glucamine; NPA, N-propyl noramorphomine; Kᵢ, low affinity agonist dissociation constant; Kᵢ, high affinity agonist dissociation constant; 3-PPP, 3-(3-hydroxyphenyl)-N-propylpiperidine.
dopamine receptor together with the G proteins G\textsubscript{o} and G\textsubscript{i2} in insect cells, using the baculovirus system (17). This system provides a powerful tool for the reconstitution of receptor/G protein interactions. Insect cells do not contain endogenous dopamine receptors, and interaction between recombimant receptors and the endogenous G proteins of the cells is minimal.

**EXPERIMENTAL PROCEDURES**

**Materials**

|phenyl-4-\textsuperscript{3}H|Spiiperone (25Ci/mmol) was from Amersham Pharmacia Biotech, and \[^{35}\text{S}]/\text{GTP}\textsubscript{S} (1,250 Ci/mmol) was from PerkinElmer Life Sciences. Antibodies specific for different G protein subunits were from Chemicon and Santa Cruz as indicated. Other reagents were obtained as indicated or were of the highest purity available from commercial suppliers.

Methods

**Cell Culture—**SF21 cells were grown either in monolayers or in suspension, using shaker flasks (25–100-ml cultures) agitated at 116 rpm. Cells were cultured at 26 °C in TC100 medium supplemented with 8% fetal calf serum and 0.1% Pluronic F-68 (Life Technologies, Inc.). CHO cells expressing the long form of the rat D\textsubscript{2} dopamine receptor (18, 19) were grown in B11 medium containing 5% fetal calf serum, 2 mM l-glutamine, and 2 mM active Geneticin.

**Construction and Isolation of Recombinant Baculovirus and Expression of the D\textsubscript{2} Dopamine Receptor and G Protein Subunits in SF21 Cells—**The baculovirus transfer vector, containing the cDNA for the FLAG-tagged D\textsubscript{2} dopamine receptor, was constructed from three DNA fragments (20). The first fragment consisted of the generic baculovirus transfer vector fusion vector, pVL1392 (PharMingen), digested with Pst\textsubscript{I} and BamHI. The second fragment was generated by polymerase chain reaction and comprised at its 5’-end, a Pst\textsubscript{I} restriction site, to facilitate ligation to the vector, an ATG start codon, immediately followed by DNA encoding the FLAG epitope and the first 116 amino acids of the rat D\textsubscript{2} dopamine receptor sequence, and at its 3’-end, an Alw\textsubscript{II} restriction site to allow ligation to the final DNA fragment. The final fragment was a 1.0-kilobase cDNA fragment, coding for the remaining amino acids of the dopamine receptor, and was excised from an existing baculovirus transfer vector containing receptor cDNA (pVL1392D2), using Alw\textsubscript{II} and BamHI. The sequence of the DNA corresponding to the polymerase chain reaction fragment and the three ligation sites was confirmed by dideoxy DNA sequencing using the Sequenase version 2.0 DNA sequencing kit (United States Biochemical). Transfer of the protein sequences were constructed as described (21).

**Preparation of Washed Cell Homogenates—**All operations were carried out at 0–4 °C. SF21 cells were harvested 48 h after incubation by centrifugation at 3,000 × g for 10 min and resuspended at ∼5 × 10\textsuperscript{7} cells/ml in 20 mM HEPES, pH 7.4, 6 mM MgCl\textsubscript{2}, 1 mM EDTA, 1 mM EGTA, and protease inhibitors (Boehringer COMPLETE\textsuperscript{TM}). SF21 cells were homogenized with 50 strokes of a Dounce homogenizer and centrifuged at 3,000 × g for 10 min. The supernatant was collected and centrifuged at 48,000 × g for 60 min, and the pellet was resuspended in 20 mM HEPES, pH 7.4, 10 mM EDTA, 1 mM EGTA, and protease inhibitors (Boehringer COMPLETE\textsuperscript{TM}). The resulting washed membrane homogenates were stored at ∼80 °C until used for Western blot analysis or ligand binding assays.

Membrane preparations from CHO cells expressing D\textsubscript{2} dopamine receptors were made as described by Castro and Strange (18, 19).

