0026-895X/95/060988-07$3.00/0

MOLECULAR PHARMACOLOGY, 48:988–994 (1995).

Evidence for the Coupling of G_q Protein to D_1-Like Dopamine Sites in Rat Striatum: Possible Role in Dopamine-Mediated Inositol Phosphate Formation

HOAU-YAN WANG, ASHIWEL S. UNDIE[1] and EITAN FRIEDMAN

Division of Molecular Pharmacology, Departments of Pharmacology and Psychiatry, The Medical College of Pennsylvania and Hahnemann University, Philadelphia, Pennsylvania 19129

Received June 14, 1995; Accepted August 30, 1995

SUMMARY

The role of G proteins in mediating the coupling of D_1 dopamine receptors to inositol phosphate formation was investigated in rat brain striatum. Pertussis toxin-activated ADP-ribosylation (≥95%) did not affect the ability of the D_1 agonist SKF38393 to stimulate the generation of inositol phosphates in striatal slices. Stimulation of striatal membranes with dopamine in the presence of [^{35}S]GTPγS or [α-^{32}P]GTP increased guanine nucleotide binding to $G_{\alpha s}$, $G_{\alpha i}$, and $G_{\alpha q}$ in a concentration-dependent fashion. The activation of $G_{\alpha s}$ and $G_{\alpha q}$ was mimicked by the D_1 agonist SKF38393 and blocked by the D_1 antagonist SCH23390. In contrast, the $D_{2/3}$ dopamine receptor agonist quinpirole stimulated guanine nucleotide binding to $G_{\alpha i}$, and dopamine-stimulated activation of $G_{\alpha i}$ was attenuated by the D_2 antagonist l-sulpiride. Furthermore, antisera directed against $G_{\alpha s}$ or $G_{\alpha q}$ but not $G_{\alpha i}$, $G_{\alpha o}$, or $G_{\alpha z}$ precipitated specific D_1-like binding sites labeled with [^{3}H]SCH23390. The D_1-like receptors that coprecipitated with $G_{\alpha s}$- but not with $G_{\alpha q}$ can be recognized by a specific D_1 dopamine receptor antibody. The data provide evidence to suggest that in addition to coupling to G_s/adenylyl cyclase, D_1-like dopamine sites that couple to G_q may mediate dopamine-stimulated formation of inositol phosphates in the rat striatum.

The coupling of dopamine D_1 receptors to diverse signal transduction pathways is supported by many functional and neurochemical studies (1–5). The dopamine D_1 receptor is a member of the G protein-coupled receptor superfamily and thus is linked to its effector systems via G proteins. Stimulation of G protein-coupled receptors results in the activation of specific heterotrimeric G proteins, leading to the exchange of GTP for GDP on the α subunit and dissociation of the trimer. Both βγ heterodimer and the activated α subunit ($G_{\alpha\text{-GTP}}$) can subsequently interact with intracellular effectors (6–9). The D_1 dopamine receptor-mediated stimulation of adenylyl cyclase is regulated by G_s proteins (10, 11). Recent investigations have demonstrated the involvement of a D_1-like receptor in mediating dopamine-stimulated phosphoinositide hydrolysis in brain tissue (12–16). Evidence showing differential order of potencies and efficacies for a series of benzazepine compounds in stimulating phosphoinositide metabolism and in activating adenylyl cyclase suggested that the D_1 receptor that is linked to PLC differs from that coupled to adenylyl cyclase (17). Other G protein-coupled receptors were shown to regulate PLC via $G_{\alpha q/11}$ proteins or the βγ dimer (9, 18–20). However, the nature of the G protein that couples the D_1-like dopamine receptor to PLC has not been previously characterized. The data presented in this report demonstrate that D_1-like dopamine receptors in rat striatal membranes couple to both $G_{\alpha s}$ and $G_{\alpha q}$ proteins. The results also show that $G_{\alpha s}$ couples to the D_1 dopamine receptor and suggest that a D_1-like site that couples to G_q may mediate dopamine-stimulated inositol phosphate formation in striatum.

