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Pharmacological Characterization of Dopamine-stimulated [^{35}S]-Guanosine 5′-(γ-thiotriphosphate) ([^{35}S]GTPγS) Binding in Rat Striatal Membranes

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ABSTRACT. Functional activation of dopamine receptors in the crude membranes from rat striatum was studied by a [^{35}S]-guanosine 5′-*O*-(γ-thiotriphosphate) ([^{35}S]GTPγS) binding assay. Binding of [^{35}S]GTPγS could be characterized with a dissociation constant (K_d) = 14.6 ± 0.8 nM and this did not depend on the presence of dopamine. The displacement of [^{35}S]GTPγS binding by GDP could be characterized with an inhibition constant (K_i) = 78 ± 15 μM in the presence of 10μM of butaclamol, while the presence of 100 μM of dopamine decreased it to a K_i = 0.13 ± 0.02 mM. Dopamine increased the association rate of [^{35}S]GTPγS binding in the presence of GDP in a dose-dependent manner with an EC_{50} = 1.45 ± 0.48 μM. Other dopamine receptor agonists studied displayed a potency to stimulate the [^{35}S]GTPγS binding in the order R(−)-10,11-dihydroxy-*N*-*n*-propylnorapomorphine (NPA) > pergolide ≥ apomorphine > dopamine ≈ quinpirole > R(+)-1-phenyl-2,3,4,5-tetrahydro-(1H)-3-benzazepine-7,8-diol hydrochloride (SKF-38393) > S(+)-(4aR,10bR)-3,4,4a,10b-tetrahydro-4-propyl-2H,5H-[1]benzopyrano[4,3-b]-1,4-oxazin-9-ol hydrocholoride (PD 128,907). The dopamine-induced stimulation of [^{35}S]GTPγS binding was inhibited by different dopamine receptor antagonists in the potency order: (+)butaclamol > haloperidol ≈ clorpromazine ≥ raclopride > (−)-sulpride > remoxipride > 5,6-dimethoxy-2-(dipropylamine)indan (U 991944A) > R(+)-7-chloro-8-hydroxy-3-methyl-1-phenyl-2,3,4,5-tetrahydro-1H-3-benzazepine (SCH-23390). Comparison of the obtained data with the dissociation constants of these ligands to different subtypes of dopamine receptors gave a good correlation only with constants for the D_2 subtype, supporting the idea that this subtype is most likely responsible for the dopaminergic activation of [^{35}S]GTPγS binding in rat striatal membranes. BIOCHEM PHARMACOL **57**;2: 155–162, 1999. © 1998 Elsevier Science Inc.

KEY WORDS. rat striatum, dopamine receptors; G-protein; [^{35}S]GTPγS binding; D_2 subtype; agonist binding

Dopamine receptors are members of the G-protein-coupled receptor superfamily and on the basis of pharmacological and biochemical criteria have been classified into D_1 and D_2 subtypes [1]. Molecular cloning studies have revealed at least five different genes for dopamine receptors [2]. D_1-like receptors, which include the D_1 and D_5 subtypes, are coupled with the effector system, which activates adenylate cyclase, whereas D_2-like receptors, including the D_2, D_3 and D_4 subtypes, inhibit adenylate cyclase activity [3]. All subtypes of dopamine receptors transduce their signals into cells by interacting with heterotrimeric guanine nucleotide-binding regulatory proteins (G-proteins). Receptors activate G-proteins by catalyzing the exchange of GDP with GTP at the α subunit of the interacting G-protein. Bound GTP activates the G-protein, causing the dissociation of the α subunit from the receptor and from the βγ subunits [4]. The activated state lasts until the GTP is hydrolyzed to GDP by the intrinsic GTPase activity of the α subunit, which then reassociates with the βγ subunits and the nonactivated receptor. The free subunits α and βγ are the activators which transfer the response to the target effectors, and the rate of GTP hydrolysis is a timing mechanism which controls the duration of the activation [4]. There is a wide variety of effectors that respond to the activated G-proteins, including adenylate cyclase (activation/inhibition), phospholipase C, G-protein-coupled receptor kinases, PI3 kinases, ion channels, etc. [5].