**Protein Determination—**Protein was determined using the Lowry method (22), with bovine serum albumin as the standard.

**Ligand Binding Assays—**Binding to washed membrane homogenates (15–50 μg of protein) was assayed in triplicate using [\(^{3}H\)]spiperone (25Ci/mmol; 0.1–5 nM for saturation analyses and 1 nM for competition assays). Except where indicated, assays were performed in a final volume of 1 ml of assay buffer: 20 mM HEPES, 1 mM EDTA, 1 mM EGTA, 6 mM MgCl\textsubscript{2}, pH 7.4. In agonist binding assays, 100 μM dithiothreitol was added as an antioxidant. For substituted benzamide antagonists, the standard assay buffer was supplemented with 100 μM NaCl or N-methyl-D-glucamine (NMDG) as indicated. Binding was measured in the presence of 3 μM (−)-butaclamol and (−)-butaclamol to define total and nonspecific binding, respectively, over a period of 180 min at 25 °C. Bound and free radioligands were separated by rapid filtration through GF/B glass fiber filters (0.14 μM NaCl, 3 mM KCl, 1.5 mM KH\textsubscript{2}PO\textsubscript{4}, and 5 mM Na\textsubscript{2}HPO\textsubscript{4}, pH 7.4). Bound radioactivity was determined by liquid scintillation counting. Ligand binding data were analyzed by nonlinear least squares regression using the computer program GraphPad Prism (GraphPad Software Inc.).

In some saturation assays a total assay volume of 10 ml was employed. The protein amount was the same as in the 1-ml assays so that the protein concentration was 10-fold lower. The concentrations of other substances were the same as in the 1-ml assays, but the time of incubation was 7 h.

[^{3}H]/\text{GTP}\textsubscript{S} Binding Assays—In agonist stimulation experiments, 50 μg of cell membranes were incubated in triplicate with 10 μM GDP and increasing concentrations of agonist in a final volume of 0.9 ml of buffer (20 mM HEPES, 10 mM MgCl\textsubscript{2}, 100 mM NaCl, pH 7.4) for 30 min at 30 °C as described by Gardner et al. (23–25). 0.1 ml of \[^{3}H]/\text{GTP}\textsubscript{S} (1,250 Ci/mmol) was added in a final concentration of 100 pM and the incubation continued for a further 20 min. Basal levels of \[^{3}H]/\text{GTP}\textsubscript{S} binding were defined as that in the absence of agonist. Incubations were terminated by rapid filtration through Whatman GF/B glass fiber filters using Brandel cell harvester with four washes of 4 ml of phosphate-buffered saline, and radioactivity determined as above. When different agonists were tested, a 1 mM dopamine control was always present in the assay to allow relative efficacy determinations to be made.

In saturation binding experiments, 40 μg of cell membranes was incubated in triplicate with 10 μM GDP, 100 pM \[^{3}H]/\text{GTP}\textsubscript{S}, 100 pM-100 nM \[^{3}H]/\text{GTP}\textsubscript{S} in the absence or presence of 1 mM dopamine in a final volume of 1 ml of buffer for 2 h at 30 °C. Dopamine-stimulated \[^{3}H]/\text{GTP}\textsubscript{S} binding was determined by subtraction, and total dopamine-stimulated \[^{3}H]/\text{GTP}\textsubscript{S} binding was determined as dpm bound × (total GTPI/\text{S1-[^{3}H]/GTP-S}).

**Determination of G Protein Level Using Quantitative Western Blot—**Before analysis, proteins (SF21 membranes or pure G protein subunits) were solubilized by the addition of 10 μl of electrophoresis buffer (100 mM Tri-s-Cl, 200 mM dithiothreitol, 4% SDS, 0.2% bromophenol blue, 20% glycerol) and heated at 90 °C for 5 min. SF21 membranes protein (20–40 μg) and G protein standards were separated by SDS-polyacrylamide gel electrophoresis on 12% acrylamide gels. Samples were then transferred to nitrocellulose membranes using the Bio-Rad semidyed transfer system. Nitrocellulose membranes were incubated for 1 h with rabbit anti-mouse (α1–3, 1 μg/ml; Santa Cruz, see Fig. 2); monoclonal antibody 3077 anti-α\textsubscript{i2}, 1 μg/ml (Chemicon, see Fig. 3); C-16 anti-β\textsubscript{i2}, 0.4 μg/ml (Santa Cruz); A-16 anti-γ\textsubscript{i2}, 0.4 μg/ml (Santa Cruz) in buffer containing 5% dried milk (w/v). Membranes were washed five times with buffer (10 min each) and then incubated with secondary antibody (anti-mouse α\textsubscript{i2}, α\textsubscript{i3}, β\textsubscript{i2}, γ\textsubscript{i2}) immunoglobulin horseradish peroxidase conjugate (Sigma, 1:5,0000) for 1 h. Membranes were then washed three times (10 min each) with buffer before exposure to equal volumes of Enhanced Chemiluminescence (ECL) detection reagents (Amersham Pharmacia Biotech). Membranes 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 GEST10 calibrated imaging densitometer (Bio-Rad), and data were analyzed 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.

In some experiments membranes were extracted with 1% cholate, 1% NaCl (10 mg of membranes/ml of cholate/NaCl) for 1 h at 4 °C. The mixture was centrifuged at 4,500 × g for 5 min at 4 °C, the supernatant and pellet were collected. These were then analyzed using Western blotting as above, the pellet having been dissolved in 1% cholate, 1% Nonidet P-40, and 1% NaCl.

