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FUNCTIONAL COUPLING OF DOPAMINE D_2 AND MUSCARINIC CHOLINERGIC RECEPTORS TO THEIR RESPECTIVE G PROTEINS ASSESSED BY AGONIST-INDUCED ACTIVATION OF HIGH-AFFINITY GTPase ACTIVITY IN RAT STRIATAL MEMBRANES

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Abstract—Agonist-induced high-affinity GTPase activity was investigated using the crude membrane preparation from rat striatum. High-affinity GTPase activity was stimulated by dopamine and carbachol in a Mg^{2+}-dependent manner and with possible optimum NaCl concentrations of 50–100 mM to detect the percent increase induced by each agonist. Dopamine and selective (as well as non-selective) D_2 receptor agonists, but not selective D_1 receptor agonists, stimulated activity in a concentration-dependent manner, with affinities which were significantly correlated with those for adenylate cyclase inhibition as previously reported in the literature. Maximal percent stimulation above basal high-affinity GTPase activity was 9.8 ± 0.6% and 4.4–7.6% for dopamine and other synthetic dopamine D_2 receptor agonists, respectively. Dopamine-stimulated activity was inhibited by several dopamine receptor antagonists with the following rank order of potency: (+)-butaclamol > spiperone > raclopride > *S*(−)-sulpiride; but not by (−)-butaclamol or SCH 23390. High-affinity GTPase activity was also stimulated by carbachol and acetylcholine through the pirenzepine-insensitive muscarinic receptors. Preincubation of the membranes with *AS/7*, a specific antiserum to G_{i1} and G_{i2}, appeared to attenuate dopamine-sensitive activity, suggesting that G_{i1} and/or G_{i2} may be at least partially involved. These results indicate that high-affinity GTPase activity in rat striatal membranes is activated through dopamine D_2-like receptors and pirenzepine-insensitive muscarinic receptors, both of which are negatively coupled to adenylate cyclase via G_i proteins.

Key words: GTP hydrolysis; G protein; dopamine D_2 receptors; muscarinic acetylcholine receptors; adenylate cyclase; striatum

Many hormones and neurotransmitters interact with cell-surface specific receptors coupled to intracellular second messenger generating systems and ion channels via intermediary guanine nucleotide-binding regulatory (G)† proteins [1, 2]. G proteins are a family of heterotrimeric proteins composed of α, β, and γ subunits. When an agonist binds to the membrane receptor, the rate of release of GDP from the nucleotide binding site of the α subunit is enhanced and the released GDP replaced by GTP. The activated α subunit (α_{GTP}) dissociates from the $\beta\gamma$ subunits and interacts with effector molecules. The GTP bound to the α subunit is hydrolysed to GDP by the intrinsic GTPase (EC 3.6.1.–) of the α subunits, α_{GDP} then reassociating with $\beta\gamma$ subunits to end the activation cycle.

These fundamental characteristics of G proteins allow several useful methods, i.e. agonist-induced activation of GTP binding, GDP release, and GTPase activity, to evaluate the functional activation/deactivation process of G protein or receptor–G protein interactions. These techniques have been applied, in many cases, to reconstitutional systems, in which purified proteins of interest are artificially mixed with each other in phospholipid vesicles. Although many fruitful results have been obtained by virtue of reconstitution experiments on potential structure–function relationships, another series of experiments using native membrane preparations is necessary to reach conclusive consideration of the real interactions between the proteins occurring in a natural milieu. In spite of the theoretical feasibility of such experiments and the simplicity of the experimental protocol, however, it has not been necessarily easy to apply these methods to the membrane system, probably owing to the high non-specific background activity making the signal-to-noise ratio too low to be unequivocally detected [3].

DA-sensitive high-affinity GTPase activity in rat striatum was demonstrated for the first time by Onali *et al.* [4], who subsequently investigated its

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† Abbreviations: G protein, guanine nucleotide-binding regulatory protein; DA, dopamine; NPA, N-*n*-propylnorapomorphine; TED buffer, 5 mM Tris-HCl, 1 mM EDTA, 1 mM dithiothreitol, pH 7.4; $\%E_{max}$, maximal percent stimulation above basal activity; pEC_{50} and pIC_{50}, negative logarithmic value of EC_{50} and IC_{50}, respectively; CCh, carbachol; ACh, acetylcholine; IAP, islet-activating protein (pertussis toxin); 5-HT, 5-hydroxytryptamine; GABA, γ-aminobutyric acid.

pharmacological characteristics and concluded that this response was mediated by activation of the DA D_2 receptors coupled with inhibition of adenylate cyclase (EC 4.6.1.1) through G_i proteins [5]. This conclusion is, however, opposed to that drawn by Tirone *et al.* [6], who insisted on the involvement of the DA D_1 receptors associated with stimulation of adenylate cyclase via G_s in DA-dependent GTP hydrolysis.

In the present study, we developed the method of measuring DA-stimulated high-affinity GTPase activity in rat striatal membranes and tried to elucidate which receptor subtype(s) and G protein subclass(es) are responsible for the response. As muscarinic receptor-mediated high-affinity GTPase activity has been frequently reported [7–13], GTP hydrolysis stimulated by muscarinic agonists were examined and characterized as well.

MATERIALS AND METHODS

Chemicals. [γ-^{32}P]GTP (30 Ci/mmol) was purchased from New England Nuclear (Boston, MA, U.S.A.). The following drugs were donated by the indicated companies: pergolide and quinpirole hydrochloride, Eli Lilly & Sons (Indianapolis, IN, U.S.A.); bromocriptine, Sandoz Ltd (Basel, Switzerland); spiperone, Janssen Pharmaceutica (Beerse, Belgium); raclopride tartrate, Astra Alab AB (Sweden); *S*(−)-sulpiride, Ravizza (Muggiò, Italy); SCH 23390, Schering Corp. (Bloomfield, NJ, U.S.A.). (−)-NPA hydrochloride, *R*(+)-SKF 38393 hydrochloride, SKF 82958 hydrobromide, and (+)-/(−)-butaclamol hydrochloride were obtained from Research Biochemical Inc. (Natick, MA, U.S.A.). All other chemicals were from Sigma Chemical Co. (St. Louis, MO, U.S.A.).

Animals. Male Sprague–Dawley rats (Alab, Stockholm, Sweden) weighing 200–250 g were used. The animals were kept under regular lighting conditions (lights on at 06:00 hr and off at 20:00 hr) in a temperature-controlled environment and had free access to food pellets and tap water.