The finding that G-proteins play a key role in receptor signal transduction underlined the importance of characterizing the receptors in the active complex with G-proteins. The most widely used method for the characterization of interactions between receptors and G-proteins is the determination of GTP-sensitive high-affinity agonist binding to the receptor; however, this method indicates how G-proteins influence ligand-binding properties to the receptors, but provides no information about the activation of the effector system [6, 7]. Receptor–G-protein interaction has also been examined by measurement of high-affinity GTPase activity [8, 9], but it is proposed that this reflects both activation and deactivation processes of G-

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proteins [10]. A more direct characterization of receptor–G-protein interactions can be obtained by measuring agonist-dependent exchange of GDP for GTP at G-proteins. A useful tool for these studies is a radiolabeled nonhydrolyzable analogue of GTP such as [^{35}S]GTPγS*, which has high affinity for G-proteins and is resistant to GTPase activity [11]. This method has been successfully utilized for a number of G-protein-coupled receptors such as muscarinic, serotoninergic, opioid, and metatropic glutamate receptors [12–15], but mainly in reconstituted systems or in recombinant cell lines. Data for different subtypes of dopamine receptors have mainly been obtained using membranes from specifically transfected cell lines [16–20]. The situation is much more complicated when the effect is studied in membranes from the mammalian brain, where several receptor systems are coupled with different subtypes and pools of G-proteins [21]. This phenomenon may also be the reason why earlier attempts to characterize dopaminergic receptors in brain membranes by direct binding of [^{35}S]GTPγS have failed [22]. As an alternative, the method of immunoprecipitation has been proposed for the detection of α subunits of G-proteins bound with [^{35}S]GTPγS [23]. This method has been successfully utilized for characterization of activation of dopaminergic receptors in brain membranes [24, 25].

The aim of the present study was to develop an assay for direct characterization of dopamine receptor activation in rat striatal membranes based on the determination of the acceleration of [^{35}S]GTPγS binding. The obtained functional efficiencies of dopaminergic ligands in striatal membranes gave a high correlation with binding affinities of these ligands to D_2 dopamine receptors.

MATERIALS AND METHODS

Materials

[^{35}S]GTPγS (1250 Ci/mmol) was obtained from DuPont-New England Nuclear; GTPγS, GDP, DTT, 3-hydroxytyramine hydrochloride (dopamine), and apomorphine hydrochloride were from Sigma Chemical Co. NPA, 8-[(methylthio)methyl]-6-propylergoline methanesulfonate (pergolide), *trans*-(−)-4aR-4,4a,5,6,7,8,8a,9-octahydro-5-propyl-1H-pyrazolo[3,4-g]quinoline hydrochloride (quinpirole), SKF-38393, PD 128,907, haloperidol, chlorpromazine hydrochloride, S(−)-raclopride L-tartrate, S(−)-sulpiride, and SCH-23390 were from Research Biochemicals Inc. U99194A was from Pharmacia Upjohn and S(−)-3-bromo-*N*-[(1-ethyl-2-pyrrolidinyl)methyl]-2,6-dimethoxy-benzamide (remoxipride) from Astra Läkemedel.

Membrane Preparation

The rat brains were rapidly removed after decapitation and the corpus striatum was dissected out. The tissue was homogenized in 1:100 volume (wet weight/volume) of ice-cold K-HEPES buffer (20 mM, pH = 7.6), containing 7 mM of $MgCl_2$, 100 mM of NaCl, 1 mM of EDTA, 1 mM of DTT (buffer A) and centrifuged at 45,000 *g* for 10 min at 4° (Sorvall RC-5C, DuPont Instruments). The membrane pellet was resuspended in 100 volumes (ww/v) of fresh buffer A and recentrifuged under the same conditions. The final pellet was suspended in buffer A and used for the following experiments at a protein concentration of 0.2 mg/mL, determined by the modified method of Lowry [26] using BSA as standard.

[^{35}S]GTPγS Binding Assay

Unless otherwise indicated, [^{35}S]GTPγS binding was assayed in a reaction mixture (final volume 300 μL) containing 20 mM K-HEPES (pH = 7.6), 7 mM $MgCl_2$, 100 mM NaCl, 1 mM EDTA, 1 mM DTT and 0.2 mM GDP. Membranes were preincubated with GDP and the ligand for 15 min at 21° and the incubation, started by addition of [^{35}S]GTPγS (final concentration 0.2–0.3 nM), lasted for 90 min at 30°. The assay was terminated by rapid filtration through glass fiber filters (GF/B, Whatman Int. Ltd.) using a Brandel cell harvester with three washings of 5 mL of ice-cold washing buffer, containing 20 mM K-HEPES and 100 mM NaCl (pH = 7.6). The radioactivity content of the filters was counted in 5 mL scintillation cocktail Flo-Scint™ V (Packard Instrument Company Inc.) by a Beckman LS 1800 liquid scintillation counter.

In the case of kinetic studies, the suspension of striatal membranes in the reaction medium with dopamine or butaclamol (3.5 mL) was preincubated for 15 min at 30° and the association reaction was started by rapid addition of 200 μL [^{35}S]GTPγS (final concentration 0.2–0.3 nM). At time moment *t*, aliquots of the reaction mixture (200 μL) were filtered through glass fibre filters (GF/B) and washed, and the radioactivity content was determined as described above.

