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Mechanisms of Agonist Action at D_2 Dopamine Receptors

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ABSTRACT

In this study, we investigated the biochemical mechanisms of agonist action at the G protein-coupled D_2 dopamine receptor expressed in Chinese hamster ovary cells. Stimulation of guanosine 5′-*O*-(3-[^{35}S]thio)triphosphate ([^{35}S]GTPγS) binding by full and partial agonists was determined at different concentrations of [^{35}S]GTPγS (0.1 and 10 nM) and in the presence of different concentrations of GDP. At both concentrations of [^{35}S]GTPγS, increasing GDP decreased the [^{35}S]GTPγS binding observed with maximally stimulating concentrations of agonist, with partial agonists exhibiting greater sensitivity to the effects of GDP than full agonists. The relative efficacy of partial agonists was greater at the lower GDP concentrations. Concentration-response experiments were performed for a range of agonists at the two [^{35}S]GTPγS concentrations and with different concentrations of GDP. At 0.1 nM [^{35}S]GTPγS, the potency of both full and partial agonists was dependent on the GDP concentration in the assays. At 10 nM [^{35}S]GTPγS, the potency of full agonists exhibited a greater dependence on the GDP concentration, whereas the potency of partial agonists was virtually independent of GDP. We concluded that at the lower [^{35}S]GTPγS concentration, the rate-determining step in G protein activation is the binding of [^{35}S]GTPγS to the G protein. At the higher [^{35}S]GTPγS concentration, for full agonists, [^{35}S]GTPγS binding remains the slowest step, whereas for partial agonists, another (GDP-independent) step, probably ternary complex breakdown, becomes rate-determining.

There is much interest in understanding the mechanisms of action of agonists at receptors (agonist efficacy) and the mechanistic distinction between full and partial agonists ((Black and Leff, 1983; Clarke and Bond, 1998; Colquhoun, 1998; Strange, 1999; Kenakin, 2002). For the G protein-coupled receptors (GPCRs), an influential biochemical model of GPCR action has been the ternary complex model and its recent extensions (De Lean et al., 1980; Samama et al., 1993; Weiss et al., 1996). The model describes a ground state of the receptor (R) which can isomerize to a partially activated form (R*) which is able to couple better to the G protein to form the active (R*G) state.

The ternary complex model accounts for differences in the relative efficacy of full and partial agonists in terms of different extents of stabilization of active (AR*G) and inactive (AR) states of the receptor. Full agonists stabilize R*G better than partial agonists so that relative efficacy is explained in terms of the differential stabilization of a single activated state. G protein activation and GDP/GTP exchange follow accordingly.

This model has been examined using ligand-binding studies to determine affinities of agonists for G protein-coupled (higher affinity, K_h) and -uncoupled (lower affinity, K_l) forms of the receptor. Some studies report a correlation between the K_l/K_h ratio for agonists and their relative efficacy (De Lean et al., 1980; Kearn et al., 1999; Egan et al., 2000; Watson et al., 2000; Payne et al., 2002; Alder et al., 2003), whereas other studies do not (Gardner et al., 1997; Gardner and Strange, 1998; Payne et al., 2002). It seems that there may be additional factors influencing relative efficacy such as differential abilities of some agonists to induce G protein activation within AR*G. Different agonists may stabilize different activated states of receptors leading to differential activities (Seifert et al., 2001; Waelbroeck, 2001).

G protein activation occurs, however, as part of a cycle of reactions (Fig. 1) (Waelbroeck, 2001; Mosser et al., 2002; Zhong et al., 2003), and the overall rate of G protein activation may be dependent on several of the component processes, although the slowest of these will limit the overall rate. The reactions of the cycle are as follows: 1) agonist (A) binds to receptor to stabilize AR*; 2) AR* and G_{GDP} combine to form AR*G: for some agonists AR*G stability is a guide to agonist relative efficacy; some agonists can produce a stable AR*G complex but are partial agonists (Gardner et al., 1997; Gardner and Strange, 1998; Payne et al., 2002) and so their activity must be limited by another event; 3) GDP release:

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ABBREVIATIONS: GPCR, G protein-coupled receptor; (±)-7-OH DPAT, 7-hydroxy-2-dipropylaminotetralin; NPA, *N*-propylnorapomorphine; CHO, Chinese hamster ovary; [^{35}S]GTPγS, guanosine 5′-*O*-(3-[^{35}S]thio)triphosphate; R, receptor; R*, partially activated form of the receptor; R*G, receptor coupled to the G protein forming the active state; AR*G, active state of the receptor; AR, inactive state of the receptor.

this is typically considered to be the rate-determining step in GPCR activation in the absence of agonist (Ross, 1989). In the presence of agonist, GDP dissociation is accelerated, and GDP association decreased (Florio and Sternweis, 1989). GDP release could, however, be the slowest step in the cycle for some agonists, despite strong stabilization of AR*G; 4) GTP binding: cells contain high concentrations of GTP (~50 μM) (Otero, 1990; Jinnah et al., 1993) so that this step will be fast, and another step is rate-determining. This step may be examined using the GTP analog ([^{35}S]GTPγS). In general, these assays are performed at low concentrations of [^{35}S]GTPγS, and this step may become rate-determining (Waelbroeck, 2001); 5) AR*G dissociates, releasing AR, G_αGTP, and $G_{\beta\gamma}$: this step may be agonist-dependent for some receptors (Hausdorff et al., 1990; Van Koppen et al., 1994) and could be rate-determining if an agonist were unable to mediate rapid breakdown of AR*G; and 6) the intrinsic GTPase of the G protein hydrolyzes GTP to GDP and deactivates G_α: this step itself is independent of agonist because it is an intrinsic activity of the G protein but is unlikely to be rate-determining because, in the presence of proteins with GTPase-accelerating activity, this step is fast (Ross and Wilkie, 2000).