Membrane preparation. Rats were killed by decapitation and their brains quickly removed. The striata dissected from each rat were homogenized in 5 mL of ice-cold TED buffer (5 mM Tris-HCl, 1 mM EDTA, 1 mM dithiothreitol, pH 7.4) containing 10% (w/v) sucrose using a motor-driven Teflon/glass tissue grinder (20 strokes). After centrifugation of the homogenate at 1000 *g* for 10 min, the supernatant was kept on ice while the pellet was resuspended in 5 mL of TED/sucrose buffer followed by another centrifugation at 1000 *g* for 10 min. The combined supernatant was then centrifuged at 9000 *g* for 20 min. The pellet was resuspended in 10 mL of TED buffer and centrifuged again at 9000 *g* for 20 min. The pellet was resuspended in 10 mL of TED buffer and maintained on ice for 30 min. The suspension was centrifuged at 35,000 *g* for 10 min, resulting in the final pellet which was then resuspended in 1 mL of 50 mM Tris-HCl buffer (pH 7.4). This homogenate (1.6–3.2 mg protein/mL) was frozen on powdered dry ice and stored at −70° for no longer than 5 weeks without deterioration in GTPase activity.

Protein concentration was measured by the method of Lowry *et al.* [14] using BSA as a standard.

GTPase assay. GTPase activity was assayed, at least in duplicate, by measuring $^{32}P_i$ released from [γ-^{32}P]GTP essentially as described previously [15]. Unless otherwise indicated, the reaction mixture (final volume 100 μL) contained the following constituents: 50 mM Tris-HCl (pH 7.4), 0.3 μM GTP ([γ-^{32}P]GTP plus unlabelled GTP), 2 mM $MgCl_2$, 0.5 mM ATP, 0.5 mM adenylylimidodiphosphate, 5 mM phosphocreatine, creatine phosphokinase (50 U/mL), BSA (50 μg), 0.1 mM EDTA, 0.2 mM EGTA, 0.2 mM dithiothreitol, 0.5 mM cyclic AMP, 0.5 mM 3-isobutyl-1-methylxanthine, 100 mM NaCl, and membrane protein (4–8 μg). The striatal membranes (25 μL) and drug(s) (agonist and/or antagonist; 25 μL) were put into 1.5 mL microcentrifuge tubes (Eppendorf) placed in an ice-cold water bath. The enzyme reaction was initiated by addition of assay mixture (50 μL) containing [γ-^{32}P]GTP and other reagents and subsequent immersion of the tubes in a 30° water bath. When the tubes contained both agonist and antagonist, they were kept in ice-cold water for 60 min before initiation of the reaction. After incubation of the tubes at 30° for 15 min (except for the time course experiment), the reaction was stopped by transferring the tubes to an ice bath followed by the addition of 500 μL of 5% (w/v) activated charcoal in 20 mM phosphoric acid (pH 2.5). The tubes were kept chilled for approx. 30 min and centrifuged at 11,000 *g* for 10 min. An aliquot (200 μL) from the supernatant fraction was mixed with 5 mL of scintillation fluid and the radioactivity count measured using liquid scintillation spectrometer. The high-affinity GTPase activity was routinely calculated by subtracting the amount of $^{32}P_i$ released from [γ-^{32}P]GTP in the presence of 100 μM unlabelled GTP from the total activity.

Data analysis. All results except those of application of AS/7, a specific antiserum to G_{i1} and G_{i2}, were presented as means ± SEM. Agonist-induced stimulation of high-affinity GTPase activity was expressed as percent increase above unstimulated basal value, and the concentration–response curves were analysed by single factor repeated measures ANOVA followed by Scheffe *F*-test. Maximal percent stimulation above basal activity ($\%E_{max}$) and EC_{50} values were calculated using computer-assisted non-linear regression analysis, according to a one site model. For inhibition by antagonists, IC_{50} values were also determined by using computer-assisted non-linear regression analysis assuming a one-site model. Because of the limited number of concentrations used and the variability of values, analysis assuming two sites was not tested in either case. EC_{50} and IC_{50} values were converted to their negative logarithmic values (pEC_{50} and pIC_{50}, respectively). Correlation coefficients were calculated by the method of least squares. Statistical significance was defined as $P < 0.05$.

RESULTS

Time course and isotopic dilution experiments

The hydrolysis of [γ-^{32}P]GTP (0.1 μM) was

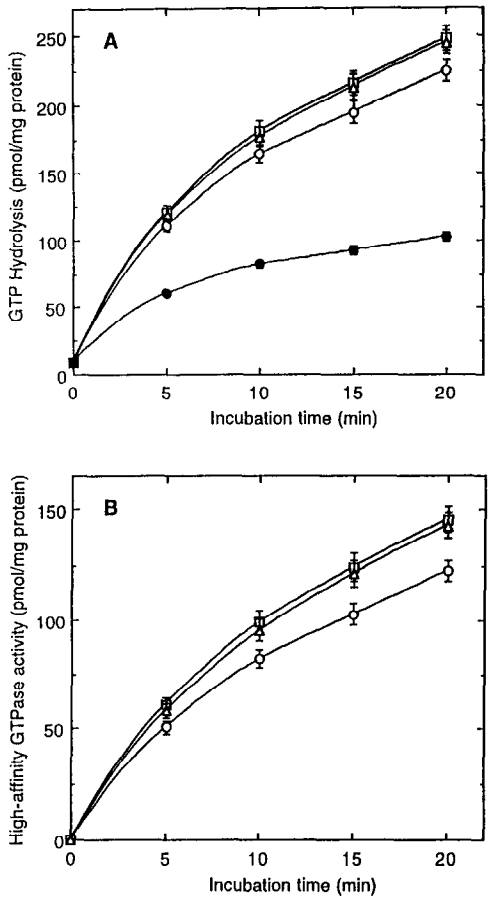

Fig. 1. Time course of GTP hydrolysis in rat striatal membranes. The amounts of $^{32}P_i$ released from 0.1 μM [γ-^{32}P]GTP were determined after different incubation times at 30°. (A) GTP hydrolysing activity was measured in the absence of agonist (○), in the presence of 1 mM DA (△) and 1 mM CCh (□), and in the presence of 50 μM unlabelled GTP (●). (B) Nonspecific activity in the presence of 50 μM unlabelled GTP was subtracted from total activity to define high-affinity GTPase activity. Basal activity in the absence of agonist (○) and agonist-stimulated activity (△, 1 mM DA; □, 1 mM CCh) are shown at different incubation times. Values are means ± SEM of five separate experiments, each determined in duplicate. DA- and CCh-stimulated activities are both significantly different from unstimulated basal activity at all time points except 0 min ($P < 0.001$ by single factor repeated measures ANOVA followed by Scheffe F-test).