**RESULTS**

**Expression of D\textsubscript{2} Dopamine Receptors in SF21 Cells—**D\textsubscript{2} dopamine receptors were expressed in SF21 insect cells using the baculovirus expression system. The expressed receptors were characterized using ligand binding with \[^{3}H]\)spiperone. Saturation analyses of \[^{3}H]\)spiperone binding (1-ml assay volume) gave a K\textsubscript{D} of 145 pm (K\textsubscript{D} 9.84 ± 0.03, mean ± S.E., n = 3) and
a $B_{\text{max}}$ of $-2\,\text{pmol/mg}$. When these assays were repeated in a 10-nl format a similar $K_d$ was observed (171 pm ($pK_d = 9.77 \pm 0.21$, mean $\pm$ S.E., $n = 3$)). The similarity of the $K_d$ values from 1-ml and 10-nl assays demonstrates that radioligand depletion artifacts are absent from the assays (26).

A series of antagonists exhibited competition curves versus $[^{3}H]$spiperone which were best fitted by one-binding site models. The derived $K_d$ values are given in Table I for experiments using buffer containing sodium ions and where the sodium had been replaced by NMDG to maintain ionic strength. The rank order of $K_d$ values is similar to that observed for the D2 receptor expressed in other systems, so the receptor is being expressed with fidelity in the present experiments. The substituted benzamide antagonists, e.g. sulphide, are sensitive to the removal of sodium ions in these assays. Some data are also given for these drugs when binding to D2 receptors expressed in CHO cells. In the presence of sodium ions $K_d$ values are similar for the receptor expressed in the two cell backgrounds, whereas upon removal of sodium ions, binding of substituted benzamide drugs is of lower affinity for the receptors expressed in Sf21 cells.

Competition binding experiments were also performed with the agonists N-propyl norapomorphine (NPA) and dopamine and, except in one experiment with NPA, the data fitted best to a single-binding site model, and there was no significant effect of the addition of 100 $\mu\text{M}$ GTP (see Table III). Formation of receptor-G protein complexes cannot, therefore, be detected in this way. Also, dopamine stimulation of [35S]GTPyS binding could not be detected in membranes expressing the D2 receptor without exogenous G proteins, whereas (see below) this activity was clearly present in membranes expressing exogenous G protein. The D2 dopamine receptor does not, therefore, interact strongly with the endogenous G proteins of Sf21 cells. Similarly, when the formyl peptide receptor was expressed in SF 9 insect cells no agonist stimulation of [35S]GTPyS binding to the endogenous G proteins could be detected (27).

Coexpression of $D_2$ Dopamine Receptors and G Proteins in $Sf_{21}$ Cells—In these experiments the $D_2$ dopamine receptor was expressed in Sf21 cells together with G protein $\alpha$, $\beta$, and $\gamma$ subunits. The G protein $\alpha$ subunits ($\alpha$ $\delta$ and $\alpha_2$) were used because the $D_2$ receptor has been reported to interact with these (13). The $\beta_1$ and $\gamma_2$ subunits were used for all of the studies here because these subunits support coupling between several receptors and G protein $\alpha$ subunits (27–31). In preliminary experiments, different multiplicity of infection values for the different baculoviruses containing the four proteins were tested to obtain similar receptor expression levels and a high [35S]GTPyS binding response to dopamine. Based on these findings (data not shown), in the experiments described below, multiplicity of infection values were used as follows: for membranes expressing $G_{\alpha_D}$, receptor/1/$\beta_1/\gamma_2$–2/1/1; for membranes expressing $G_{\alpha_\delta}$, receptor/1/$\beta_1/\gamma_2$–3/1/1.

The levels of $D_2$ receptor were determined in the membranes using saturation analyses with $[^{3}H]$spiperone (Fig. 1 and Table II). Levels of $D_2$ dopamine receptor were similar in the two preparations, and there was no significant difference in the radioligand affinity. The affinity for $[^{3}H]$spiperone binding was unaffected by the addition of 100 $\mu\text{M}$ GTP in both preparations and was not significantly different from that for the receptor expressed alone.

The levels of G protein $\alpha$, $\beta$, and $\gamma$ subunits were determined by quantitative Western blot, and the levels were not significantly different in the two preparations (Table II). Representative blots are shown in Fig. 2. Levels of $\alpha$ subunits were also determined after extraction of the membranes with 1% cholate. In each preparation 60–70% of the $\alpha$ subunit was found in the cholate extract, suggesting that the majority of the expressed subunits were fully active (Fig. 3). [35S]GTPyS saturation binding assays in the presence of dopamine were also performed in the two preparations, and these showed $B_{\text{max}}$ and $K_d$ values for [35S]GTPyS binding which were not significantly different (Table II and Fig. 1).