There are, therefore, several steps in the cycle that are regulated by agonists and that could determine the relative efficacy of agonists. It is not known whether the rate-determining step in the cycle is the same for all agonists. In this study, therefore, we examined the ability of a range of full and partial agonists to mediate G protein activation via the D_2 dopamine receptor. We have perturbed the function of the G protein cycle by altering the concentrations of both GDP and GTPγS to understand which step in the cycle is rate-limiting for different agonists.

Materials and Methods

Materials. [^{35}S]GTPγS (~37 TBq/mmol) and [^{3}H]spiperone (~600 GBq/mmol) were purchased from Amersham Biosciences UK, Ltd. (Little Chalfont, Buckinghamshire, UK). Optiphase HiSafe-3 scintillation fluid was purchased from PerkinElmer Life and Analytical Sciences (Cambridge, UK). Dopamine, bromocriptine, and (±)-7-OH-DPAT were purchased from Tocris Cookson Inc. (Bristol, UK). NPA, β-phenylethylamine, *m*-tyramine, and *p*-tyramine were purchased from Sigma Chemical (Poole, Dorset, UK).

Cell Culture. CHO cells stably expressing native D_{2short} dopamine receptors (Wilson et al., 2001) were grown in Dulbecco's modified Eagle's medium containing 5% fetal bovine serum and 400 μg/ml active geneticin (to maintain selection pressure). Cells were grown at 37°C in a humidified atmosphere of 5% CO_2.

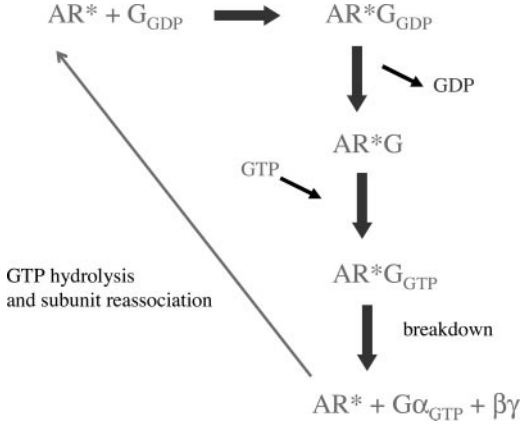

Fig. 1. An illustration of the G protein cycle is shown. See the text for a discussion of the constituent steps.

Membrane Preparation. Membranes were prepared from CHO cells expressing D_{2short} dopamine receptors as described previously (Castro and Strange, 1993). In brief, confluent 175-cm^2 flasks of cells were washed once with 5 ml HEPES buffer (20 mM HEPES, 1 mM EGTA, 1 mM EDTA, and 10 mM $MgCl_2$, pH 7.4). Cells were then removed from the surface of the flasks using 5 ml of HEPES buffer and glass balls (2 mm in diameter) and were then homogenized using an Ultra-Turrax homogenizer (two 5-s treatments). The homogenate was centrifuged at 1700*g* (for 10 min at 4°C), after which the supernatant was centrifuged at 48,000*g* (for 60 min at 4°C). The resulting pellet was resuspended in HEPES buffer at a concentration of 3 to 5 mg of protein/ml as determined by the method of Lowry et al. (1951) and stored in aliquots at −70°C until use.

Radioligand Binding Experiments. Cell membranes (25 μg) were incubated with [^{3}H]spiperone (0.35 nM) and competing drugs in HEPES buffer (20 mM HEPES, 1 mM EGTA, 1 mM EDTA, 10 mM $MgCl_2$, and 100 mM NaCl, pH 7.4, using KOH, containing 0.1 mM dithiothreitol) in a final volume of 1 ml for 3 h at 25°C. The assay was terminated by rapid filtration (through Whatman GF/C filters) using a Brandel cell harvester (Brandel Inc., Gaithersburg, MD) followed by four washes with 4 ml of ice-cold phosphate-buffered saline (0.14 M NaCl, 3 mM KCl, 1.5 mM KH_2PO_4, and 5 mM Na_2HPO_4, pH 7.4) to remove unbound radioactivity. Filters were soaked in 2 ml of scintillation fluid for at least 5 h, and bound radioactivity was determined by liquid scintillation counting. Nonspecific binding of [^{3}H]spiperone was determined in the presence of 3 μM (+)-butaclamol.

[^{35}S]GTPγS Binding Assays. Cell membranes (25 μg) were incubated with various concentrations of GDP and agonist for 20 min before the addition of 0.1 nM [^{35}S]GTPγS for 30 min or 10 nM [^{35}S]GTPγS (0.5 nM [^{35}S]GTPγS with 9.5 nM GTPγS) for 3 min in HEPES buffer at 30°C containing 0.1 mM dithiothreitol. In the absence of GDP, incubation times with 0.1 nM [^{35}S]GTPγS were reduced to 15 min. The assay was terminated by rapid filtration (through Whatman GF/C filters) using a Brandel cell harvester followed by four washes with 4 ml of ice-cold phosphate-buffered saline (0.14 M NaCl, 3 mM KCl, 1.5 mM KH_2PO_4, and 5 mM Na_2HPO_4, pH 7.4) to remove unbound radioactivity. Filters were soaked in 2 ml of Optiphase HiSafe-3 for at least 5 h, and bound radioactivity was determined by liquid scintillation counting.