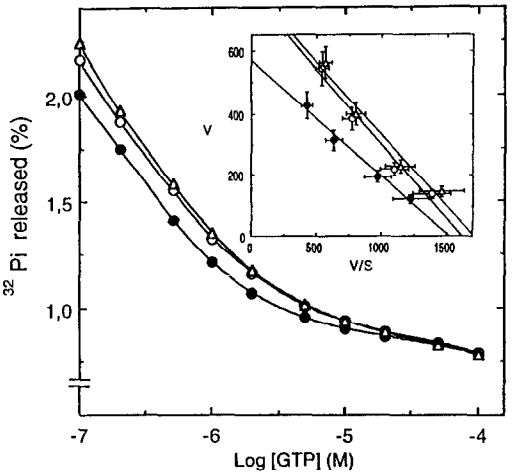

Fig. 2. Hydrolysis of [γ-^{32}P]GTP (0.1 μM) by rat striatal membranes in the presence of different concentrations of unlabelled GTP. The radioactivity of $^{32}P_i$ released from 0.1 μM [γ-^{32}P]GTP in the absence of agonist (●) and in the presence of 1 mM DA (○) and 1 mM CCh (△) is expressed as percent of radioactivity of added [γ-^{32}P]GTP per μg of membrane protein. The abscissa indicates the concentration of GTP (0.1 μM [γ-^{32}P]GTP plus unlabelled GTP). The mean values of four separate experiments are presented without SEM bars (less than 10% of the respective mean values) for the purpose of clarity. DA- and CCh-stimulated activities are significantly different from basal activity at lower concentrations of GTP ($P < 0.001$ at 0.2, 0.5, 1.0, 5.0 μM, $P < 0.01$ at 0.1, 2.0, 10 μM, $P < 0.05$ at 50 μM for DA-stimulated activity; $P < 0.001$ at 0.1, 0.2, 0.5, 1.0, 2.0, 5.0 μM, $P < 0.01$ at 10 μM for CCh-stimulated activity, single factor repeated measures ANOVA followed by Scheffe F-test). Inset: Eadie–Hofstee analysis of high-affinity GTPase activity. Enzyme activity was determined in the absence of agonist (●) and in the presence of 1 mM DA (○) and 1 mM CCh (△) at various GTP concentrations (0.1–1.0 μM). Values are means ± SEM of four separate experiments.

determined at 30° after different incubation times of 0, 5, 10, 15, and 20 min. The rate of hydrolysis decreased gradually according to the incubation time, which gave a curvilinear pattern as demonstrated in Fig. 1A. The high-affinity GTPase activity, defined as the difference between the total and nonspecific (in the presence of 50 μM unlabelled GTP) GTP hydrolysis, was also curvilinear (Fig. 1B). Despite the considerable variability in unstimulated basal high-affinity GTPase activity in experiments in which separate membranes prepared from different rats were used, both agonists stimulated the activity unexceptionally to a constant percentage extent as compared with the respective unstimulated basal activity. At an incubation time of 15 min, for instance, unstimulated basal activities varied from 92 to 121 pmol/mg protein (103 ± 5 pmol/mg protein, N = 5, Fig. 1B) depending on the experiment, whereas the percent increases above the respective unstimulated basal values by addition of DA (1 mM) and carbachol (CCh) (1 mM) were 18.3 ± 0.8% (range: 16.1–20.0%) and 21.0 ± 0.5% (range: 19.0–21.9%), respectively. The following experiments were all performed using an incubation time of 15 min. As GTP hydrolysing activity was proportional to the added membrane protein at least up to 10 μg/assay (data not shown), enzyme activity was, if necessary, expressed as pmol released $^{32}P_i$/mg protein/15 min.

Isotope dilution experiments were then performed by measuring the amounts of $^{32}P_i$ released from 0.1 μM [γ-^{32}P]GTP in the presence of increasing concentrations of unlabelled GTP (Fig. 2). The differences between agonist-stimulated and unstimulated basal activities were statistically significant at

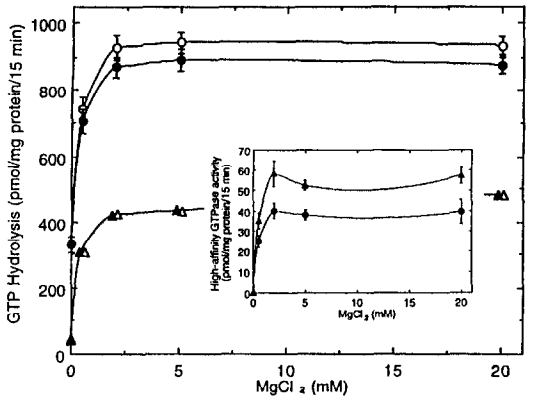

Fig. 3. Effects of $MgCl_2$ on GTP hydrolysis in rat striatal membranes. Hydrolysis of [γ-^{32}P]GTP (0.3 μM) was determined in the absence (●, ▲) and presence (○, △) of 1 mM CCh. Nonspecific, low-affinity GTP hydrolysing activity was defined in the presence of 100 μM unlabelled GTP (▲, △). Values are means ± SEM of four separate experiments, each determined in duplicate. Total activity in the presence of CCh was significantly different from unstimulated activity at $MgCl_2$ concentrations of 0.5–20 mM ($P < 0.01$ at 2 and 5 mM, $P < 0.05$ at 0.5 and 20 mM; Student's paired two-tailed t-test adjusted by the Bonferroni procedure). Results similar to those of DA-stimulated GTP hydrolysis were obtained. Inset: Effects of $MgCl_2$ on DA- and CCh-stimulated increase in high-affinity GTPase activity. Increment of high-affinity GTPase activity by addition of 1 mM DA (●) and 1 mM CCh (▲) was determined at various concentrations of $MgCl_2$. Values are means ± SEM of four separate experiments, each performed in duplicate.

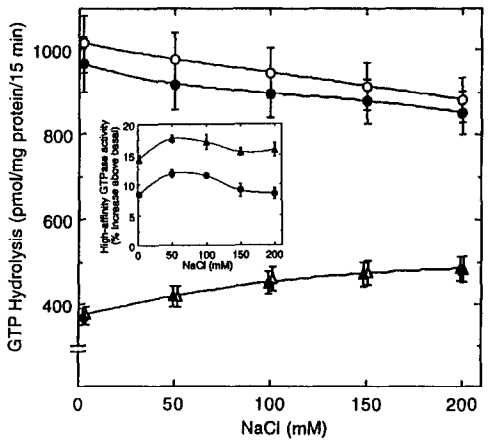

Fig. 4. Effects of NaCl on GTP hydrolysis in rat striatal membranes. Hydrolysis of [γ-^{32}P]GTP (0.3 μM) was determined in the absence (●, ▲) and presence (○, △) of 1 mM DA. Nonspecific, low-affinity GTP hydrolysing activity was defined in the presence of 100 μM unlabelled GTP (▲, △). Values are means ± SEM of six separate experiments, each determined in duplicate. Total activity in the presence of DA was significantly different from unstimulated activity at all concentrations of NaCl ($P < 0.001$ at 0, 50, 100, 200 mM, $P < 0.01$ at 150 mM; Student's paired two-tailed t-test adjusted by the Bonferroni procedure). Results similar to those of CCh-stimulated GTP hydrolysis were obtained. Inset: Percent increase in high-affinity GTPase activity by DA (●) and CCh (▲) at various concentrations of NaCl. Values are means ± SEM of six separate experiments, each performed in duplicate. The percent increase by DA at 50 and 100 mM NaCl was significantly different than the value in the absence of NaCl ($P < 0.01$ for both, Student's paired two-tailed t-test). The percent increase by CCh at 50 mM NaCl was significantly different from the value in the absence of NaCl ($P < 0.001$, Student's paired two-tailed t-test).