Materials and Methods

Animals. Male Sprague-Dawley rats (275–450 g) purchased from Zivic-Miller (Zelienople, PA) were housed in groups and maintained at an ambient temperature of 22 ± 1°C with a 12-hr photoperiod with the light period beginning at 6:00 a.m. All rats had ad libitum access to rat chow pellets (Agway Pellets 3000, Agway, Syracuse,

[1]Current affiliation: Program in Pharmacology, Department of Pharmaceutical Sciences, University of Maryland School of Pharmacy, Baltimore, MD 21201-1180.

This work was supported by National Institutes of Health Grants NS29514 and DA06871.

ABBREVIATIONS: HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; KRB, Krebs-Ringers solution; TBS, phosphate-buffered saline/Tween 20; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; ANOVA, analysis of variance; PLC, phospholipase C; PTX, pertussis toxin (islet-activating protein); PBS, phosphate-buffered saline; *S*-(−)-sulpiride, (−)5-aminosulfonyl)-*N*-[(1-ethyl-2-pyrrolidinyl)methyl]2-methoxybenzamide; *cis*-(*Z*)-flupenthixol dihydrochloride, (*Z*)-4-[3-[2-(trifluormethyl)-9*H*-thioxanthen-9-ylidene]propyl]-1-piperazine-ethanol dihydrochloride.

NY) and tap water. The animals were habituated to this environment for at least 1 week before experiments were conducted.

PTX treatment and measurement of inositol phosphates in brain striatal slices. Brains were removed immediately after decapitation and placed in HEPES bicarbonate buffer composed of (in mM) NaCl 122, $MgCl_2$ 1.2, KCl 4.9, KH_2PO_4 1.2, $CaCl_2$ 1.3, $NaHCO_3$ 3.6, HEPES 30, and glucose 10 and bubbled for 30 min with 95% O_2/5% CO_2, with pH adjusted to 7.4 at 37°. The striata were dissected, and 350 × 350-μm slices prepared as previously described (12). Slices were washed several times, preincubated in a shaking water bath at 37° for 30 min, washed again, and divided into two portions, with each containing four volumes of fresh buffer. To one portion we added 500 μg/ml PTX, and to the second portion we added an equal volume of the PTX solvent (500 μl of 0.01 M $NaPO_4$ buffer, pH 7.0, with 0.05 M NaCl). The tubes were incubated in a shaking water bath at 37° and aerated every 30 min with 95% O_2/5% CO_2 for up to 3 hr. Each portion of slices was distributed in 25-μl aliquots (200–250 μg of protein) into 5-ml polypropylene culture tubes containing 150 μl of buffer. The remainder of each set of slices was used to assess *in vitro* PTX catalyzed ADP-ribosylation. The distributed slices were incubated in a shaking water bath at 37°. After 10 min, 25 μl of 9 μM 2-[^{3}H]inositol (17 Ci/mmol, American Radiolabeled Chemicals, St. Louis, MO) was added to each tube, and incubations continued for 30 min. At this point, 5 mM LiCl was added, followed 5 min later by the addition of SKF38393. The final incubation volume was adjusted to 250 μl by the addition of buffer. Incubation was continued for an additional 60 min or as indicated, and the reaction was stopped by mixing the slices with 1 ml of chloroform/methanol/1 M HCl (100:200:1). Inositol phosphates were extracted, separated, and quantified as previously described (12).