**Agonist Binding to $D_2$ Dopamine Receptors Coexpressed with G Proteins**—The binding of agonists to $D_2$ dopamine receptors

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**D$_2$ Dopamine Receptor/G Protein Specificity**

**TABLE I**

*Binding of drugs to $D_2$ dopamine receptors expressed in Sf21 and CHO cells*

Competition experiments versus $[^{3}H]$spiperone for various substances were performed as described under “Experimental Procedures,” and $K_d$ values ($pK_d \pm$ S.E., $K_d$ from three or more experiments) were derived from the best fit curves to one-binding site models.

| Ligand | $pK_d \pm$ S.E. | Fold affinity change | $pK_d \pm$ S.E. | Fold affinity change |
|--------|----------------|---------------------|----------------|---------------------|
|        | NMDG | Na$^+$ |         | NMDG | Na$^+$ |         |
| [$^{3}H$]Spiperone | 9.46 ± 0.04 | 9.72 ± 0.05 | 1.8 † | 10.02 ± 0.08 | 10.08 ± 0.13 | 1.0 |
| (+)-Butacloam | 8.38 ± 0.10 | 8.37 ± 0.14 | 1.0 | ND | ND | ND |
| Nemonapride | 7.83 ± 0.15 | 9.57 ± 0.09 | 55 † | 8.89 ± 0.19 | 10.05 ± 0.19 | 14 † |
| Clozapine | 6.32 ± 0.03 | 6.63 ± 0.07 | 2 † | 6.58 ± 0.13 | 6.29 ± 0.05 | 2.0 |
| Clebopride | 5.89 ± 0.07 | 8.11 ± 0.05 | 165 † | 7.07 ± 0.09 | 8.40 ± 0.11 | 21 † |
| Raclopride | 5.79 ± 0.09 | 7.44 ± 0.27 | 45 † | ND | ND | ND |
| (-)-Sulphide | 5.15 ± 0.20 | 6.48 ± 0.16 | 22 † | ND | ND | ND |
| Metoclopramide | 4.81 ± 0.05 | 6.68 ± 0.14 | 73 † | 5.26 ± 0.13 | 6.93 ± 0.04 | 47 † |
| Tiapride | <4.11 | 6.01 ± 0.15 | 73 † | ND | ND | ND |
| Remoxipride | <4.0 | 5.66 ± 0.22 | >29 † | <5.1 | 5.95 ± 0.14 | >7 |
| Dopamine | 3.98 ± 0.13 | 4.51 ± 0.10 | 3 † | ND | ND | ND |
was determined in competition with \(^{3}H\)spiperone in the preparations containing \(G_0\) and \(G_{12}\). Competition curves for several agonists (NPA, dopamine, (+)-3-PPP, \(m\)-tyramine), in both preparations, were best fitted by a two-site binding model with 20–30% higher affinity sites (Figs. 4 and 5; Table III). The proportion of higher affinity sites for a ligand did not differ significantly between the two preparations. Competition experiments for dopamine and NPA were also performed in the presence of 100 \(\mu M\) GTP, and competition curves under these conditions were best described by one-binding site models; the affinity in the presence of GTP was similar to that of the lower affinity site observed in the absence of GTP and also similar to that observed in preparations expressing receptor alone. For (−)-3-PPP, data obtained in the preparation containing \(G_0\) were also fitted best to a two-site model. For other agonists in both preparations (bromocriptine and \(p\)-tyramine) and for (−)-3-PPP in the preparation containing \(G_{12}\) the competition curves were best fitted by a one-binding site model. When \(K_i\) values for the different sites were compared between the two preparations there were significant differences for some agonists (NPA, \(m\)-tyramine) at the higher affinity site, but for other agonists affinities at this site were not significantly different. Affinities at the lower affinity site and for the single affinity site seen for some agonists were not significantly different between the two preparations.

Stimulation of \(^{35}S\)GTP\(_S\) Binding by Agonists—G protein activation by agonists in the two preparations was assessed by determining agonist-stimulated \(^{35}S\)GTP\(_S\) binding (Fig. 6).
D₂ dopamine receptors were expressed together with G protein subunits as described under "Experimental Procedures," and saturation binding analyses using [³H]spiperone were performed to determine levels of D₂ receptor. Binding parameters (Kₐ and Bₘₐₓ) were derived from the data, and values are expressed as the mean ± S.E. (3–4). Neither D₂ receptor nor G protein levels were significantly different in the two preparations (p > 0.05). [³⁵S]GTP₆ saturation binding assays were performed, and Kₐ and Bₘₐₓ values are given; these were not significantly different between the two preparations (p > 0.05).