submicromolar concentrations of GTP, but not at concentrations higher than 10 μM, indicating that both agonists increased high-affinity GTPase activity without affecting low-affinity GTP hydrolysis. When the nonspecific low-affinity GTPase(es), defined as the activity in the presence of 100 μM unlabelled GTP, were subtracted from the total amount of released $^{32}P_i$, the high-affinity GTPase derived from G proteins could be determined. Analysis of the data using Eadie–Hofstee plots gave the apparent K_m value of 0.38 ± 0.02 μM and the V_{max} of 571 ± 54 pmol/mg protein/15 min for basal high-affinity GTPase activity (Fig. 2, inset). The V_{max} values were significantly increased by addition of 1 mM DA (means ± SEM: 781 ± 72 pmol/mg protein/15 min, $P < 0.01$) and 1 mM CCh (800 ± 58 pmol/mg protein/15 min, $P < 0.01$), with a slight increase in the apparent K_m values to 0.49 ± 0.02 and 0.48 ± 0.03 μM for DA- and CCh-stimulated activity, respectively. Negative logarithmic values of K_m were 6.42 ± 0.02 for basal, 6.32 ± 0.02 ($P < 0.01$ versus basal) for DA-stimulated, and 6.33 ± 0.03 ($P < 0.05$ versus basal) for CCh-stimulated activity.

Effects of $MgCl_2$ and NaCl on DA- and CCh-stimulated GTPase activity

In order to clarify the significance of Mg^{2+} in agonist-stimulated GTPase activity, GTP hydrolysis was determined at various concentrations of $MgCl_2$. As shown in Fig. 3, total and nonspecific GTPase activities were both augmented dramatically as the concentrations of $MgCl_2$ were increased, reaching a plateau at concentrations of 2 mM. The stimulating effects of DA and CCh became manifest only when $MgCl_2$ was present in an assay mixture in total GTP hydrolysis but not in nonspecific low-affinity activity. The increase in high-affinity GTPase activities by both agonists were null in the absence of $MgCl_2$ and maximal at and above 2 mM of $MgCl_2$ (Fig. 3, inset).

The effects of NaCl on GTP hydrolysis were then investigated. In the presence of 2 mM $MgCl_2$, addition of increasing concentrations of NaCl gradually decreased total activity but increased non-specific low-affinity components (Fig. 4), resulting in a lowering of basal high-affinity GTPase activity to 369 ± 26 pmol/mg protein/15 min ($N = 6$) at 200 mM NaCl from 595 ± 46 pmol/mg protein/15 min ($N = 6$) in the absence of NaCl. Although the stimulating effects of DA and CCh were statistically significant at all concentrations of NaCl, the percent increase by both agonists above basal high-affinity GTPase activity showed an inverted U-shape with the highest percent activation at 50–100 mM NaCl (Fig. 4, inset).

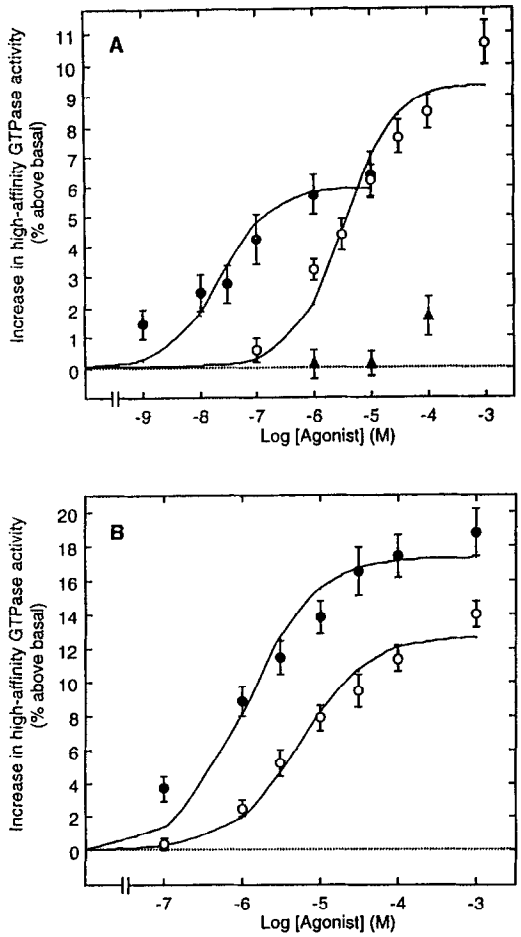

Fig. 5. Percent stimulation of high-affinity GTPase activity by several agonists in rat striatal membranes. (A) Hydrolysis of [γ-^{32}P]GTP (0.3 μM) was measured after incubation of striatal membranes for 15 min at 30° in the absence and presence of increasing concentrations of DA (○), pergolide (●), and *R*(+)-SKF 38393 (▲). Nonspecific, low-affinity GTP hydrolysing activity was defined in the presence of 100 μM unlabelled GTP and was subtracted from total activity to define high-affinity GTPase activity. Stimulation was expressed as percent increase above basal high-affinity GTPase activity. Basal high-affinity GTPase activities in the absence of agonist were 436 ± 23 (N = 17), 629 ± 60 (N = 6), and 412 ± 32 (N = 8) pmol/mg protein/15 min for DA-, pergolide-, and *R*(+)-SKF 38393-stimulation, respectively. Values are means ± SEM of the respective number of separate experiments, each performed in duplicate. (B) Percent increase in high-affinity GTPase activity was measured by increasing concentrations of CCh (○) and ACh (●). Acetylcholine-stimulated GTPase assay was performed in the presence of 10 μM eserine. Basal high-affinity GTPase activities were 406 ± 26 (N = 16) and 347 ± 29 (N = 6) pmol/mg protein/15 min for CCh- and ACh-stimulation, respectively. Values are means ± SEM of the indicated number of separate experiments, each performed in duplicate.

Effects of agonists on high-affinity GTPase activity

Figure 5A shows the concentration-response relationship as to DA-, pergolide-, and *R*(+)-SKF 38393-stimulated high-affinity GTPase activity. DA stimulated the activity in a concentration-dependent manner with a mean EC_{50} value of 3.7 μM (calculated from the mean pEC_{50} value listed in Table 1) and with $\%E_{max}$ of 9.8 ± 0.6%. In a representative experiment in which [γ-^{32}P]GTP added into an assay mixture was 889,243 cpm, the actually measured values of the released $^{32}P_i$ in an aliquot (200 μL) from the supernatant fraction were 38,269 and 19,774 cpm for total and nonspecific GTP hydrolysis activity, respectively, which resulted in 18,495 cpm for basal high-affinity GTPase activity. Radioactivity in the presence of 1 mM DA was 40,841 cpm, from which the unstimulated value (38,269 cpm) was subtracted to determine the increase by 1 mM DA. This difference (2572 cpm) was divided by the basal high-affinity GTPase activity (18,495 cpm) to determine the percent increase by 1 mM DA (13.9%). Pergolide stimulated enzyme activity with a higher affinity (mean EC_{50} = 23 nM) but with a lower efficacy (% E_{max} = 5.9 ± 0.6%) as compared with the DA-stimulated response. The selective DA D_1 agonist, *R*(+)-SKF 38393, on the other hand, did not activate high-affinity GTPase in a broad concentration range from 0.1 nM up to 100 μM.