ADP-ribosylation with [α-^{32}P]NAD. To determine the magnitude of PTX-catalyzed ADP ribosylation, membranes were prepared from striatal slices previously incubated with buffer or 500 μg/ml of PTX for 3 hr. The *in vitro* ADP-ribosylation was carried out according to a modification of a method previously described by Gill and Woolkalis (21). Brain slices were homogenized in 10 volumes of TMES buffer containing 50 mM Tris·HCl, pH 7.6, 1 mM EDTA, 2 mM $MgCl_2$, 0.01 unit/ml soybean trypsin inhibitor, and 10% sucrose and centrifuged at 1000 × *g* for 5 min. The supernatant was saved, and the pellet was resuspended in 5 volumes of TMES. After centrifugation at 1000 × *g* for 5 min, the supernatant was combined with the first supernatant and centrifuged at 25,000 × *g* for 10 min. The resultant pellet was resuspended in HNT buffer (10 mM HEPES, pH 7.3, 130 mM NaCl, 0.01 unit/ml soybean trypsin inhibitor, and 0.2% Triton X-100), and 1-ml aliquots were frozen and stored at −70°. At the time of the assay, an aliquot was thawed, and the protein concentration was determined according to the method of Lowry *et al.* (22). Fifty micrograms of membrane protein were incubated in a total volume of 10 μl at 25° for 10 min. Ten microliters of [^{32}P]NAD mixture and 2 μl of PTX were then added (final concentration in each assay in total of 22 μl containing 10 mM HEPES, pH 7.3, 130 mM NaCl, 0.01 unit/ml soybean trypsin inhibitor, 10 mM thymidine, 10 mM dithiothreitol, 20 mM isonicotinic acid hydrazide, 1 mM 3-acetylpyridine adenine dinucleotide, 20 μM [^{32}P] NAD [10,000 cpm/pmol], 200 μg/ml activated PTX, 0.1% Triton X-100, and 1 mM ATP). Incubation was carried out at 25° for 60 min. The reaction was stopped by the addition of 0.1 ml of ice-cold HEPES buffer, pH 7.3, containing 130 mM NaCl (HN buffer) followed by centrifugation at 16,000 × *g* for 20 min. To separate $G_{\alpha i}$ from $G_{\alpha o}$ in PTX-treated samples, the pellets were resuspended and heated at 75° for 5 min in 15 μl of buffer containing 40 mM Tris·HCl, pH 6.8, 1 mM dithiothreitol, and 2% SDS. Ten microliters of 100 mM *N*-ethylmaleimide were then added, and reaction was performed at 25° for 15 min followed by 2 min of boiling. The samples were solubilized by boiling for 5 min in sample preparation buffer containing 62.5 mM Tris·HCl, pH 6.8, 10% glycerol, 2% SDS, 5% 2-mercaptoethanol, and 0.1% bromphenol blue and subjected to SDS-PAGE. The Coomassie blue-stained gels were dried and subjected to autoradiography. ADP-ribosylation was quantified by densitometric scanning of the autoradiograms (Zeineh Soft Laser Scanning Densitometer, Fullerton, CA).

[^{35}S]GTPγS and [α-^{32}P]GTP binding to G proteins and immunoprecipitation. Crude membrane preparations were obtained from rat brain striata, and GTP binding studies were performed by a previously described procedure (23). All procedures were carried out at 4° unless otherwise indicated. Brain striatal tissues were homogenized in 10 volumes of ice-cold homogenization buffer containing 25 mM HEPES, pH 7.4, 100 mM sucrose, 1 mM EGTA, 0.2% 2-mercaptoethanol, 50 μg/ml leupeptin, 25 μg/ml pepstatin A, 0.01 unit/ml soybean trypsin inhibitor, and 0.04 mM phenylmethylsulfonyl fluoride using glass/glass homogenizer. The tissue homogenate was centrifuged for 5 min at 750 × *g* (4°). The resultant supernatant was then centrifuged for 10 min at 48,200 × *g* (4°). The pellet was resuspended in 10 volumes of KRB containing 20 mM HEPES, pH 7.4, 154 mM NaCl, 4.8 mM KCl, 1.2 mM $KHPO_4$, 1.2 mM $MgCl_2$, 0.5 μg/ml leupeptin, 25 μg/ml pepstatin A, 0.01 unit/ml soybean trypsin inhibitor, and 0.04 mM phenylmethylsulfonyl fluoride and used as the crude membrane preparation. Protein concentrations were determined according to the method of Lowry *et al.* (22). The assay mixture (250 μl) containing 200 μg of membrane protein and 2 nM [^{35}S]GTPγS was incubated for 5 min at 25° followed by incubation in the absence or presence of agonist for 5 min. In the experiments in which receptor antagonists were applied, membranes were incubated with antagonists for 10 min before exposure of [^{35}S]GTPγS (15 min before exposure to the agonist). The reaction was terminated by the addition of 750 μl ice-cold KRB containing 1 mM EGTA followed by centrifugation at 16,000 × *g* for 5 min. The pellet was solubilized and immunoprecipitated with antisera directed against specific G_α peptides as described previously (23). The resulting pellet containing the antigen/antibody complex was resuspended in KRB by brief sonication, and radioactivity was measured by liquid scintillation spectrometry. The radioactivity precipitated by normal rabbit serum was considered background and subtracted from all agonist-stimulated values.