**Table II**

| Preparation     | [³H]Spiperone binding | G protein Western blot | [³⁵S]GTP₆ saturation binding |
|-----------------|-----------------------|------------------------|-----------------------------|
|                 | pKₐ ± S.E. (Kₐ) | Bₘₐₓ control pmol/mg | α-subunit pmol/mg | β₁-subunit pmol/mg | γ₂-subunit pmol/mg | pKₐ ± S.E. (Kₐ) | Bₘₐₓ pmol/mg |
| D₂/α₁/β₁/γ₂     | 10.01 ± 0.12 (98 pm) | 1.16 ± 0.08 | 410 ± 140 | 110 ± 50 | 38 ± 13 | 8.80 ± 0.17 (1.6 nm) | 1.34 ± 0.30 |
| D₂/α₁/β₁/γ₂     | 9.78 ± 0.02 (160 pm) | 1.11 ± 0.07 | 180 ± 60 | 130 ± 40 | 34 ± 15 | 8.34 ± 0.12 (4.6 nm) | 2.61 ± 0.91 |

**DISCUSSION**

In this study we have expressed the D₂ dopamine receptor together with the G proteins Gₐ and G₁₂ in insect cells using the
baculovirus system. We show that the D2 receptor interacts more strongly with Gα than Gβγ and that this influences the functional selectivity of agonist signaling. We also show that the extent of the selectivity of the interaction between the D2 receptor and either Gα (panel A) or Gβγ (panel B) was determined in competition versus [3H]spiperone as described under "Experimental Procedures." Data shown are from representative experiments replicated as in Table II, and the curves are the best fit curves to one-site (R/Gα, ○; R/Gβγ, ▼) or two-site models (R/Gα, ○; R/Gβγ, ●).

The levels of receptor (R) and G protein (G) subunits (αα/αβγ, and β1 and γ2) in the preparations expressing Gα and Gβγ were determined using saturation analysis with the radioligand [3H]spiperone and quantitative Western blot, respectively. The levels of α subunit detected were similar to those reported in other studies on expression of receptors and Gα or Gβγ proteins in insect cells (27, 32). The γ2 subunit was expressed at lower levels than either the β1 or α subunits and so may limit the levels of G protein heterotrimeric G protein to receptor of 20-fold in both preparations. This shows that the expression of the radioligand substantially. At Gα/Gβγ (panel B) was determined in competition versus [3H]spiperone in the absence (○) or presence (●) of 100 μM GTP as described under "Experimental Procedures." Data shown are from representative experiments replicated as in Table II, and the curves are the best fit curves to one-site (●) or two-site models (○).

[35S]GTPγS saturation binding assays gave similar values for the level of dopamine-stimulated [35S]GTPγS binding in the two preparations. The levels determined by [35S]GTPγS binding are low compared with the numbers of G proteins measured by quantitative Western blot. This is probably because there is a high concentration of GDP in the [35S]GTPγS binding assays which reduces the binding of the radioligand substantially. At the highest concentrations of GTPγS used, there is more than a 100-fold excess of GDP, and therefore it is not surprising that the levels of α subunits detected by Western blotting are roughly 100-fold higher than detected in the [35S]GTPγS binding assays. Nevertheless, in the present study, based on these different determinations, the levels of receptor and G protein subunits were similar in the membranes expressing Gα and Gβγ. The two preparations are, therefore, comparable, and any differences between the preparations are unlikely to be caused either by receptor or G protein numbers.

Agonist binding in the membranes expressing receptor and G protein (Gα, Gβγ) could, for many agonists, be resolved into contributions from sites of higher and lower affinity in similar proportions in the two preparations. This shows that the expressed D2 receptor and G proteins are able to interact. For two of the agonists (dopamine, NPA), GTP abolished the higher affinity binding site. The affinity seen in the presence of GTP was similar to both the lower affinity site seen in the absence of GTP and the affinity for these agonists seen in a preparation containing receptor alone. These data follow the predicted be-
The stimulation of \[^{35}S\]GTP\(^{2}\) expressing D\(_2\) receptor and either Go (\(\alpha\)2) or Gi\(_2\) (\(\alpha\)G) using the baculovirus coding for D\(_2\) receptor, the following values were obtained: dopamine – GTP (4.80 \pm 0.13 (16 \mu M) + GTP (4.88 \pm 0.08 (15 \mu M)), NPA – GTP (7.51 \pm 0.19 (31 nM)) + GTP (7.11 \pm 0.10 (77 nM)) (competition curves fit best to a one binding site model).