The results from several DA-related compounds including these three reagents are summarized in Table 1. The selective D_2 receptor agonists, (−)-NPA, bromocriptine, and quinpirole, as well as the non-selective DA agonist apomorphine, stimulated high-affinity GTPase activity in a concentration-dependent manner with intrinsic activities less than unity (approximately 0.6, 0.8, 0.4, and 0.6 for (−)-NPA, bromocriptine, quinpirole, and apomorphine, respectively, as compared with DA-stimulated activity). (−)-Norepinephrine also activated high-affinity GTPase to the same extent as DA with an approximately four-fold reduced affinity (mean EC_{50} = 14 μM). In contrast with these agonists, another selective and full D_1 agonist, SKF 82958 [16], did not stimulate the activity up to high concentrations (0.1 nM–10 μM).

When the pEC_{50} values (means ± SEM) of agonists for stimulation of high-affinity GTPase activity listed in Table 1 were plotted against mean pEC_{50} values for inhibition of adenylate cyclase activity in rat striatum reported previously by Onali *et al.* [17], there was a statistically significant correlation between the two parameters ($r = 0.87$, N = 6, $P < 0.05$; Fig. 6).

High-affinity GTPase activity was also stimulated concentration-dependently by CCh and ACh as shown in Fig. 5B. The mean EC_{50} value for CCh was 6.6 μM (pEC_{50} = 5.18 ± 0.13, N = 16) and for ACh was 1.2 μM (pEC_{50} = 5.91 ± 0.13, N = 6). The $\%E_{max}$ values for CCh and ACh were 13.3 ± 0.7 and 17.3 ± 1.3%, respectively.

Effects of antagonists on DA- and CCh-stimulated high-affinity GTPase activity

The increase in high-affinity GTPase activity by a sub-maximally effective concentration (30 μM, see Fig. 5A) of DA was antagonized by a non-selective DA blocker, (+)-butaclamol with a mean IC_{50} value of 75 nM (pIC_{50} = 7.12 ± 0.10, N = 6), but not by the inactive enantiomer, (−)-butaclamol (Fig. 7A). The potent D_2 receptor antagonist, spiperone,

Table 1. Effects of several DA-related agonists on high-affinity GTPase activity in rat striatal membranes

Compound	N	pEC_{50}	Mean $EC_{50}(\mu M)$	$\%E_{max}$
(−)-NPA	6	7.64 ± 0.36	0.023	5.4 ± 0.9
Pergolide	6	7.64 ± 0.17	0.023	5.9 ± 0.6
Bromocriptine	6	6.47 ± 0.05	0.34	7.6 ± 0.5
Apomorphine	6	6.28 ± 0.36	0.52	6.1 ± 0.9
Quinpirole	6	6.09 ± 0.24	0.81	4.4 ± 0.7
Dopamine	17	5.43 ± 0.13	3.7	9.8 ± 0.6
(−)-Norepinephrine	10	4.85 ± 0.15	14.1	10.3 ± 0.6
R(+)-SKF 38393	8	—	—	Inactive up to 100 μM
SKF 82958	8	—	—	Inactive up to 10 μM

Striatal membranes were incubated with 0.3 μM [γ-^{32}P]GTP for 15 min at 30° in the absence and presence of increasing concentrations of agonists as described in the Materials and Methods section. The concentration response curves were analysed by single factor repeated measures ANOVA followed by Scheffe *F*-test.

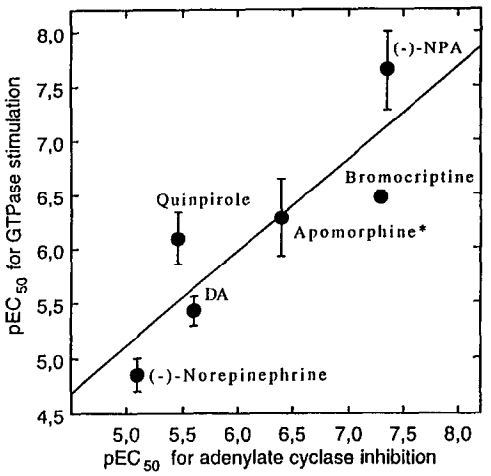

Fig. 6. Relationship between the pEC_{50} values (means ± SEM) of DA-related agonists for stimulation of high-affinity GTPase activity (Table 1) and their pEC_{50} values for inhibition of adenylate cyclase activity, which were converted from the mean EC_{50} values reported previously by Onali *et al.* [17]. The correlation coefficient (*r*) of the fit of the line to the data was 0.87 ($P < 0.05$), with a slope of 0.85. *Apomorphine was used in racemic form in the present study whereas (−)-apomorphine was used in the investigation reported by Onali *et al.* [17].

inhibited DA (30 μM)-sensitive stimulation with an IC_{50} value of 110 nM ($pIC_{50} = 6.98 \pm 0.39$, N = 11; Fig. 7B). Raclopride and *S*(−)-sulpiride, both selective D_2 receptor blockers, also antagonized DA-stimulated activity with IC_{50} values of 1.2 μM ($pIC_{50} = 5.92 \pm 0.19$, N = 7) and 16 μM ($pIC_{50} = 4.79 \pm 0.17$, N = 7), respectively (Fig. 7B). In contrast, SCH 23390, a selective D_1 antagonist, failed to inhibit DA-stimulated activity at least up to 10 μM (Fig. 7B).

Activation of high-affinity GTPase by 30 μM CCh was antagonized by atropine and pirenzepine as shown in Fig. 8. The potency of pirenzepine to inhibit CCh-stimulated GTPase activity (mean IC_{50} = 6.8 μM, $pIC_{50} = 5.17 \pm 0.14$, N = 6) was, however, 260-fold less than that of atropine (mean IC_{50} = 26 nM, $pIC_{50} = 7.59 \pm 0.13$, N = 6).

Trials for identification of the G proteins involved

In order to clarify whether the G proteins involved in DA- and CCh-stimulated GTPase activity were sensitive to pertussis toxin (islet-activating protein, IAP), the membranes were preincubated with IAP (preactivated by incubation for 30 min at 30° with 5 mM ATP and 25 mM dithiothreitol) for ADP-ribosylation before GTPase assay. In spite of several experimental conditions applied to IAP-catalysed ADP-ribosylation (e.g. incubation time, 30 or 60 min; IAP concentration, ~10 μg/mL; β-nicotineamidodinucleotide concentration, 100 μM–4 mM; GTP concentration, 0 or 100 μM), we could not obtain any indication that agonist-induced activation of high-affinity GTPase was IAP-sensitive in any experiment with the two agonists (data not shown).