In a separate experiment, membranes (200 μg) were incubated with KRB containing 5 nM GTP for 2 min followed by an additional incubation with 50 nM of [α-^{32}P]GTP (20 μCi) for 3 min in the absence (control) or presence of 10 μM of dopamine (total incubation volume, 250 μl). The reaction was terminated by dilution with 750 μl of ice-cold Mg^{2+}-free, KRB containing 1 mM EGTA, mixed, placed on ice, and immediately centrifuged at 16,000 × *g* for 5 min. The obtained pellets were solubilized and subjected to immunoprecipitation with specific anti-G_α antisera (24, 25) using the procedure described previously (23, 26). The [α-^{32}P]guanine nucleotide bound proteins were separated by SDS-PAGE (10% polyacrylamide gel) according to Laemmli (27). The Coomassie blue-stained gels were dried and subjected to autoradiography. The autoradiograms were analyzed by densitometric scanning.

Coprecipitation of [^{3}H]SCH23390-bound receptor with discrete G_α proteins. Determination of the linkage between receptor and G proteins was carried out according to a modification of a method previously described by Matesic *et al.* (28). Crude striatal membranes were prepared by homogenizing brain striata in 10 volumes of 25 mM HEPES, pH 7.5, buffer containing 2 mM $MgCl_2$, 1 mM EDTA, 0.2% 2-mercaptoethanol, 50 μg/ml leupeptin, 25 μg/ml pepstatin A, 0.01 unit/ml soybean trypsin inhibitor, and 0.04 mM phenylmethylsulfonyl fluoride using glass/glass homogenizer. Homogenate was centrifuged at 750 × *g* for 5 min, and supernatant was centrifuged for 10 min at 48,200 × *g*. Membranes were washed, resuspended in immunoprecipitation buffer containing 100 mM Tris·HCl, pH 7.5, 200 mM NaCl, 2 mM $MgCl_2$, 1 mM EDTA, 0.2% 2-mercaptoethanol, 50 μg/ml leupeptin, 25 μg/ml pepstatin A, 0.01 unit/ml soybean trypsin inhibitor, and 0.04 mM phenylmethylsulfonyl fluoride, and the concentration of membrane proteins was determined according to the method of Lowry *et al.* (22). Two hundred micrograms of membrane proteins were solubilized in 1 ml of the immunoprecipitation buffer with 0.2% cholate and 0.5% digitonin.

Immunoprecipitation with antisera against specific G_α proteins was carried out according to a procedure described previously (23). Solubilized tissues were precleared by the addition of normal rabbit serum (1:100 dilution) at 4° for 60 min followed by a 30-min incubation with 100 μl of a 10% suspension of protein A-bearing *Staphylococcus aureus* cells (Pansorbin cells, Calbiochem). The suspension was centrifuged (at 4°), and the supernatant combined with antiserum (1:1000 dilution) raised against specific peptides of G_α proteins (DuPont) for 3 hr at 4° followed by an additional 30-min incubation with 100 μl Pansorbin. After centrifugation and washing, the pellet obtained from each tube was suspended in 500 μl of binding buffer (50 mM Tris·HCl, pH 7.5, 5 mM $MgCl_2$, 1 μM mesulergine) and incubated for 30 min with 1 nM [^{3}H]SCH23390 at 30°. Nonspecific binding was defined by the addition of 1 μM *cis*-(*Z*)-flupenthixol. The reaction was terminated by the addition of 9 ml of ice-cold binding buffer and immediately vacuum filtered over Whatman GF/F filters. The amount of radioactivity on filter was assessed by liquid scintillation spectrometry, and specific [^{3}H]SCH23390 binding was determined.