Data Analysis

All binding and kinetic data were analyzed by means of the nonlinear least squares regression method, using a commercial program GraphPad PRISM™ (GraphPad). The kinetic data were fitted to the first order rate equation: $B_t = B_0 + B_{max}[1 - \exp(-k_{obs}t)]$, where B_t corresponds to the total bound [^{35}S]GTPγS at time moment *t*, B_0 to the basal level and B_{max} the maximal specific binding of [^{35}S]GTPγS. The effect of butaclamol was studied according to Schild analysis, where the log [(DR)-1] was plotted as function of

* *Abbreviations:* [^{35}S]GTPγS, [^{35}S]-guanosine 5′-(γ-thiotriphosphate); DTT, dithiotreitol; NPA, R(−)-10,11-dihydroxy-*N*-*n*-propylnorapomorphine; SFK-38393, R(+)-1-phenyl-2,3,4,5-tetrahydro-(1H)-3-benzazepine-7,8-diol hydrocholoride; PD128,907, S(+)-(4aR,10bR)-3,4,4a,10b-tetrahydro-4-propyl-2H,5H-[1]benzopyrano[4,3-b]-1,4-oxazin-9-ol hydrochloride; SCH-23390, R(+)-7-chloro-8-hydroxy-3-methyl-1-phenyl-2,3,4,5-tetrahydro-1H-3-benzazepine; U99194A, 5,6-dimethoxy-2-(dipropylamine) indan; k_{obs}, apparent rate constant of reaction; and K_b, potency constant of antagonists to inhibit the dopamine stimulation of [^{35}S]GTPγS binding.

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antagonist concentration [27]. K_b values for other antagonists were calculated according to the equation: response = $(B_{max} - \text{basal})/(1 + \{EC_{50} \cdot ([L]^S/K_b + 1)/[A]\}^H + \text{basal}$, where B_{max} corresponds to maximal binding of [^{35}S]GTPγS in the presence of agonist and basal is the binding without agonist; EC_{50} is the concentration of agonist causing half-maximal response; [A] is the concentration of agonist; H is the slope factor of agonist, [L] is the concentration of antagonist, and S is the Schild slope factor as described by Lazareno and Birdsall [28], assuming competitive interactions between agonist and antagonist. All data are presented as means ± SEM of at least two independent determinations. Statistical significance of effects was determined by the Student–Newman–Keuls test using the GraphPad InStat™ (GraphPad Software). $P \leq 0.05$ was taken as the criterion for statistical significance.

RESULTS

Binding of [^{35}S]GTPγS to Rat Striatal Membranes

Binding of [^{35}S]GTPγS with rat striatal membranes was relatively fast and with high affinity. At the concentration of 0.27 nM of [^{35}S]GTPγS, the binding at 30° was completed within one hour (k_{obs} = 0.083 ± 0.027 min^{-1}). Nonradioactive GTPγS displaced [^{35}S]GTPγS in a concentration-dependent manner, with pIC_{50} = 7.84 ± 0.12 (n = 4), which according to the equation of Cheng–Prusoff [29] gives an affinity constant of K_d = 14.6 nM, which is in good agreement with constants for GTPγS from similar studies in reconstituted systems [12, 30, 31]. The presence of dopaminergic agonist or antagonists had no influence on the binding parameters of [^{35}S]GTPγS (data not shown).

Influence of GDP on [^{35}S]GTPγS Binding

GDP displaced the binding of [^{35}S]GTPγS in a concentration-dependent manner, while the apparent potency depended on the incubation time. This is likely caused by the slow dissociation of GDP, producing lower binding of [^{35}S]GTPγS than predicted by the equilibrium [32]. Thus, after 30 min incubation time, the pIC_{50} = 5.42 ± 0.21 and approached the value of pIC_{50} = 4.03 ± 0.21 exponentially (Fig. 1), which corresponds to an inhibition constant of K_i = 92 μM at 0.21 nM of [^{35}S]GTPγS. The decrease in pIC_{50} values of GDP can also be caused by the degradation of GDP, but in this case the plateau could not be achieved. The rate of the change of apparent constants of GDP could be characterized by an apparent rate constant k_{obs} = 0.014 ± 0.009 min^{-1}, which corresponds to a half-life of $\tau_{1/2}$ = 48 min and indicates that at least 4 hr incubation is required to reach equilibrium. However, in this kind of experiment, incubation time is limited by inactivation of G-proteins, as the ability of rat striatal membranes to specifically bind [^{35}S]GTPγS was lost in the absence of GDP, with a half-life of $\tau_{1/2}$ = 315 ± 43 min. Dopamine (100 μM) caused a decrease in pIC_{50} values for GDP in all cases studied and reached the value pIC_{50} = 3.86 ± 0.16