The specific antiserum to the C-terminal part of the α subunits of G_{i1} and G_{i2}, AS/7 [18], did not seem to inhibit the stimulation of high-affinity GTPase activity by 1 mM DA, at least in a 1:100 dilution (data not shown). When the membranes were incubated with AS/7 for 60 min at 30° at a 1:10 dilution, however, the stimulating effect of 10 μM DA on high-affinity GTPase activity was attenuated as compared with the respective response in the membranes preincubated under the same conditions without the antiserum (percent increase above basal high-affinity GTPase activity: 6.2 to 2.9% and 10.8 to 5.7%, Fig. 9). The normal nonimmunized rabbit serum, on the other hand, failed to attenuate the percent increase in the activity by 10 μM DA at a 1:10 dilution (5.2 ± 1.2 to 4.3 ± 1.5%, N = 4, $P > 0.05$, Fig. 9). Unstimulated basal high-affinity GTPase activity was markedly reduced by the incubation of the membranes with AS/7 as well as with normal serum (625 to 219 pmol/mg protein/15 min, and 589 to 210 pmol/mg protein/15 min, in the membranes preincubated without and with AS/7; 434 ± 37 to 365 ± 33 pmol/mg protein/

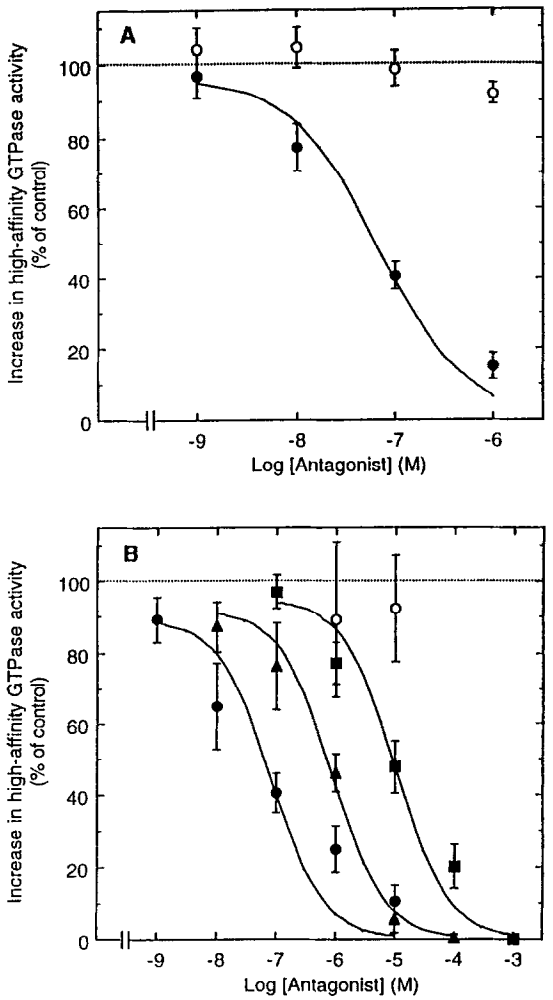

Fig. 7. Inhibition of DA-stimulated high-affinity GTPase activity by antagonists. (A) The increase in high-affinity GTPase activity by 30 μM DA was determined in the absence and presence of increasing concentrations of (+)-butaclamol (●) and (−)-butaclamol (○). Values are means ± SEM of six experiments for both antagonists. The increase in enzyme activity by 30 μM DA in the absence of antagonist is considered as 100%, and the values in the presence of the indicated concentrations of antagonists are presented as percentages. (B) The increase in high-affinity GTPase activity by 30 μM DA was determined in the absence and presence of increasing concentrations of spiperone (●), raclopride (▲), *S*(−)-sulpiride (■), and SCH 23390 (○). Values are means ± SEM of 11 experiments for spiperone and seven experiments for the other antagonists.

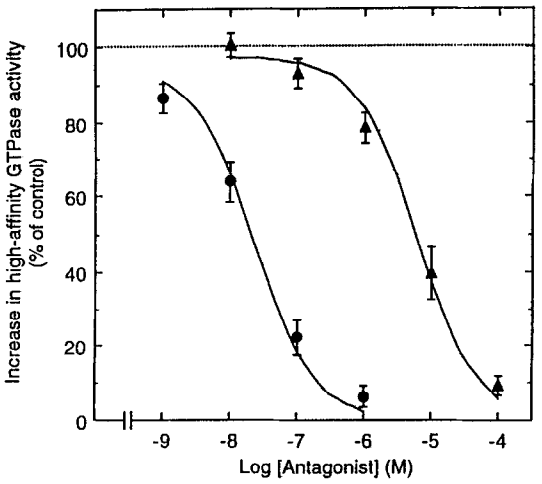

Fig. 8. Inhibition of CCh-stimulated high-affinity GTPase activity by atropine (●) and pirenzepine (▲). The increase in enzyme activity by 30 μM CCh was determined in the absence and presence of increasing concentrations of antagonists. Values are means ± SEM of six experiments for both antagonists. The increase in activity by 30 μM CCh in the absence of antagonist is considered as 100%, and the values in the presence of the indicated concentrations of antagonists are presented as percentages.

15 min, in the membranes preincubated without and with normal rabbit serum, N = 4, $P < 0.01$).

DISCUSSION

In the present study, we established a method for detecting the agonist-induced stimulation of high-affinity GTPase activity using crude membrane preparation from rat striatum. The pharmacological profile of this response has also been investigated, especially the characterization of the receptor subtype involved in DA-induced activation. The time course experiments showed a curvilinear pattern, which did not allow us to present the absolute GTP hydrolysing activity as pmol/mg protein/min. This may be due to excessive consumption of the substrate during incubation, though usually no more than 15–20% of the added [γ-^{32}P]GTP was hydrolysed after incubation for 15 min at 30°. Such a curvilinear time-dependent pattern has sometimes been demonstrated by other investigators [12, 19, 20]. In spite of the curvilinear time course, the percent increase in high-affinity GTPase by 1 mM DA or 1 mM CCh appeared constant at all time points investigated. The activating effects of agonists were then expressed routinely as percent increase above basal high-affinity GTPase activity at an indicated concentration.