Data Analysis. Radioligand binding and [^{35}S]GTPγS binding data were analyzed by nonlinear regression using Prism (GraphPad Software Inc., San Diego, CA). Statistical significance over multiple data sets was determined using an unpaired two-way analysis of variance followed by a Bonferroni post-test, whereas that for two groups was determined using a *t* test. Statistical significance was determined as $P < 0.05$.

Results

Effects of Different Concentrations of GDP and [^{35}S]GTPγS on Agonist Stimulation of [^{35}S]GTPγS Binding

Maximal Agonist-Stimulated Effect and Relative Agonist Efficacy. The maximal agonist-stimulated effect and relative agonist efficacy were determined from the stimulation of [^{35}S]GTPγS binding by agonists in membranes of CHO cells expressing the D_2 receptor (Wilson et al., 2001) (expression level of D_2 receptor, 1–1.5 pmol/mg of protein). Agonist-stimulated [^{35}S]GTPγS binding is caused by the D_2 receptor because there is no stimulation in untransfected cells (Gardner et al., 1996). The agonist-stimulated response is completely inhibited after pertussis toxin treatment (100 ng/ml for 18 h; data not shown), indicating a role for $G_{i/o}$

proteins. The principal $G_{i/o}$ proteins in CHO cells are G_{i2} and G_{i3} (Raymond et al., 1993; Gettys et al., 1994).

The stimulation of [^{35}S]GTPγS binding by two full agonists (dopamine and NPA) and two partial agonists (*p*-tyramine and (±)-7-OH-DPAT) was assessed in the presence of increasing concentrations of GDP (0.3–30 μM) and using two concentrations of [^{35}S]GTPγS (0.1 and 10 nM). The four agonists were used at maximally stimulating concentrations, and total [^{35}S]GTPγS binding was corrected for the agonist-independent binding to give the agonist-stimulated binding (Fig. 2). The association rate of [^{35}S]GTPγS binding stimulated by dopamine was much faster at the higher [^{35}S]GTPγS concentration (10 nM) ($t_{1/2}$, 1–2 min; data not shown) compared with the rate at the lower concentration of [^{35}S]GTPγS (0.1 nM) ($t_{1/2}$, 10–15 min) (Gardner et al., 1996). Therefore, assays were terminated after 3 min for experiments at 10 nM [^{35}S]GTPγS compared with after 30 min at 0.1 nM [^{35}S]GTPγS to ensure that the determinations were on the linear part of the time course. In this way, the determinations of agonist-stimulated [^{35}S]GTPγS binding correspond to rate measurements.

Maximal stimulated [^{35}S]GTPγS binding (over basal) was decreased for both full and partial agonists with increasing GDP concentration, although full agonists required higher concentrations of GDP than partial agonists to reduce their stimulation below that observed at the lowest concentration of GDP (Fig. 2, A and C). Maximal agonist-stimulated [^{35}S]GTPγS bound was also increased by ~10-fold by increasing the [^{35}S]GTPγS concentration from 0.1 to 10 nM, and given the difference in assay time for the two concentrations of [^{35}S]GTPγS, this corresponded to a ~100-fold increase in the rate of [^{35}S]GTPγS binding.

Increasing the concentration of GDP reduced the relative efficacy of partial agonists compared with full agonists in an almost linear fashion (from ~75 to ~50% relative efficacy at 0.1 nM [^{35}S]GTPγS and from ~60 to ~30% relative efficacy at 10 nM [^{35}S]GTPγS) (Fig. 2, B and D). When relative agonist efficacies were compared at the two concentrations of [^{35}S]GTPγS, it was seen that the relative efficacy of the partial agonists was lower at the higher [^{35}S]GTPγS concentration (Fig. 2, B and D).

Given that the relative efficacy of partial agonists was higher at the lower GDP concentrations, we performed some assays in the absence of GDP. Under these conditions, both full and partial agonists were still able to promote [^{35}S]GTPγS binding over basal levels (Table 1 and Fig. 3). Both full and partial agonists, however, thus stimulated [^{35}S]GTPγS binding to the same extent. There was no change in the rate or extent of basal [^{35}S]GTPγS binding in the presence of (+)-butaclamol if the membranes were pretreated with an agonist, suggesting that there is no prebound GDP present in the preparation (data not shown). Therefore, the stimulation of [^{35}S]GTPγS binding in the absence of GDP is not a reflection of the release of bound GDP.