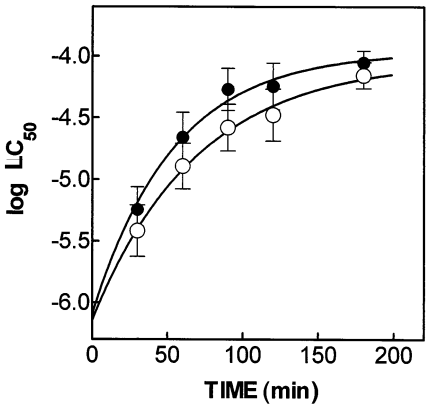

FIG. 1. Influence of incubation time on the apparent binding constant of GDP in competition with [^{35}S]GTPγS in rat striatal membranes in the presence of 100 μM of dopamine (○) or 1 μM of (+)butaclamol (●). Binding of 0.21 nM of [^{35}S]GTPγS was studied at different concentrations of GDP and incubation times at 30° as described in Materials and Methods. The log IC_{50} values were calculated from inhibition curves by means of nonlinear least squares regression and presented as means ± SEM (as error bar) of three independent experiments carried out in duplicate.

(Fig. 1). In the presence of dopamine, the apparent rate constant was also higher (k_{obs} = 0.019 ± 0.004 min^{-1}), making it possible to measure the dopaminergic effect on [^{35}S]GTPγS binding under pre-equilibrial conditions (Fig. 1).

Influence of Dopamine on [^{35}S]GTPγS Binding

In the absence of GDP, dopamine had no influence on [^{35}S]GTPγS binding (data not shown), while the presence of 200 μM of GDP caused a concentration-dependent acceleration of binding (Fig. 2). The GDP concentration of 200 μM, which is above the apparent IC_{50} value, was found to be optimal to obtain reasonable dopamine-dependent effects on [^{35}S]GTPγS binding in rat striatal membranes

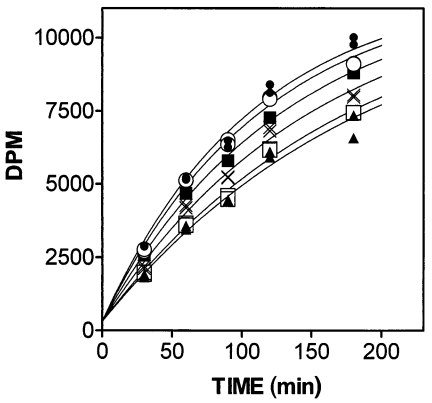

FIG. 2. Time-course of dopamine-stimulated [^{35}S]GTPγS binding to rat striatal membranes. Binding of 0.26 nM of [^{35}S]GTPγS was studied without dopamine (▲) and in the presence of 0.1 μM (□), 1 μM (X), 10 μM (■), 100 μM (○) and 10 mM (●) of dopamine as described in Materials and Methods. The data are representative of two independent experiments carried out in duplicate.

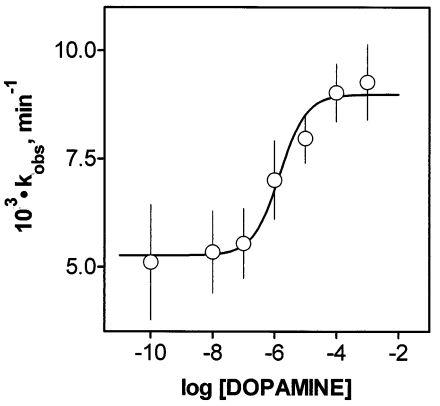

FIG. 3. Influence of the concentration of dopamine on the association rate constants for [^{35}S]GTPγS binding to rat striatal membranes. Kinetics of binding of 0.26 nM of [^{35}S]GTPγS to rat striatal membranes was measured and apparent rate constants (k_{obs}) calculated as described in Materials and Methods. The data are means ± SEM of two independent experiments carried out in duplicate.

(data not shown). The association of [^{35}S]GTPγS to the membranes was well described by the equation of one-phase exponential association, and allowed calculation of apparent association rate constants (k_{obs}) for this process. The dependence of these constants on dopamine concentration revealed a dose-response curve (Fig. 3) characterized by the parameter pEC_{50} = 5.84 ± 0.18 (EC_{50} = 1.45 μM). Similar dose-response curves were obtained by measuring [^{35}S]GTPγS binding at different incubation times. In this case, the level of [^{35}S]GTPγS binding was different, but the EC_{50} value for dopamine remained the same (pEC_{50} = 5.57 ± 0.15) (Fig. 4). As the apparent potency of dopamine did not depend on incubation time, the experimental conditions for characterization of other ligands were selected so that the maximal effect on [^{35}S]GTPγS binding could be achieved. Thus, all the following experiments