**Fig. 6.** Stimulation of \[^{35}S\]GTP\(^{2}\) binding by agonists in membranes of SF21 cells expressing D\(_2\) dopamine receptors and G proteins. The stimulation of \[^{35}S\]GTP\(^{2}\) binding by agonists was determined as described under "Experimental Procedures" in membranes expressing D\(_2\) receptor and either G\(_{\alpha}\) (panel A) or G\(_{\alpha}\) (panel B). Agonists used were as follows: bromocriptine (\(\square\)), dopamine (\(\circ\)), NPA (\(\blacksquare\)), quinpirole (\(\heartsuit\), m-tyramine (\(\triangle\)), p-tyramine (\(\heartsuit\), (\(\pm\))-3-PPP (\(\bigcirc\)), and (+)-3-PPP (\(\blacktriangle\)). The data are representative stimulation curves replicated as in Table IV.

### Table III

**Agonist binding to D\(_2\) dopamine receptors expressed in S21 cells**

| Ligand         | Preparation | \(pK_{A}\) (mean \pm S.E.) | \(pK_{G}\) (mean \pm S.E.) | % High affinity sites | \(pK_{A}\) (mean \pm S.E.) | \(pK_{G}\) (mean \pm S.E.) | % High affinity sites |
|----------------|-------------|----------------------------|----------------------------|-----------------------|----------------------------|----------------------------|-----------------------|
|                | \(D_{2}\)   | (\(K_{A}\))                | (\(K_{G}\))                |                       | (\(K_{A}\))                | (\(K_{G}\))                |                       |
|                |             | (\(nM\))                   | (\(nM\))                   |                       | (\(nM\))                   | (\(nM\))                   |                       |
| Bromocriptine  |             | 9.09 \pm 0.20 (0.82)        | 4.68 \pm 0.13 (21,000)     | 23 \pm 8              | 7.15 \pm 0.17 (70)         | 4.40 \pm 0.24 (40,000)     | 19 \pm 5              |
| Dopamine       |             | 7.12 \pm 0.20 (76)          | 4.69 \pm 0.14 (20,000)     |                       | 7.44 \pm 0.08 (0.36)       | 7.22 \pm 0.16 (61)         | 33 \pm 6              |
| Dopamine + GTP (100 \(\mu M\)) |             | 10.24 \pm 0.23 (0.06)       | 7.47 \pm 0.37 (34)        |                       | 7.06 \pm 0.36 (170)        | 4.13 \pm 0.04 (74,000)    |                       |
| NPA            |             | 7.40 \pm 0.17 (40)          | 4.65 \pm 0.09 (25,000)     |                       | 7.49 \pm 0.32 (32)         | 4.29 \pm 0.10 (48,000)     |                       |
| NPA + GTP (100 \(\mu M\)) |             | 7.40 \pm 0.17 (40)          | 4.65 \pm 0.09 (25,000)     |                       | 7.49 \pm 0.32 (32)         | 4.29 \pm 0.10 (48,000)     |                       |
| (+)-3-PPP      |             | 7.97 \pm 0.29 (11)          | 4.56 \pm 0.09 (25,000)     | 22 \pm 6              | 7.74 \pm 0.29 (18)         | 4.20 \pm 0.10 (48,000)     | 19 \pm 4              |
| (\(\pm\))-3-PPP |             | 7.73 \pm 0.10 (19)          | 4.96 \pm 0.01 (11,000)     | 19 \pm 5              | 4.80 \pm 0.03 (16,000)     | 19 \pm 4              |
| m-Tyramine     |             | 7.60 \pm 0.18 (25)          | 4.79 \pm 0.30 (16,000)     | 36 \pm 9              | 6.76 \pm 0.36 (170)        | 4.13 \pm 0.04 (74,000)     |                       |
| p-Tyramine     |             | 4.27 \pm 0.03 (54,000)      | 4.27 \pm 0.03 (54,000)     |                       | 4.13 \pm 0.04 (74,000)     | 4.13 \pm 0.04 (74,000)     |                       |

Behavior of a system that conforms to a ternary complex model with an excess of receptor over G protein (40, 41). The data on the levels of receptor and G protein in the membranes show, however, that there is an excess of G protein over receptor of about 20-fold. Similar discrepancies between inferred and measured R/G ratios have been noted in other systems. It has been proposed (37, 42) that receptors and G proteins may not interact freely and that there may be microdomains with different amounts of receptor and G protein. Alternatively, the ternary complex models are an oversimplification of receptor and G protein may form oligomers with properties different from the predictions of the models (43).

Two observations from the ligand binding studies suggest that there may be a greater affinity of the D\(_2\) receptor for G\(_{\alpha}\) than for G\(_{\alpha}\) when occupied by several agonists. First, the affinity of the higher affinity site is higher in the preparation containing G\(_{\alpha}\) for m-tyramine and NPA. This affinity difference should reflect the affinity of R/G coupling, given that the ground state affinity of the receptor is similar in the two preparations. Also, (\(\pm\))-3-PPP is unable to stabilize the higher affinity state in the preparation containing G\(_{\alpha}\) but can do so in the preparation containing G\(_{\alpha}\). In agreement with these findings, differences in agonist affinity for one receptor coupled to different G proteins have been described by others (11, 44, 45).