Agonist Potency. To probe the relationship between the EC_{50} for agonist stimulation of [^{35}S]GTPγS binding and GDP concentration, a series of agonist-concentration experiments were performed at different concentrations of GDP. The four agonists used above were assessed at two different concentrations of [^{35}S]GTPγS (0.1 and 10 nM), and additional agonists were also tested at the lower concentration of [^{35}S]GTPγS. Representative data are shown in Fig. 4, and the derived EC_{50} values are given in Table 3 and Fig. 5. At 0.1 nM [^{35}S]GTPγS, increasing concentrations of GDP caused a similar rightward shift in agonist EC_{50} values for all of the agonists tested, with the exception of bromocriptine. This shift falls between the agonist binding affinities calculated for G protein-coupled and -uncoupled receptors determined under the conditions used for the [^{35}S]GTPγS binding assays (see below and Table 2). In contrast, bromocriptine, which

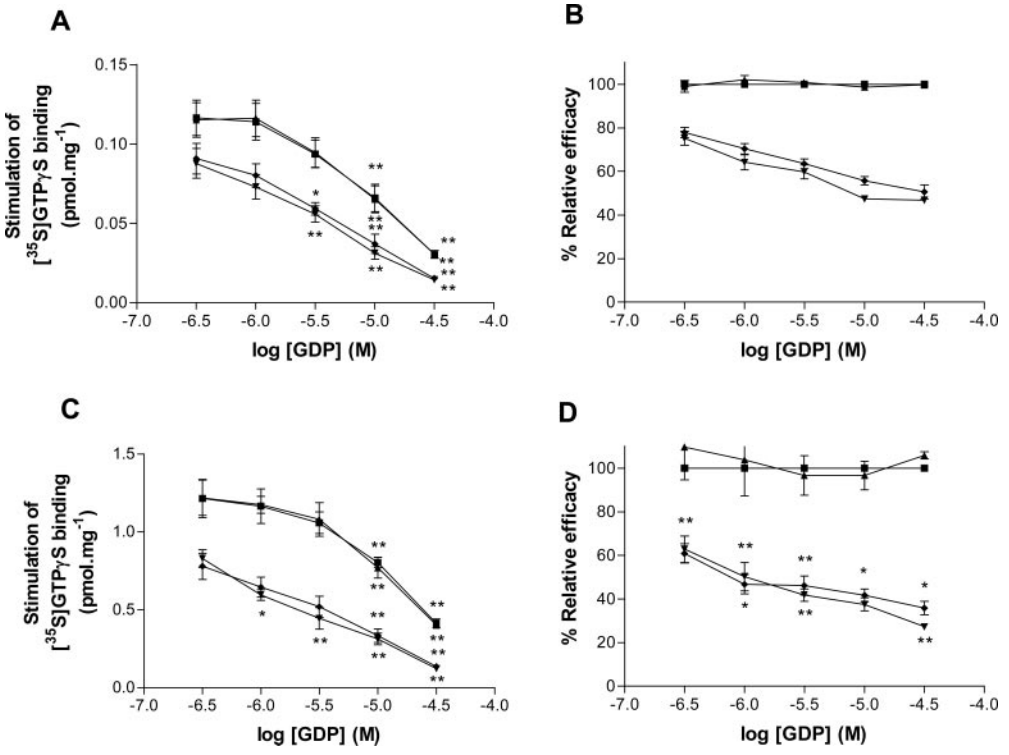

Fig. 2. Stimulation by agonists of [^{35}S]GTPγS binding to membranes of CHO cells expressing the D_2 dopamine receptor. The effects of GDP on maximal [^{35}S]GTPγS binding and relative agonist efficacy for full and partial agonists are shown. Membranes were incubated with either 0.1 (A and B) or 10 nM (C and D) [^{35}S]GTPγS in the presence of varying concentrations of GDP and maximal stimulatory concentrations of agonist as described under *Materials and Methods*. Agonist concentrations were 100 μM dopamine (■), 10 μM (±)-7-OH-DPAT (◆), 10 μM NPA (▲), and 1 mM *p*-tyramine (▼). Incubation times were 30 (0.1 nM [^{35}S]GTPγS) or 3 min (10 nM [^{35}S]GTPγS). Data shown are mean ± S.E.M. of three to four experiments with basal subtracted. ⋆, $P < 0.05$; ⋆⋆, $P < 0.01$ for comparison of relative efficacy between 0.1 and 10 nM [^{35}S]GTPγS (B and D) or for comparison of picomole per milligram of [^{35}S]GTPγS binding from that observed at 300 nM GDP (A and C).

displays no observable affinity preference for coupled or uncoupled receptors (Gardner et al., 1997), shows no change in EC_{50} value with changing GDP concentration. With the exclusion of bromocriptine, when the pEC_{50} was plotted against log[GDP], there was no significant difference between the slopes of the lines ($P > 0.05$), and a mean slope of −0.31 was obtained.

When the [^{35}S]GTPγS concentration was increased to 10 nM, the EC_{50} value for the two full agonists tested was shifted rightward as before as the GDP concentration was increased, whereas for the two partial agonists, the EC_{50} value was much less affected by GDP. This effect is emphasized in the pEC_{50} versus log[GDP] plots (Fig. 5). Linear relationships between pEC_{50} and log[GDP] were still observed, but there were significantly greater slopes for the full agonists, NPA and dopamine, compared with the partial agonists (±)-7-OH-DPAT and *p*-tyramine ($P < 0.05$) (Fig. 5 and Table 3).

Binding of Full and Partial Agonists to D_2 Dopamine Receptors. The binding of the agonists (dopamine, NPA, *p*-tyramine, and (±)-7-OH-DPAT) was determined in competition versus the binding of [^{3}H]spiperone using assay buffer containing 100 mM NaCl as in the [^{35}S]GTPγS binding experiments (see above). In each case, the competition data were fitted best by a two-binding site model, and the derived dissociation constants (K_h, K_l) are given in Table 2.