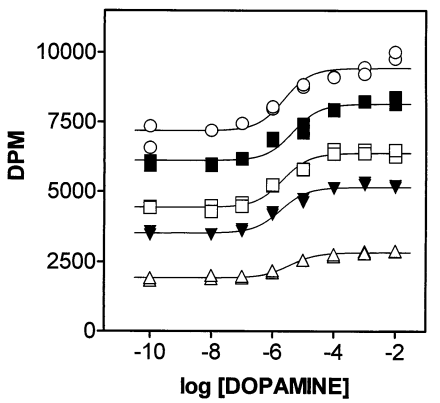

FIG. 4. Influence of time on the dose-response curves of dopamine/[^{35}S]GTPγS binding. Rat striatal membranes were incubated with different concentrations of dopamine and 0.26 nM of [^{35}S]GTPγS for 30 min (△), 60 min (▼), 90 min (□), 120 min (■), and 180 min (○) at 30° and bound radioactivity was measured as described in Materials and Methods. The data are representative of two independent experiments carried out in duplicate.

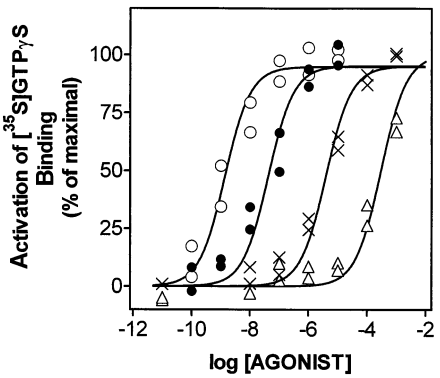

FIG. 5. Activation of [^{35}S]GTPγS binding by NPA (○), apomorphine (●), quinpirole (X), and SKF 38393 (△). Binding of [^{35}S]GTPγS (0.27 nM) to rat striatal membranes was carried out in the presence of different concentrations of dopaminergic agonists determined after incubation for 90 min at 30° as described in Materials and Methods. Data are representative of at least 3 independent experiments carried out in duplicate.

were carried out in the presence of 200 μM GDP and the incubation time was 90 min at 30°.

Influence of Other Dopaminergic Ligands on [^{35}S]GTPγS Binding

In addition to dopamine, several other dopaminergic agonists were effective in stimulation of [^{35}S]GTPγS binding to rat striatal membranes (Fig. 5). Among the ligands studied, NPA, pergolide, and apomorphine were the most effective, exhibiting no significant difference in the level of maximal effect; however, with 1 mM of SKF-38393, only 72 ± 15% of the maximal dopamine effect was achieved and 3 mM of PD 128,907 had no influence on the [^{35}S]GTPγS binding (Table 1).

The dopaminergic antagonist (+)butaclamol caused a concentration-dependent rightward shift of the dopamine dose-response curve of the stimulation of [^{35}S]GTPγS binding (Fig. 6). The Schild plot yielded a pK_b value of 9.88 ± 0.16 with slope 0.86 ± 0.17. Using a fixed concentration of dopamine (100 μM) at different concentrations of (+)butaclamol, a dose-response curve was obtained, which according to the Schild model [33] could be characterized by pK_b = 10.12 ± 0.46 (N = 5). Similar inhibition curves were obtained for several dopaminergic antagonists (Fig. 7) and corresponding constants are listed in Table 2. None of the antagonists studied affected the basal level of [^{35}S]GTPγS binding.

Comparison of the constants for dopaminergic ligands listed in Tables 1 and 2 with dissociation constants of these ligands for different subtypes of dopamine receptors determined in radioligand binding assay [34–38] revealed a good correlation for both agonists and antagonists only for D_2 receptors, with the correlation coefficients $r^2 > 0.97$ and slopes significantly different from zero ($P < 0.001$) (Fig. 8). A similar correlation was obtained with agonist affinities to D_4 receptors (r^2 = 0.98, P = 0.01) (Fig. 8a) and with antagonist affinities to D_3 receptors (r^2 = 0.83, P =

TABLE 1. Effect of dopaminergic agonists on the stimulation of [^{35}S]GTPγS binding to rat striatal membranes

No.	Agonist	pEC_{50}	EC_{50}	N
1.	NPA	8.43 ± 0.45	3.7 nM	3
2.	Pergolide	7.72 ± 0.48	19.1 nM	3
3.	Apomorphine	7.56 ± 0.39	27.9 nM	4
4.	Dopamine	5.51 ± 0.32	3.1 μM	18
5.	Quinpirole	5.43 ± 0.23	3.7 μM	7
6.	SKF-38393	3.22 ± 0.11*	603 μM	3
7.	PD 128,907	>2.5	>3 mM	2

Rat striatal membranes were incubated with different concentrations of ligand and 0.2–0.3 nM [^{35}S]GTPγS for 90 min at 30° as described in Materials and Methods. pEC_{50} values were calculated from the obtained dose-response curves and correspond to the agonist concentration causing half-maximal activation of [^{35}S]GTPγS binding. Data are means ± SEM of n independent experiments carried out in duplicate.