Isotopic dilution experiments indicate the existence of nonspecific low-affinity GTPase(s) and high-affinity GTPase activity with a K_m value lower than 1 μM. Agonists such as DA and CCh stimulated high-affinity GTPase activity, which is probably intrinsic to G proteins functionally coupled to the respective cell-surface receptors. Eadie–Hofstee analysis indicated that both agonists significantly increased V_{max} values with slight, but significant increases in apparent K_m values. Although an activation of the receptor may theoretically lead to an increase only in the V_{max} value without any alteration in K_m, a slight increase in apparent K_m values due to the addition of an agonist was also noticed in our previous experiments on epinephrine-stimulated GTPase activity in human blood platelet

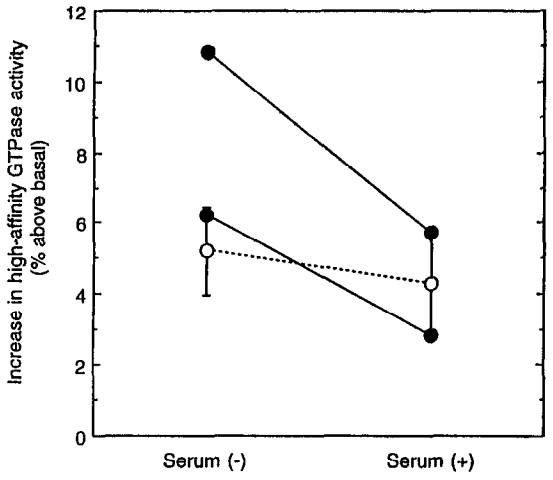

Fig. 9. Effect of AS/7 (a specific antiserum to G_{i1} and G_{i2}) and normal rabbit serum on DA-stimulated high-affinity GTPase activity. Thawed striatal membranes were preincubated for 60 min at 30° without and with rabbit serum (AS/7 (●) or normal nonimmunized rabbit serum (○)) in a volume of 40 μL. After preincubation, the tubes were transferred into an ice-cold water bath and 10 μL of water or DA (final concentration = 10 μM) and 50 μL of assay mixture containing [γ-^{32}P]GTP were added. GTP hydrolysing activity was assayed according to the routine protocol as described in the Materials and Methods section. Rabbit serum was added in a final dilution of 1:10. Non-specific, low-affinity GTP hydrolysing activity was defined in the presence of 100 μM unlabelled GTP and subtracted from total activity. Values are presented as percent increase above basal high-affinity GTPase activity by 10 μM DA. Experiments on the effect of AS/7 were performed twice using different membrane preparations. The values were 6.2 and 2.9% in one experiment, and 10.8 and 5.7% in another for the membranes preincubated without and with AS/7, respectively. In the case of normal rabbit serum, the values represent means ± SEM of four experiments.

membranes [21]. The same phenomenon, or at least a tendency, has sometimes been observed in other investigators' reports on agonist-induced high-affinity GTPase activity [7, 11, 19, 22–25].

GTP hydrolysing activity was affected dramatically by the concentrations of $MgCl_2$ in an assay mixture and neither DA- nor CCh-induced activation of high-affinity GTPase was detected in the absence of $MgCl_2$. This absolute requirement of Mg^{2+} for a detection of the stimulating effects of both agonists supports the notion that the agonist-induced stimulation of high-affinity GTPase derives from the receptor-coupled functionally relevant G proteins, since it is known that the activation process of G proteins involved in the transmembrane signal transduction is strictly dependent on Mg^{2+} [1, 2].

In contrast with the marked effects of Mg^{2+}, NaCl affected enzyme activity to a limited extent. Increasing concentrations of NaCl in an assay medium led to a gradual decrease in high-affinity GTPase activity, with optimum NaCl concentrations of 50–100 mM for detecting percent increases in the two agonist-sensitive activities. This result is essentially the same as seen in CCh-stimulated GTPase activity in porcine atrial membranes [26], although more profound effects of NaCl were reported with optimum concentrations of 100–150 mM in this investigation. The sensitivity of agonist-induced GTPase activity to NaCl appears to vary depending on the receptors and G proteins involved in transmembrane signalling, since opioid receptor-mediated high-affinity GTPase activity has been shown to be absolutely dependent on the presence of NaCl [6, 22, 27, 28].

The agonist-induced increase in high-affinity GTPase activity in the present study was not remarkable as compared with high basal activity. The percent increase above unstimulated basal activity by maximally effective concentrations of CCh was at most 10–20%, and usually even lower by DA. However, this is to be expected when considering that brain synaptic membranes contain abundant background GTP hydrolysing activity (originating from many subtypes of G proteins as well as non-G protein components) and that stimulation of one receptor type should lead to an activation of a limited pool of G proteins which could be functionally coupled with the receptor. The percent increase in high-affinity GTPase activity by an agonist at maximally effective concentrations varies to a great extent according to experiments, probably depending on the method of membrane preparation, the receptor and G protein subtypes involved, the experimental condition applied, the brain region examined, etc. For example, it was reported that DA at 100 μM augmented high-affinity GTPase activity by 30–40% [4], referred to the GTP hydrolysis of the IAP-sensitive G proteins coupled with DA D_2 receptors [5]. This augmentation, far higher than the value obtained in the present study, probably derived from the more purified synaptic membranes used in these experiments [4, 5], which necessitated more devised and complicated methods of membrane preparation. In the experiments where habitual crude membrane preparation has been used, the percent increase by an agonist has been relatively small, and this is why few researchers have successfully reported agonist-induced high-affinity GTPase activity in brain membranes except for muscarinic cholinergic receptor-mediated stimulation [7–13]. In fact, Treisman *et al.* [29] mentioned that 100 μM DA stimulated GTPase activity by 10–20%, which was not always statistically significant and led them to abandon further characterization of the response. Although the percent increase by both agonists in our assay system did not exceed the value reported by Treisman *et al.* [29], we were able to detect DA- and CCh-stimulated activity constantly and reliably, allowing us to further characterize the response pharmacologically.

The DA receptors have been subdivided into five classes, namely D_1–D_5, by utilizing molecular cloning and sequencing techniques [30]. From a pharmacological and physiological point of view, however, the dichotomy of DA receptors originally defined by Kebabian and Calne [31] is still helpful and has thus been retained. The pharmacological profile of DA-sensitive high-affinity GTPase activity in striatal membranes in the present study

demonstrates clearly that this response is mediated by the activation of pharmacologically defined D_2-like, but not D_1-like, receptors. Thus, selective D_2 receptor agonists as well as nonselective agonists including DA stimulated the activity in a concentration-dependent manner and with apparent EC_{50} values compatible with previously reported affinities for D_2-mediated responses, whereas the selective D_1 agonists *R*(+)-SKF 38393 and SKF 82958 had no effect up to high concentrations (~10 or 100 μM). Stimulation by 30 μM DA was potently antagonized by (+)-butaclamol, but not by (−)-butaclamol, an inactive enantiomer, indicating the stereospecificity of the response. Furthermore, DA antagonists including selective D_2 blocking agents such as raclopride and *S*(−)-sulpiride inhibited the response with a rank order of potency demonstrating the involvement of the D_2 receptor subtype. On the contrary, the selective D_1 receptor antagonist, SCH 23390, did not block the response even at a high concentration of 10 μM. In addition, there was a significant correlation between the affinities of agonists for stimulation of high-affinity GTPase activity and those for inhibition of adenylate cyclase activity previously reported in rat striatal membranes [17], suggesting that the D_2 receptors involved in both biological responses are the same and that DA-sensitive GTPase activity is functionally relevant for adenylate cyclase inhibition.