TABLE 1
Stimulation of [^{35}S]GTPγS binding by agonists in the absence of GDP
The stimulation of [^{35}S]GTPγS binding by agonists was determined as described under *Materials and Methods* in the absence of GDP. pEC_{50} values were determined, and maximal effects were expressed as the stimulation of [^{35}S]GTPγS binding over basal. Maximal effects were not statistically different ($P > 0.05$).

	pEC_{50} (EC_{50})	Stimulation of [^{35}S]GTPγS Binding
	nM	*pmol · mg protein*$^{-1}$
Dopamine	7.84 ± 0.04 (14)	0.056 ± 0.012
(±)-7-OH-DPAT	8.21 ± 0.09 (6)	0.067 ± 0.013
NPA	10.00 ± 0.12 (0.1)	0.067 ± 0.018
p-Tyramine	5.48 ± 0.10 (3310)	0.057 ± 0.013

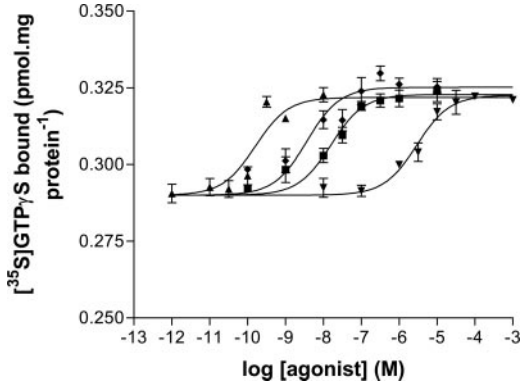

Fig. 3. Stimulation of [^{35}S]GTPγS binding by agonists in the absence of GDP. Membranes of CHO cells expressing the D_2 dopamine receptor were incubated with either dopamine (■), (±)7-OH-DPAT (♦), NPA (▲), or *p*-tyramine (▼) in the presence of 0.1 nM [^{35}S]GTPγS and the absence of added GDP as described under *Materials and Methods*. The data shown represent a single experiment replicated as in Table 1. Concentration-response curves are fitted best by sigmoidal curves with Hill coefficients of 1.

Discussion

In this study, we have examined some basic mechanisms of agonist action using the D_2 dopamine receptor as a model GPCR, with the aim of understanding the mechanistic distinction between full and partial agonists. For the GPCRs, differences in the relative efficacy of agonists have been explained using the ternary complex model (De Lean et al., 1982; Lefkowitz et al., 1993; Weiss et al., 1996), whereby partial agonists stabilize the ternary complex (AR*G) less well than full agonists. The model does not always account for relative efficacy, and this may relate to the fact that GPCR activation depends on a cycle of reactions (Fig. 1) (Waelbroeck, 2001; Mosser et al., 2002; Zhong et al., 2003). During receptor activation, the reactions of the cycle will not be at equilibrium, and different agonists may influence steps in the cycle differentially. In this report, we examined how agonists with different relative efficacies influence the steps in the cycle using the D_2 dopamine receptor as a model system. From the data, we have shown that full and partial agonists differ in their abilities to modulate different reactions in the G protein cycle. The study therefore provides a mechanistic basis for the distinction between full and partial agonists.

We used the stimulation of [^{35}S]GTPγS binding by agonists as a measure of their relative efficacy and examined the effect of different concentrations of GDP on both the maximal [^{35}S]GTPγS binding and relative efficacy. Assays were performed at two concentrations of [^{35}S]GTPγS (0.1 and 10 nM) with several full and partial agonists. In the [^{35}S]GTPγS binding assays, two principal parameters were determined for each agonist: the concentration of agonist achieving half the maximal stimulated effect (potency, EC_{50}) and the maximal rate of [^{35}S]GTPγS binding stimulated by saturating agonist concentrations.

The effects of GDP on the potency (EC_{50}) for agonists to stimulate [^{35}S]GTPγS binding were assessed. At low concentrations of [^{35}S]GTPγS (0.1 nM), the potency of each of the agonists tested, with the exception of bromocriptine, was reduced as the GDP concentration was increased. The effect of GDP was similar for each agonist, independent of its relative efficacy, as shown by the similar slopes of the lines relating pEC_{50} and log[GDP]. The effects of GDP here reflect the binding of GDP to the AR*G state, leading to its breakdown and sequestration of G protein as G_{GDP}. Higher concentrations of agonist are then required to stabilize AR*G in which [^{35}S]GTPγS binding occurs, and the EC_{50} value for the agonist is increased. Simulations of these effects have been reported (McLoughlin and Strange, 2000). The slope of the line relating pEC_{50} and log[GDP] reflects the affinities of the agonist for the G protein-coupled and -uncoupled states of the receptor and the sensitivity of the agonist/receptor/G protein complex to GDP. Bromocriptine has been shown have similar affinities for the coupled and uncoupled states (Gardner et al., 1997), so it is not surprising that it is insensitive to GDP.