*Estimated value is based on the assumption of similar maximal response as dopamine.

0.005) (Fig. 8b), but the correlation was poor with antagonists for D_4 and agonists for D_3 ($r^2 < 0.7$, $P > 0.1$). Neither the affinities of agonists nor antagonists to D_1 and D_5 receptors revealed reasonable correlation with constants presented in Tables 1 and 2 ($r^2 < 0.5$). These data indicate that the activation of [^{35}S]GTPγS binding in rat striatal membranes by dopamine is most likely mediated by the D_2 subtype of dopamine receptors.

DISCUSSION

In the present study, a method for the determination of agonist-induced dopaminergic stimulation of [^{35}S]GTPγS binding in crude membrane preparation from rat striatum was established. As little information on the dopaminergic activation of [^{35}S]GTPγS binding in brain membranes was available, proper experimental conditions for this response had to be established.

A slight difference in IC_{50} values in the presence and absence of dopamine as well as the long incubation time required complicated the use of the influence of dopamine on GDP affinity as a measure of the efficacy of dopaminergic ligands. Clearer data were obtained by studying the time dependence of [^{35}S]GTPγS binding in the presence of GDP (Fig. 3), but the determination of the kinetic parameters of [^{35}S]GTPγS binding is material- and time-consuming. A compromise between these two approaches was the determination of [^{35}S]GTPγS binding in a pre-equilibrium state, when the influence of dopamine is greatest. Optimization of experimental conditions revealed that the effect of dopamine caused its clearest effect on the [^{35}S]GTPγS binding in rat striatal membranes after an incubation of 90 min at 30°, so these conditions were employed in the experiments which followed.

The dopaminergic activation of [^{35}S]GTPγS was found only in the presence of submillimolar concentrations of GDP. In most of the studies where receptor-stimulated [^{35}S]GTPγS binding has been studied, the presence of GDP in the reaction medium was required [12, 17, 30, 39]. The requirement of additional GDP supports the theory that G-proteins, which are activated by dopamine in the rat striatal membranes, belong to the G_i or G_0 family [11]. However, receptor-stimulated binding of [^{35}S]GTPγS to transducin and G_S can also be determined, but due to slow dissociation of GDP the additional GDP only complicates

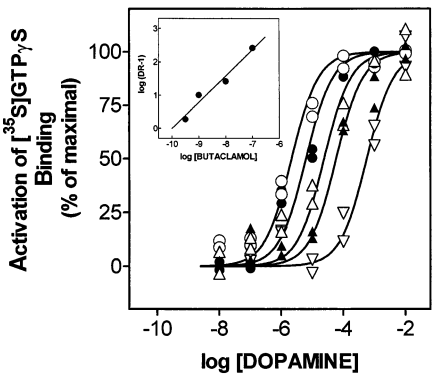

FIG. 6. Inhibition of dopamine stimulation of [^{35}S]GTPγS binding by (+)butaclamol. Binding of [^{35}S]GTPγS (0.27 nM) to rat striatal membranes was measured at the indicated concentration of dopamine in the absence (○) and in the presence of 0.3 (●), 1 (△), 10 (▲), and 100 nM (▽) of butaclamol as described in Materials and Methods. Data are representative of 5 independent experiments carried out in duplicate. Inset: Schild plot of (+)butaclamol antagonism.

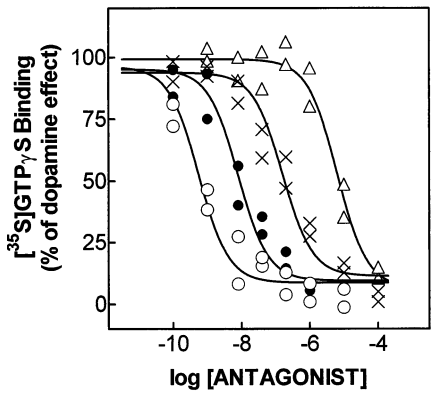

FIG. 7. Inhibition of the activation of [^{35}S]GTPγS binding by haloperidol (○), (−)sulpride (●), remoxipride (X), and SCH 23390 (△). Binding of [^{35}S]GTPγS (0.27 nM) to rat striatal membranes was activated by 100 μM of dopamine and inhibition was determined in the presence of antagonists after incubation for 90 min at 30° as described in Materials and Methods. Data are representative of 3–4 independent experiments carried out in duplicate.