In a separate experiment, total specific [^{3}H]SCH23390 binding was determined in striatal membranes. Two hundred micrograms of membrane proteins were solubilized in 100 μl of the immunoprecipitation buffer with 0.2% cholate and 0.5% digitonin and precleared with normal rabbit serum (1:100 dilution) by incubation at 4° as described above. The resultant supernatant was combined with 400 μl of binding buffer (final concentrations in the assay mixture were 50 mM Tris·HCl, pH 7.5, 5 mM $MgCl_2$, 1 μM mesulergine) and incubated for 30 min with 1 nM [^{3}H]SCH23390 at 30°. Nonspecific binding was defined by the addition of 1 μM *cis*-(*Z*)-flupenthixol. The reaction was terminated by filtering over 10-kDa molecular weight cutoff filters (Cole-Parmer instrument Co., Niles, IL). The amount of radioactivity on filter was measured by liquid scintillation spectrometry, and specific [^{3}H]SCH23390 binding was determined.

Immunoblot analysis. Immunoprecipitants derived from 200 μg of membrane proteins or 25 μg of membrane proteins were solubilized in sample preparation buffer, and proteins were separated by SDS-PAGE (12% polyacrylamide gel) according to Laemmli (27). Proteins were transferred electrophoretically to a nitrocellulose membrane. The completeness of transfer was checked by Coomassie blue staining of the gel. The membranes were incubated at 4° overnight with 10% nonfat dry milk in phosphate buffered saline to block nonspecific sites, washed with 0.1% TBS, and incubated for 2 hr with monoclonal antibody against D_1 dopamine receptors (29) (1:1000 in 1% TBS), and unbound antibody was washed out with 1% TBS. After a 60-min incubation with horseradish peroxidase-conjugated anti-mouse IgG (Amersham) (1:10,000 in 1% TBS), the blots were washed with 3% TBS for 20 min followed by four 5-min washes. The immunoreactive proteins were detected by the enhanced chemiluminescence Western blot detection system (Amersham/Searle Corporation, Des Plaines, IL) and visualized by 2-min exposure to film.

Materials. For these studies, dopamine HCl, soybean trypsin inhibitor, 3-acetylpyridine adenine dinucleotide, dithiothreitol, *N*-ethylmaleimide, thymidine, isonicotinic acid hydrazide, and the buffer reagents were purchased from Sigma Chemical Co. (St. Louis, MO). The chemicals used for inositol phosphate isolation and determination were purchased from Fisher Scientific (Pittsburgh, PA). Mesulergine HCl, *S*-(−)-sulpiride, *cis*-(*Z*)-flupenthixol dihydrochloride, and anti-human dopamine D_1 receptor monoclonal antibody were purchased from RBI (Natick, MA). PTX was purchased from List Biological Laboratories (Campbell, CA). Normal rabbit serum and Pansorbin were purchased from Calbiochem (La Jolla, CA). SKF38393 HCl, quinpirole HCl (LY171555), and SCH23390 hemimaleate were generously supplied by SmithKline Beecham (King of Prussia, PA), Lilly Pharmaceutical (Indiana, IN) and Schering Corp. (Bloomfield, NJ), respectively. [^{35}S]GTPγS (1311 Ci/mmol), [α-^{32}P]GTP (3000 Ci/mmol), SCH23390 [*N*-methyl-^{3}H](71.3 Ci/mmol), and the antisera to $G_{\alpha s}$ (RM/1), $G_{\alpha i(1,2)}$ (AS/7), $G_{\alpha o}$ (GC/2), and $G_{\alpha q}$ (QL) were purchased from DuPont (Boston, MA). Antisera to $G_{\alpha z}$ (#2919) was kindly provided by Dr. David Manning (University of Pennsylvania).

Drugs used for GTP binding studies were dissolved in KRB buffer. Radiolabeled probes and antisera were stored at −70° until needed. All drug solutions were freshly prepared and stored on ice for the duration of the experiment.