When higher concentrations of [^{35}S]GTPγS (10 nM) were used, the potencies of the two full agonists (NPA and dopamine) tested were sensitive to the effects of GDP. Indeed, the pEC_{50} was more sensitive to log[GDP] than at the lower concentration of [^{35}S]GTPγS ($P < 0.05$). In contrast, the potencies of the two partial agonists [(±)-7-OH-DPAT and *p*-tyramine] were virtually independent of log[GDP] when

assays were performed at the higher [^{35}S]GTPγS concentration. A change in the mechanism of [^{35}S]GTPγS binding from a GDP-dependent rate-determining step to a GDP-independent rate-determining step may have occurred for the partial agonists at the higher concentration of [^{35}S]GTPγS. The 100-fold increase in [^{35}S]GTPγS concentration also leads to a substantial increase (~100-fold) in the maximal rate of [^{35}S]GTPγS binding stimulated by dopamine. This increase in the maximal rate of binding of [^{35}S]GTPγS as the concentration is increased suggests that the rate-determining step in the cycle at the lower concentration of [^{35}S]GTPγS is the [^{35}S]GTPγS-binding event. This reflects the rather low concentration of [^{35}S]GTPγS that is being used, and similar conclusions have been reached by others using other approaches (Waelbroeck, 2001). Therefore, agonists are able to modulate directly the rate of binding of [^{35}S]GTPγS, as has been suggested by Florio and Sternweis (1989). Indeed, in the present study, agonists were able to stimulate [^{35}S]GTPγS binding in the absence of GDP, supporting these ideas. This underlines the idea that agonists are able to regulate the rate of [^{35}S]GTPγS binding and suggests that the G protein associated with receptor is a better substrate for [^{35}S]GTPγS binding than free G protein. In the present system, however, in the absence of GDP, the agonists all mediated the same maximal [^{35}S]GTPγS binding (i.e., all appear as full agonists). Therefore, there may be a limit to the stimulation of [^{35}S]GTPγS binding possible in this system, and in the absence of GDP, this is achieved by all of the agonists tested here.

Therefore, if [^{35}S]GTPγS binding is the rate-determining step at the lower concentration of [^{35}S]GTPγS for all agonists, then the effect of the increase in [^{35}S]GTPγS concentration and concomitant increase in overall rate may have led to a change in the rate-determining step for some agonists. For the partial agonists, at the higher concentration of [^{35}S]GTPγS, another, GDP-independent, step may have become rate-determining. For this process to be GDP-independent, it must be subsequent to formation of AR*G, and a likely candidate could be ternary complex breakdown. For the full agonists, the rate-determining step at the higher concentration of [^{35}S]GTPγS is GDP-dependent and most likely is the [^{35}S]GTPγS-binding event, although we cannot rule out that GDP release has become rate-determining.

The maximal agonist-stimulated [^{35}S]GTPγS binding was reduced at both concentrations of [^{35}S]GTPγS as the GDP concentration was increased. The maximal [^{35}S]GTPγS binding reflects the rate of the slowest process in the cycle and the amount of the different species in the cycle. At the lower concentration of [^{35}S]GTPγS, at which the rate directly reflects the [^{35}S]GTPγS binding process, the effect of GDP on the maximal rate of [^{35}S]GTPγS binding may result from a reduction in the level of AR*G species after sequestration of G proteins as G_{GDP} when the G protein cycle is at steady state. Although the potency of bromocriptine is unaffected by changes in GDP (see above), the maximal [^{35}S]GTPγS binding for this compound is reduced by increased GDP, supporting the above ideas. At the higher [^{35}S]GTPγS concentration, for the full agonists, the binding event is still the slowest process, whereas for the partial agonists, ternary complex breakdown may be the slowest process in the cycle. The