A range of agonists was used to stimulate \[^{35}S\]GTP\(^{2}\) binding in the two preparations to assess G protein activation. Maximal agonist effects (relative to dopamine) were greater in the preparation containing G\(_{\alpha}\) and some agonists (p-tyramine, (\(\pm\))-3-PPP) were unable to stimulate \[^{35}S\]GTP\(^{2}\) binding at all in the preparation containing G\(_{\alpha}\). The potencies of agonists to stimulate \[^{35}S\]GTP\(^{2}\) binding were also generally greater in the preparation containing G\(_{\alpha}\) with the exception of bromocriptine. These data suggest that there is a more productive interaction between the D\(_2\) receptor and G\(_{\alpha}\). The affinity of the interaction between receptor and G protein may contribute to this, as suggested above from the ligand binding data. The pattern of agonist binding and potencies in \[^{35}S\]GTP\(^{2}\) binding assays is very similar in the preparation containing G\(_{\alpha}\) compared with that seen for the D\(_2\) receptor expressed in CHO cells.


The stimulation of \(^{35}\)S-GTP\(\gamma S\) binding in membranes expressing D\(_2\) receptors and G proteins was determined as described under “Experimental Procedures.” The maximum response (relative to dopamine) and the EC\(_{50}\) were determined. Data are expressed as the mean ± S.E. from three or more experiments.

For the preparation containing Go, greater effects for stimulation of \(^{35}\)S-GTP\(\gamma S\) binding were seen for several agonists, but for two of the agonists, m-tyramine and (−)-3-PPP, no agonism is seen at all. For these compounds, binding to the receptor appears to be insufficient to stabilize receptor/G protein interaction. In this preparation, therefore, receptor/G protein interaction is less efficient, and for some agonists there is a complete failure to signal. The data outlined earlier show that the affinity of the D\(_2\) dopamine receptor is greater for Go than for Gi2. This cannot be the only factor influencing the activation of the G proteins because otherwise a general reduction in signaling efficiency would be seen for all agonists tested when the lower affinity interaction is present, i.e., in the Gi2 preparation. This suggests that different agonists are able to stabilize different conformations of the receptor with different affinities for the G protein and different functional activities in the ternary complex rather than there being differential stabilization of the same activated state by different agonists. As a result, the selectivity of the D\(_2\) receptor for Gi over Go2 is dependent on the agonist used. The two agonists that show the greatest selectivity, p-tyramine and (−)-3-PPP, are both monohydroxylated compounds. It is interesting that for the \(\alpha_{2A}\)-adrenergic receptor, catechol agonists, e.g. noradrenaline, lead to stimulation of both Gi and Go2-dependent pathways, whereas monohydroxylated agonists, e.g. octopamine, lead only to activation of Gi-dependent pathways (47).

Further evidence that agonists may regulate the activity of the ternary complex comes from analysis of the K\(_i\)/K\(_o\) ratio (ratio of low affinity and high affinity agonist dissociation constants). The K\(_i\)/K\(_o\) ratio was derived from the ligand binding data (Table V) because this has been proposed to be an index of the ability of the agonist to stabilize receptor/G protein coupling (see, e.g. Ref. 40). There is no clear relationship between

### Table IV

**Agorist stimulation of \(^{35}\)S-GTP\(\gamma S\) binding via D\(_2\) dopamine receptors expressed in S\(\beta\)21 cells**

| Preparation | D\(_{2}\)/D\(_{1}\)/β/γ/\(\gamma S\) | % Maximal dopamine response | D\(_{2}\)/D\(_{1}\)/β/γ/\(\gamma S\) | % Maximal dopamine response |
|-------------|----------------------------------|-----------------------------|----------------------------------|-----------------------------|
| Go          | pEC\(_{50}\) ± S.E. (EC\(_{50}\)) | Go                          | pEC\(_{50}\) ± S.E. (EC\(_{50}\)) | Go                          |
| Bromocriptine | 8.82 ± 0.17 (1.5) | 145 ± 13 | 8.70 ± 0.09 (2.0) | 97 ± 15 |
| Dopamine     | 6.05 ± 0.29 (880) | 100   | 4.84 ± 0.10 (14,000) | 100 |
| NPA          | 8.75 ± 0.22 (1.8) | 113 ± 9 | 7.90 ± 0.08 (13) | 134 ± 15 |
| (+)-3-PPP    | 5.45 ± 0.12 (3,600) | 86 ± 1 | 64 ± 5 |
| (−)-3-PPP    | 5.83 ± 0.27 (1,500) | 53 ± 5 | ND* |
| Quinpirole   | 6.29 ± 0.15 (510) | 95 ± 11 | 5.57 ± 0.15 (2,700) | 101 ± 6 |
| m-Tyramine   | 5.73 ± 0.15 (1900) | 104 ± 13 | 4.80 ± 0.05 (16,000) | 68 ± 6 |
| p-Tyramine   | 4.40 ± 0.23 (40,000) | 52 ± 5 | ND |

* ND, not determined.