Statistical analysis. Statistical comparisons of group mean values were performed either by ANOVA followed with Dunnett's test where appropriate or by planned comparisons, as specified in the figure legends.

Results

Dopamine-activated phosphoinositide hydrolysis in striatum is not affected by PTX. Brain striatal slices incubated with 500 μg/ml PTX showed 98.3 ± 1.7% (three experiments) ADP-ribosylation of $G_{\alpha i}/G_{\alpha o}$ after 3 hr of incubation with the toxin. The basal accumulation of inositol phosphates in striatal slices was unaltered by PTX treatment. Moreover, the increase in inositol phosphates induced by the D_1 dopamine receptor agonist SKF 38393 was also unaffected by $G_{i/o}$ ADP-ribosylation (Table 1).

Effects of dopamine on GTP binding to G proteins in striatum. Incubation of striatal membranes with [α-^{32}P]GTP and Mg^{2+} followed by immunoprecipitation with specific anti-G_α antisera and separation on SDS-PAGE yielded a single ^{32}P-labeled band for each antisera. The $G_{\alpha s}$, $G_{\alpha i}$, $G_{\alpha o}$, $G_{\alpha z}$, and $G_{\alpha q}$ bands displayed apparent molecular masses of 45, 41 40, 41, and 41 kDa, respectively (Fig. 1). Stimulation of membranes with dopamine increased [α-^{32}P]GTP binding to $G_{\alpha s}$, $G_{\alpha i}$, and $G_{\alpha q}$ (Fig. 1). Western blot analyses confirmed the presence of these G_α proteins in striatum; their respective molecular masses were identical to the bands labeled by [^{32}P]guanine nucleotide (data not shown). These results together therefore suggest that dopamine receptors in striatum are coupled to G_s, G_i, and G_q.

Incubation of striatal membranes with [^{35}S]GTPγS also resulted in a Mg^{2+}-dependent labeling of the α subunits of G_s, G_i, G_o, G_z, or G_q, which were precipitated by antisera directed against the respective proteins. Dopamine increased [^{35}S]GTPγS binding to $G_{\alpha s}$, $G_{\alpha i}$, and $G_{\alpha q}$ in a concentration-dependent fashion with EC_{50} values of 10–20 nM. Maximal stimulation of G_s/G_i and G_q by dopamine was obtained at 1 and 10 μM, respectively (Fig. 2).

Pharmacological characterization of dopamine-induced potentiation of [^{35}S]GTPγS binding to $G_{\alpha q}$ in striatal membranes. Activation of the D_1 receptors with the specific D_1 agonist SKF38393 induced concentration-de-

TABLE 1

Effects of PTX treatment on basal and SKF38393-stimulated [^{3}H]inositol phosphate formation in brain striatal slices

Slices were prelabeled with [^{3}H]inositol for 30 min, 5 mM LiCl was added, and 5 min later 500 μM of SKF38393 was added. Incubations were continued for an additional 60 min, and formed inositol phosphates were determined. Values are the mean ± standard error for four rats. No differences in SKF38393-induced response was observed between saline- and PTX-treated tissues by the two-tailed Student's *t* test.

	[^{3}H]Inositol phosphate	
	Basal	SKF38393
	dpm/mg protein	
Control	4010 ± 410	10,310 ± 490
PTX	3600 ± 240	10,550 ± 610