TABLE 2. Effect of dopaminergic antagonists on the stimulation of [^{35}S]GTPγS binding to rat striatal membranes caused by 100 μM dopamine

No.	Antagonist	pK_b	K_b	N
1.	(+)-Butaclamol	10.12 ± 0.46	76 pM	5
2.	Haloperidol	9.44 ± 0.39	0.36 nM	4
3.	Chlorpromazine	9.24 ± 0.36	0.57 nM	3
4.	Raclopride	8.99 ± 0.33	1.02 nM	6
5.	(−)-Sulpiride	8.34 ± 0.41	4.6 nM	3
6.	Remoxipride	6.85 ± 0.29	0.14 μM	4
7.	U 99194A	6.15 ± 0.28	0.71 μM	2
8.	SCH-23390	5.57 ± 0.62	2.7 μM	4

Binding of [^{35}S]GTPγS (0.27 nM) to rat striatal membranes was activated with 100 μM dopamine and its inhibition was determined in the presence of different concentrations of antagonist after incubation for 90 min at 30°. The values of pK_b were calculated according to the Schild equation as described in Materials and Methods. Data are means ± SEM of n independent experiments carried out in duplicate.

the determination in these cases [11]. For G_i and G_0 the basal release of GDP is rapid and reveals a catalytic role of the receptors in the exchange of GDP to GTP only after the dissociation of GDP is suppressed by a large excess of this ligand. Thus, in the presence of 100 μM of GDP, the binding of [^{35}S]GTPγS slowed down 3 times, which was enough to make the detection of the influence of dopamine receptor activation possible.

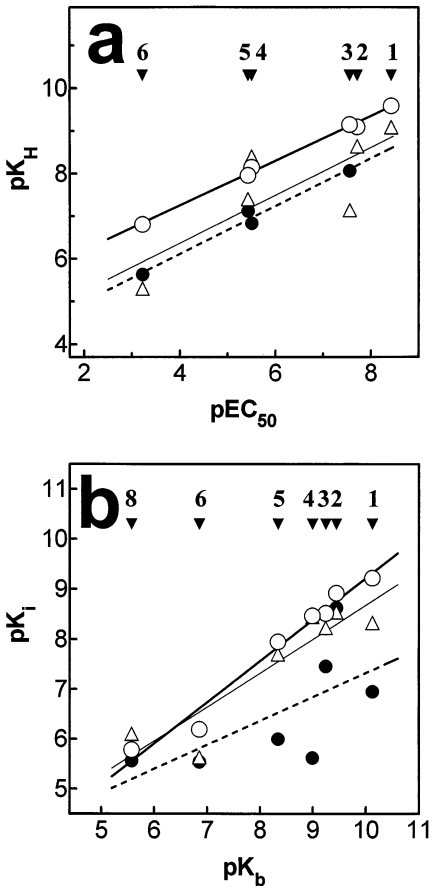

FIG. 8. Comparison of agonist potencies in stimulation of [^{35}S]GTPγS binding to rat striatal membranes pEC_{50} (a) and antagonist potencies to inhibit the dopamine stimulation of [^{35}S]GTPγS binding pK_b (b) with binding affinities of these ligands in displacement of radioactive antagonists at D_2 (○), D_3 (△), and D_4 (●) subtypes of dopamine receptors. The pK_i values (pK_H for agonists) were taken from [34–38]. Numbers correspond to the ligand number in Tables 1 and 2 for agonists and antagonists, respectively.

The optimal concentration of GDP (200 μM) for determination of dopamine-dependent stimulation of [^{35}S]GTPγS binding to rat striatal membranes was considerably higher than the micromolar concentrations used in reconstituted systems [11, 17, 40]. It can be proposed that crude brain membranes contain considerable amounts of different GTP-binding proteins, only a very small number of which are coupled to dopamine receptors. Thus, the large excess of GDP is required to suppress the binding to all these proteins so as to make apparent the slight change in GDP affinity for proteins coupled with the dopamine receptors. The high concentration of GDP also slows down the association of [^{35}S]GTPγS and a longer incubation time (90 min) was therefore required in brain membranes in comparison with the 10–30 min used in reconstituted systems [11, 12, 40].

Kinetic studies of [^{35}S]GTPγS binding revealed that the acceleration was dependent on the dopamine concentration and was saturable at higher agonist concentrations (Fig. 3), indicating that we were actually monitoring receptor-mediated events. According to the general model of G-protein-coupled receptor activation [41], agonists bound to receptors increase the rate of GDP/GTP exchange in the ternary receptor–G-protein–GDP complexes. As all the components of the ternary complex are in equilibrium, the increase in dopamine (agonist) concentrations shifted the equilibrium towards higher concentrations of the ternary complexes, which caused the apparent activation of [^{35}S]GTPγS binding. Therefore, it can be concluded that the acceleration of [^{35}S]GTPγS binding is also a determination of the activated complexes of dopamine receptors with G-proteins.