### Table V

**Agorist signaling parameters**

Parameters were derived from data in Tables III and IV. K\(_i\) and K\(_o\) are respectively the dissociation constants for the lower and higher affinity agonist binding sites as in Table III. EC\(_{50}\) is the concentration of agonist which gives a 50% maximal response in the \(^{35}\)S-GTP\(\gamma S\) binding assays as in Table IV.

| Preparation | K\(_i\)/K\(_o\) | D\(_{2}\)/D\(_{1}\)/β/γ/\(\gamma S\) | K\(_i\)/EC\(_{50}\) | D\(_{2}\)/D\(_{1}\)/β/γ/\(\gamma S\) |
|-------------|----------------|----------------------------------|------------------|----------------------------------|
| Go          | K\(_i\)/K\(_o\) | D\(_{2}\)/D\(_{1}\)/β/γ/\(\gamma S\) | K\(_i\)/EC\(_{50}\) | D\(_{2}\)/D\(_{1}\)/β/γ/\(\gamma S\) |
| Bromocriptine | 1              | 1                      | 0.6             | 0.6 |
| Dopamine     | 270             | 570                   | 23              | 2.8 |
| NPA          | 600             | 170                   | 19              | 4.8 |
| (+)-3-PPP    | 2,600           | 2,700                  | 7.9             | 3.3 |
| (−)-3-PPP    | 600             | 1                      | 7.5             | 6.4 |
| m-Tyramine   | 660             | >600                   | 8.7             | 1.3 |
| p-Tyramine   | 1               | 1                      | 8.7             | 1.3 |

(23–25). The present system is, therefore, behaving similarly to a system in which the receptor couples exclusively with endogenous mammalian G proteins.

To understand the differences between the two preparations in more detail, the data were analyzed to provide the K\(_i\)/EC\(_{50}\) ratio (ratio of agonist binding dissociation constant to agonist potency) (Table V). The K\(_i\)/EC\(_{50}\) ratio (or amplification ratio (24, 25, 46)) indicates the extent to which agonist activation of a response occurs at lower concentrations than agonist binding to the receptor and so is a measure of receptor/G protein activation. The K\(_i\)/EC\(_{50}\) ratio of the agonist is greater in the preparation containing Go than in the preparation containing Gi2, providing a further indication that there is a more productive interaction between the D\(_2\) receptor and Go.

For the preparation containing Gi2, greater K\(_i\)/EC\(_{50}\) ratios are generally observed for the agonists that give greater maximal effects for stimulation of \(^{35}\)S-GTP\(\gamma S\) binding. In this preparation, therefore, the two measures of efficacy, agonist maximal effect and K\(_i\)/EC\(_{50}\) are in agreement for a range of compounds. For the preparation containing Gi2, lower values of K\(_i\)/EC\(_{50}\) are seen for several agonists, but for two of the agonists, p-tyramine and (−)-3-PPP, no agonism is seen at all. For these compounds, binding to the receptor appears to be insufficient to stabilize receptor/G protein interaction. In this preparation, therefore, receptor/G protein interaction is less efficient, and for some agonists there is a complete failure to signal. The data
the maximal effects of the agonists in [35S]GTP\(_{\gamma}S\) binding assays and the \(K_r/K_{h}\) ratio. Therefore, stabilization of receptor/G protein coupling is not a clear predictor of agonist efficacy, and similar results were seen in other studies on the \(D_2\) receptor expressed in CHO cells (24, 25). Agonists may, therefore, influence the activity of the ternary complex as well as its formation (7, 8, 24, 25).

The behavior of bromocriptine provides further support for the idea that agonists stabilize different receptor conformations. Bromocriptine is a full agonist on both preparations, and its potency (\(EC_{50}\)) and binding affinity (\(K_r\)) are similar in each preparation, leading to identical \(K_r/EC_{50}\) ratios, in contrast to the other agonists tested. This suggests that the bromocriptine-receptor complex has a similar affinity for the two G proteins. Bromocriptine is an unusual compound in that its binding to \(D_2\) receptor complex has a similar affinity for the two G proteins.

In conclusion, we have shown that the \(D_2\) dopamine receptor has a greater affinity for the G protein \(G_o\) than for \(G_{12}\). Activation of \(G_o\) occurs with higher potencies for agonists and greater relative efficacies for partial agonists, and this is in agreement with the findings of Yang and Lanier (11) for the \(\alpha_2\)-adrenergic receptor.

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