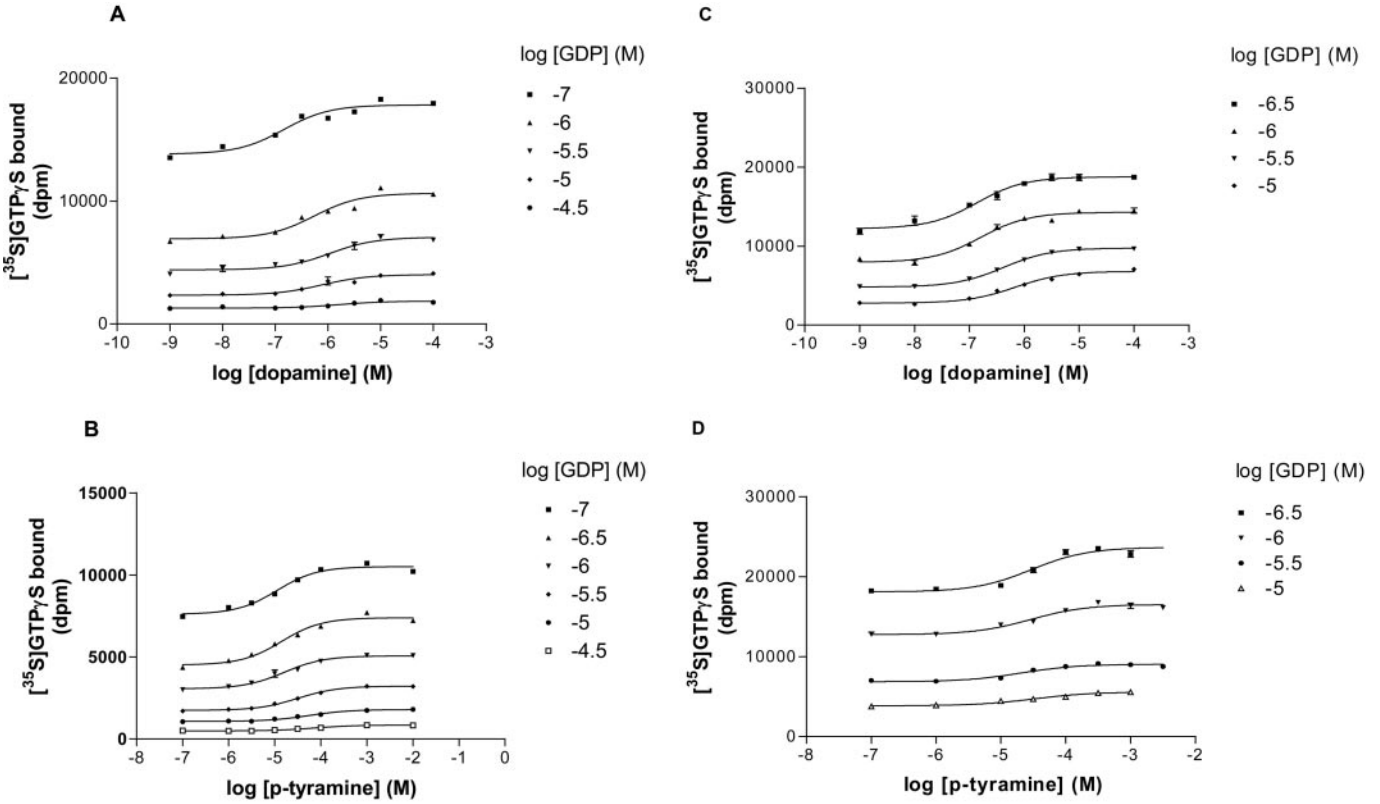

Fig. 4. Stimulation of [^{35}S]GTPγS binding to membranes of CHO cells expressing the D_2 dopamine receptor by agonists and effects of GDP on potency of dopamine (A and C) and *p*-tyramine (B and D). Membranes were incubated with either 0.1 (A and B) or 10 nM (C and D) [^{35}S]GTPγS in the presence of varying concentrations of GDP and a range of concentrations of agonist as described under *Materials and Methods*. The data shown represent single experiments replicated as shown in Fig. 5. Concentration-response curves are fitted best by sigmoidal curves with Hill coefficients of 1.

effects of GDP on maximal [^{35}S]GTPγS binding rate presumably reflect sequestration of G proteins reducing levels of AR*G and hence the overall rate of the cycle.

In addition to the effects of GDP on the maximal rates of [^{35}S]GTPγS binding, there were effects on the relative efficacies of the partial agonists. The maximal rates of [^{35}S]GTPγS binding of the partial agonists were more sensitive to GDP than those of the full agonists, resulting in a reduction in relative efficacy for the two partial agonists as GDP was increased. This suggests that differences in relative efficacy may reflect differences in GDP sensitivity of different agonist/receptor/G protein species; full agonists are able to overcome G protein sequestration more than partial agonists. For other GPCRs, it has been shown that agonists may affect the affinity of the receptor for the G protein (Tota and Schimerlik, 1990). Thus, different AR*G complexes may seem differentially sensitive to GDP. The relative efficacies of the partial agonists were also generally lower at higher [^{35}S]GTPγS concentrations, and this may reflect the change in the rate-determining step to ternary complex breakdown for which the partial agonists are deficient relative to the full agonists.

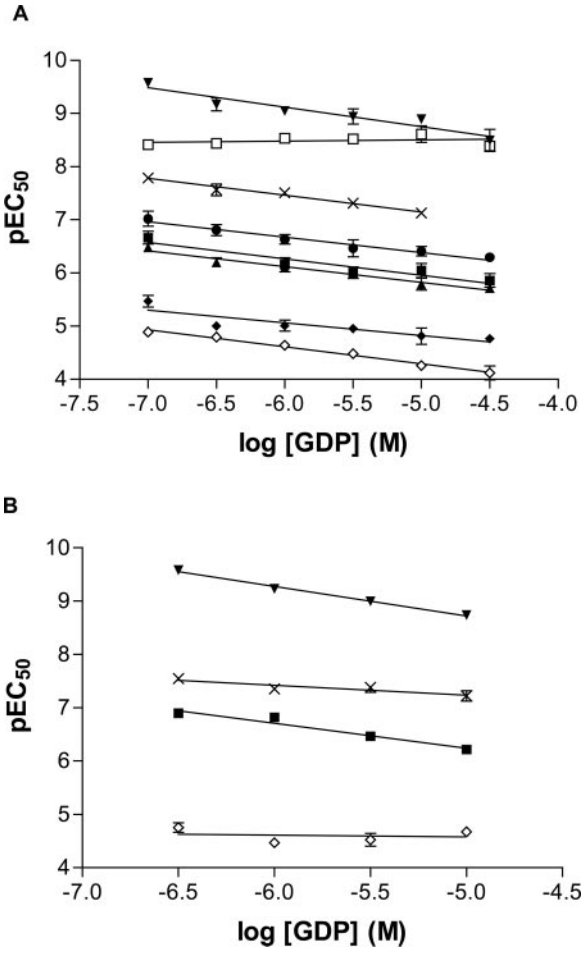

Fig. 5. Stimulation of [^{35}S]GTPγS binding to membranes of CHO cells expressing the D_2 dopamine receptor by agonists, and the relationship between GDP concentration and agonist pEC_{50} values at 0.1 (A) and 10 nM (B) [^{35}S]GTPγS. Concentration-response curves were constructed for dopamine (■), bromocriptine (□), NPA (▼), (±)-7-OH-DPAT (×), quinpirole (●), β-phenylethylamine (♦), *m*-tyramine (▲), and *p*-tyramine (◇) at the indicated GDP concentrations, and the potency (EC_{50}) was determined as described in Fig. 4 and under *Materials and Methods*. Data shown are mean ± S.E.M. of three to five experiments performed in triplicate. Statistical analysis is given in Table 3.