The efficiency of different dopaminergic antagonists in inhibiting dopamine-induced activation of [^{35}S]GTPγS binding to rat striatal membranes was in good agreement with their binding parameters to the D_2 subtype of dopamine receptors (r^2 = 0.97, slope 0.89 ± 0.06) (Fig. 8b). Among the other subtypes of dopaminergic receptors, only D_3 can be involved in the dopaminergic regulation of [^{35}S]GTPγS binding in rat striatal membranes, as the binding parameters of this subtype

also have a reasonable correlation with obtained pK_b values ($r^2 = 0.83$, slope 0.68 ± 0.14). The involvement of other subtypes is unlikely, due to the lack of correlation with their binding parameters ($r^2 < 0.5$).

The comparison of the potencies of dopaminergic agonists to stimulate [^{35}S]GTPγS binding to rat striatal membranes with their high-affinity binding constants to different subtypes of dopamine receptors gave a good correlation for the D_2 and D_4 subtypes ($r^2 > 0.98$) (Fig. 8a). The correlation for the D_3 subtype was much lower ($r^2 = 0.6$) and this subtype can be ruled out as a possible dopaminergic receptor subtype responsible for dopamine-dependent activation of [^{35}S]GTPγS binding in rat striatal membranes. Combining the results of these two independent correlations (Fig. 8a and 8b) supports the view that activation of [^{35}S]GTPγS binding in rat striatal membranes is mediated by D_2 dopamine receptors.

It is to be noted that in spite of a very good correlation between pK_H (high-affinity binding constants) and pEC_{50} (Fig. 8a), the former constants are considerably larger than those for effects, the difference increasing with an increase in affinity (the slope of correlation was significantly different from unity: 0.52 ± 0.02, 0.56 ± 0.21, and 0.56 ± 0.06 for D_2, D_3, and D_4, respectively). This indicates that the connection between the binding of agonist to receptor and the subsequent activation of G-proteins is not rigid. It can be assumed that a minimal affinity of agonist is required to produce a physiological response. The lifetime of agonist-receptor complexes lengthens with higher affinities thereby increasing the possibility for conformational changes required for the physiological responses [42]. This is also in good agreement with the proposed operational model of pharmacological agonism [43], where the agonist has a high transducer ratio τ and $pEC_{50} \ll pK_A$, whereas τ is small for antagonists and $pEC_{50} \approx pK_A$. Of course, the values of low-affinity binding constants of agonists (pK_L) are much closer to the pEC_{50} values, but obtained correlations were much weaker (best $r^2 = 0.87$ was for D_2) and slopes remained close to 0.5.

In addition, we have taken into account that monovalent salts at concentrations above 50 mM inhibit high-affinity agonist binding to receptors as well as agonist-dependent activation of [^{35}S]GTPγS binding [44–46]. Here, we used 100 mM of NaCl in the incubation buffer, a concentration which has been found optimal to decrease the basal binding of [^{35}S]GTPγS and increase the effect/noise ratio (data not shown). Usually, ligands with higher affinity are less affected by NaCl than weaker ligands [46], and this was also found to be true here. The relatively high salt concentration can also be a reason why the pEC_{50} values from the activation of [^{35}S]GTPγS binding are much lower than the pK_H values from radioligand binding (Fig. 8a).

Comparisons of the efficiency of dopaminergic agonists to stimulate [^{35}S]GTPγS binding on the basis of their ability to activate high-affinity GTPase activity in rat striatal membranes [9] revealed a reasonable correlation ($r^2 = 0.73$, slope = 0.61 ± 0.22, Y-intercept = 2.4 ± 1.5), but one that was considerably weaker than with binding affinities of these ligands. This indicates that receptor-mediated processes can be described more precisely by the [^{35}S]GTPγS binding assay than by GTPase activity. The latter includes, besides the activation of G-protein, its deactivation as well (hydrolysis of GTP) [10], while the [^{35}S]GTPγS assay determines only the activation of G-protein. Of course, both these methods play an important role in studies of signal transduction, but it is worth noting that [^{35}S]GTPγS binding depends only on a catalytic effect of receptors on G-proteins, while assays on GTPase and adenylate cyclase activities also include second (third) catalytic steps, which can be influenced by other factors than those active in receptor–G-protein complexes.

In conclusion, a simple method for the characterization of the functional coupling of dopamine receptors with G-proteins in crude membranes of rat striatum has been demonstrated in the present study. This functional assay was successfully used for characterization of the efficiencies of different agonists as well as of antagonists to inhibit these effects. The obtained data suggest that the activation of [^{35}S]GTPγS binding in rat striatal membranes by dopamine in the presence of an excess of GDP is most likely mediated by the D_2 subtype of dopamine receptors.

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