The intrinsic activities of synthetic DA agonists less than unity as compared with the DA's effect raise the possibility that receptor(s) other than DA D_2 receptors may be involved in DA-stimulated high-affinity GTPase activity. In fact, DA has been shown to stimulate high-affinity GTPase activity in hippocampal membranes via 5-HT_{1A} receptors, but not via dopaminergic receptors (submitted). However, the possibility of an involvement of 5-HT_{1A} receptors in DA-stimulated high-affinity GTPase activity in the present study can be excluded, since 5-HT itself failed to stimulate the activity in the striatal membranes [32]. Although (−)-norepinephrine stimulated enzyme activity, this response was shown to be mediated by the same DA D_2 receptors as DA-sensitive activity, and not by any adrenergic receptor subtype (submitted). Of course, it could not be completely ruled out that part of the DA-sensitive high-affinity GTPase activity in our experimental system derives from activation of receptor(s) other than the D_2-like receptors, either non-dopaminergic or dopaminergic which has(have) not been recognized or defined as yet. The most likely explanation is, however, that all of these synthetic, artificial DA agonists have characteristics as partial agonists, at least in the experimental system detecting agonist-induced high-affinity GTP hydrolysing activity.

Unfortunately, it has not been possible to characterize in detail the pharmacological profile of the muscarinic receptors involved in ACh- and CCh-stimulated high-affinity GTPase activity, since highly selective agents for each muscarinic receptor subtype were not available and since such a pharmacological characterization of muscarinic receptor subtype(s) was not the main purpose of the present investigation. It was, however, evident that pirenzepine-sensitive receptor subtype(s) was(were) not involved, since very high concentrations of pirenzepine were necessary to inhibit the CCh-stimulated response (mean IC_{50} = 6.8 μM) as compared to the inhibition curve of atropine (mean IC_{50} = 26 nM). Muscarinic receptors have been divided into four subtypes (M_1–M_4) using conventional pharmacological techniques and into five subclasses (m1–m5) based on molecular cloning experiments [33]. As pirenzepine is known to be a potent antagonist for M_1 and also, although less so, for M_4 receptors, the involvement of these two receptors appears to be excluded. It is also known that M_1 and M_3 receptors are coupled to phosphoinositide breakdown, whereas M_2 and M_4 receptors are coupled negatively to adenylate cyclase [33]. In our assay system, we were able to detect agonist-induced high-affinity GTPase activity mediated via DA D_2 receptors (shown in the present study and discussed above) and $GABA_B$ receptors (submitted) in the striatal membranes and via 5-HT_{1A} [32], $GABA_B$, and A_1 adenosine receptors in the hippocampal membranes (submitted). All these receptor subtypes have been shown to be coupled negatively to adenylate cyclase [34–36], suggesting that the assay systems adapted by us are preferential for the detection of receptor-G protein coupling associated with adenylate cyclase inhibition. Taken together, this indicates that the M_2 receptor is the most likely subtype involved in the CCh-stimulated high-affinity GTPase activity shown in the present study. Ghodsi-Hovsepian *et al.* [10] also suggested the involvement of M_2 receptors in the oxotremorine-stimulated GTPase response in the rat cerebral cortex. However, if the inhibition of cyclic AMP levels in the striatum is not mediated via M_2 receptors as reported by McKinney *et al.* [37], the possible contribution of the M_4 receptor subtype should also be considered. A further detailed pharmacological characterization using a series of compounds including *para*-fluoro-hexahydrosiladifenidol, AF-DX 116, 4-diphenyl acetoxymethyl piperidine methiodide, and methoctramine [33] is necessary to define the precise receptor subtype(s) involved in muscarinic receptor-mediated high-affinity GTPase activity.

The inhibition of adenylate cyclase activity is mediated by the activation of IAP-sensitive G proteins, in particular G_i [1, 2]. Thus, it was expected that ADP-ribosylation of the membranes catalysed by IAP would abolish the agonist-induced response in the present study, postulated to be associated with adenylate cyclase inhibition as discussed previously. We could not, however, demonstrate IAP-sensitivity in spite of different experimental conditions for the IAP-catalysed ADP-ribosylation. This negative result may be related to the difficulty of obtaining a complete *in vitro* ADP-ribosylation of all $G_{i/o}$ proteins abundantly contained in brain membranes. Onali and Olianas [5] showed that DA-sensitive high-affinity GTPase activity could be partly inhibited by the intrastriatal *in vivo* injection of IAP. There is no definite evidence as to which G protein subtype(s) is(are) responsible for receptor-mediated adenylate cyclase inhibition in native brain membranes. Reconstitution studies using crude D_2 receptors partially purified from porcine striatum

[38] and muscarinic receptors from porcine brain [39–41] have shown that both receptors are able to interact with G_o as well as G_i. On the other hand, Senogles *et al.* [42] demonstrated that the D_2 receptors purified from bovine anterior pituitary showed their most preferential interaction with G_{i2} followed by that with G_{i1} and G_{i3}, but did not couple with G_o. In the present study, AS/7, a specific antiserum to the C-terminal part of the α subunits of G_{i1} and G_{i2}, appeared to inhibit DA-sensitive high-affinity GTPase activity as compared with the effect of nonimmunized serum, although the experiments could be done only twice because of a 1:10 dilution necessary for the inhibition. In addition, the inhibiting effect of AS/7 was partial and incomplete in spite of the high concentration of AS/7 applied. The difficulty in employing antisera against G proteins to reveal *in situ* interactions between opioid receptors and G proteins has also been demonstrated in brain cortical membranes [43]. It has been reported that DA D_2 receptors are coupled not only to the adenylate cyclase inhibition system but also to other signalling systems such as phosphoinositide hydrolysis and ion channels. Lledo *et al.* [44] revealed that $G_o\alpha$ and $G_{i3}\alpha$ subunits were responsible for reduced calcium currents and increased potassium currents, respectively, both of which were mediated through DA D_2 receptors in the anterior pituitary cells. Given these results and the possibility that DA-stimulated high-affinity GTPase activity might derive partly from some undefined receptors other than DA D_2 receptors as discussed above, it is possible that other G proteins than G_{i1} and G_{i2} (e.g. G_o and G_{i3}) may also be involved in the DA-sensitive high-affinity GTPase activity noted in the present study. Although this notion should be verified in future experiments, the study using AS/7 suggests that G_{i1} and/or G_{i2} are responsible, at least partially, for the D_2 receptor-mediated high-affinity GTPase activation observed in our assay system.

In conclusion, the present study has demonstrated a simple but useful method for investigating functional receptor-G protein coupling in crude membranes prepared from rat brain. In the striatum, DA and ACh activate high-affinity GTPase activity of G proteins, in a Mg^{2+}-dependent manner, through DA D_2-like receptors and pirenzepine-insensitive muscarinic receptors, respectively. As both responses are supposed to be associated with the inhibition of adenylate cyclase activity via G_i proteins, this method may effectively measure the receptor-mediated G_i function in native membranes and may also be applied to other signal transduction systems in which G_i proteins have been known to play an important role in transducing information at the receptors (e.g. α_2-adrenergic, 5-HT_{1A}, $GABA_B$, and A_1 adenosine receptors) to the inhibition of adenylate cyclase activity.

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