In the present study, bromocriptine stands out as having unusual properties in that its potency for stimulation of [^{35}S]GTPγS binding, when measured at 100 pM [^{35}S]GTPγS, is insensitive to GDP, unlike the potencies of the other agonists tested (Fig. 5). Bromocriptine exhibits similar behavior in ligand binding assays in that its binding is insensitive to guanine nucleotides unlike other agonists (Gardner et al., 1997; Gardner and Strange, 1998). We have suggested that this reflects stabilization by bromocriptine, in the absence of G protein coupling, of a conformation of the receptor that is close to the conformation in the fully active G protein-coupled state (Strange, 1999). Hence, there is little difference in affinity between the uncoupled and G protein-coupled forms of the receptor.

This study provides new information on the biochemical basis of the distinction between full and partial agonists. Several steps in the G protein cycle are agonist-dependent: GDP release from the G protein, [^{35}S]GTPγS binding to the G protein, and breakdown of the ternary complex. In the experiments performed in this report at the low concentration of [^{35}S]GTPγS, the binding of [^{35}S]GTPγS is the slowest step, and differential effects of agonists on this step reflect full and partial agonism. This conclusion is likely to apply to all [^{35}S]GTPγS binding assays performed on all GPCRs at these low concentrations of [^{35}S]GTPγS. At the higher concentrations of [^{35}S]GTPγS, the rate of [^{35}S]GTPγS binding increases ~100-fold, and for the full agonists, ternary complex breakdown is still fast enough for a GDP-dependent event, most likely the [^{35}S]GTPγS-binding event, to be rate-determining. For the partial agonists, however, another step, probably ternary complex breakdown, is slower and becomes rate-determining. These conclusions are of some significance in that the concentration of GTP in the cell is high (~50 μM) (Otero, 1990; Jinnah et al., 1993), so that partial agonism in cells may be apparent because of this limitation of the rate of ternary complex breakdown.

Given that the experiments conducted here at the higher

TABLE 2

The binding of agonists to D_2 dopamine receptors

Agonist binding was determined in competition versus [^{3}H]spiperone (~0.3 nM) in a buffer containing Na^+ (100 mM) as described under *Materials and Methods*. Data were fitted best by a two-binding site model and analyzed by nonlinear regression to derive dissociation constants for the higher (K_h) and lower (K_l) affinity sites and the percentage of receptors in the high-affinity state ($\%R_h$). Data represent mean ± S.E.M. of three experiments performed in triplicate.

	pK_h (K_h)	pK_l (K_l)	$\%R_h$	K_l/K_h
	nM	*nM*		
Dopamine	7.61 ± 0.01 (24.5)	5.55 ± 0.02 (2820)	49 ± 7	115
(±)-7-OH-DPAT	7.83 ± 0.15 (14.8)	6.49 ± 0.08 (320)	42 ± 4	22
NPA	10.03 ± 0.16 (0.09)	8.21 ± 0.12 (6.2)	35 ± 5	69
p-Tyramine	5.10 ± 0.25 (7940)	3.61 ± 0.07 (245,000)	40 ± 10	31

TABLE 3

The relationship between GDP concentration and agonist potency

In the experiments at 0.1 nM [^{35}S]GTPγS, all compounds had similar slopes ($P > 0.05$), with the exception of bromocriptine. In the experiments at 10 nM [^{35}S]GTPγS, the slope for 7-OH-DPAT was different from 0 ($P < 0.05$) and was different from slopes for other compounds ($P < 0.05$); the slope for *p*-tyramine was not different from 0 ($P > 0.05$), and the slopes for dopamine and NPA were different from those for (±)-7-OH-DPAT and *p*-tyramine . Slopes for NPA or dopamine were different at the two concentrations of [^{35}S]GTPγS. The slopes of the lines shown in Fig. 5 were determined by linear regression.

	Slope	
	0.1 nM [^{35}S[GTPγS	10 nM [^{35}S]GTPγS
Dopamine	−0.31 ± 0.06	−0.47 ± 0.04
Bromocriptine	0.02 ± 0.04	N.D.
(±)-7-OH-DPAT	−0.31 ± 0.04	−0.20 ± 0.07
NPA	−0.37 ± 0.06	−0.55 ± 0.03
Quinpirole	−0.29 ± 0.05	N.D.
β-Phenylethylamine	−0.24 ± 0.05	N.D.
p-Tyramine	−0.32 ± 0.03	−0.04 ± 0.09
m-Tyramine	−0.30 ± 0.04	N.D.

N.D., not determined.

concentration of [^{35}S]GTPγS reflect more closely cellular conditions, it should be possible to relate effects of agonists on [^{35}S]GTPγS binding performed under these conditions to experiments performed on whole cells, such as examining effects of agonists to inhibit cAMP accumulation (Payne et al., 2002). The present set of data for the higher [^{35}S]GTPγS concentration is not extensive enough to allow this correlation to be examined, but this will be an important aim for future work in relating these in vitro assays to cellular assays.

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