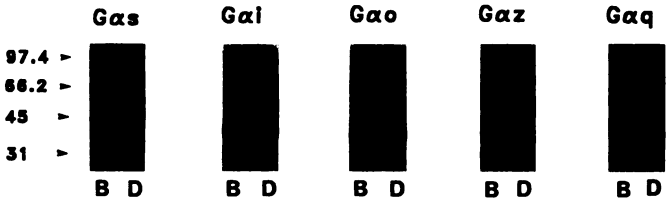

Fig. 1. Dopamine-stimulated [α-^{32}P]GTP binding to membrane G_α proteins. Membranes prepared from striata were labeled with 50 nM of [α-^{32}P]GTP (10 μCi) and incubated with 10 μM of dopamine (D) or buffer solution (B). The [α-^{32}P]guanine nucleotide-labeled G_α proteins were immunoprecipitated with the indicated anti-G_α antisera followed by separation on 10% SDS-PAGE and autoradiography. Each autoradiogram is representative of five experiments. The apparent molecular masses for $G_{\alpha s}$, $G_{\alpha i}$ $G_{\alpha o}$, $G_{\alpha z}$, and $G_{\alpha q}$ are 45, 41, 40, 41, and 41 kDa, respectively. Dopamine increased [α-^{32}P] guanine nucleotide binding to $G_{\alpha s}$, $G_{\alpha i}$, and $G_{\alpha q}$ by 350%, 320%, and 180%, respectively.

pendent increases in [^{35}S]GTPγS binding to $G_{\alpha s}$ and $G_{\alpha q}$ (Fig. 3A). In contrast, stimulation of striatal D_2 receptors with quinpirole increased [^{35}S]GTPγS binding to $G_{\alpha i}$ in a concentration-related fashion (Fig. 3B). The selective interaction of striatal D_1 and D_2 receptors with G_s/G_q and G_i proteins, respectively, was confirmed by blockade of the respective responses by specific D_1 and D_2 receptor antagonists. Although pretreatment of striatal membranes with the D_1 antagonist SCH23390 blocked dopamine-mediated activation of G_s and G_q, the dopamine-induced increase in [^{35}S]GTPγS binding to $G_{\alpha i}$ was selectively inhibited by the D_2 antagonist l-sulpiride (Fig. 3C). These results are in agreement with previous data that indicate that striatal D_1 and D_2 dopamine receptors are linked to G_s and G_i, respectively (23, 30). They also indicate that the D_1-like dopamine receptors may also couple to G_q in the rat striatum.

Coprecipitation of D_1 dopamine receptors with $G_{\alpha q}$ and $G_{\alpha s}$ proteins in striatal membranes. The possibility that D_1 dopamine receptors are directly coupled to G proteins was tested by coprecipitation of receptors with anti-G_α protein antibodies. The results summarized in Fig. 4 demonstrate that antisera against $G_{\alpha s}$ and $G_{\alpha q}$ but not $G_{\alpha i}$, $G_{\alpha o}$, or $G_{\alpha z}$ precipitated specific D_1 receptor binding sites labeled by the selective D_1 receptor ligand [^{3}H]SCH23390. Preincubation of the membranes with 100 μM of Gpp(NH)p before immunoprecipitation prevented the precipitation of specific [^{3}H]SCH23390 binding sites with the $G_{\alpha s}$ and $G_{\alpha q}$ antisera. The association of specific D_1-like dopamine sites, measured using 1 nM [^{3}H]SCH23390, with $G_{\alpha s}$ and $G_{\alpha q}$ proteins was 22% of total specific D_1 binding sites in solubilized striatal membranes. Anti-G_α antisera precipitants were also blotted with a monoclonal antibody against the D_1 dopamine receptor. Fig. 5 illustrates that the D_1 dopamine receptor antibody recognized a single protein band with an apparent molecular mass of ~60 kDa in striatal membranes, which was also found in the anti-$G_{\alpha s}$ precipitant but not in any of the other G_α antisera precipitants assessed. These results are in good agreement with those obtained in the GTP binding assays (Figs. 1–3) and demonstrate that in striatal membranes $G_{\alpha s}$ and $G_{\alpha q}$ couple to D_1-like dopamine sites and that the D_1 dopamine receptor is associated with $G_{\alpha s}$ but not with $G_{\alpha q}$ or the other tested G_α proteins (Fig. 5).

Discussion

The results demonstrate that dopamine receptors couple to a number of G proteins in a receptor-subtype-related manner. Thus, increases in [^{35}S]GTPγS or [α-^{32}P]GTP binding to $G_{\alpha s}$/$G_{\alpha q}$ and $G_{\alpha i}$ are mediated via activation of D_1 and D_2

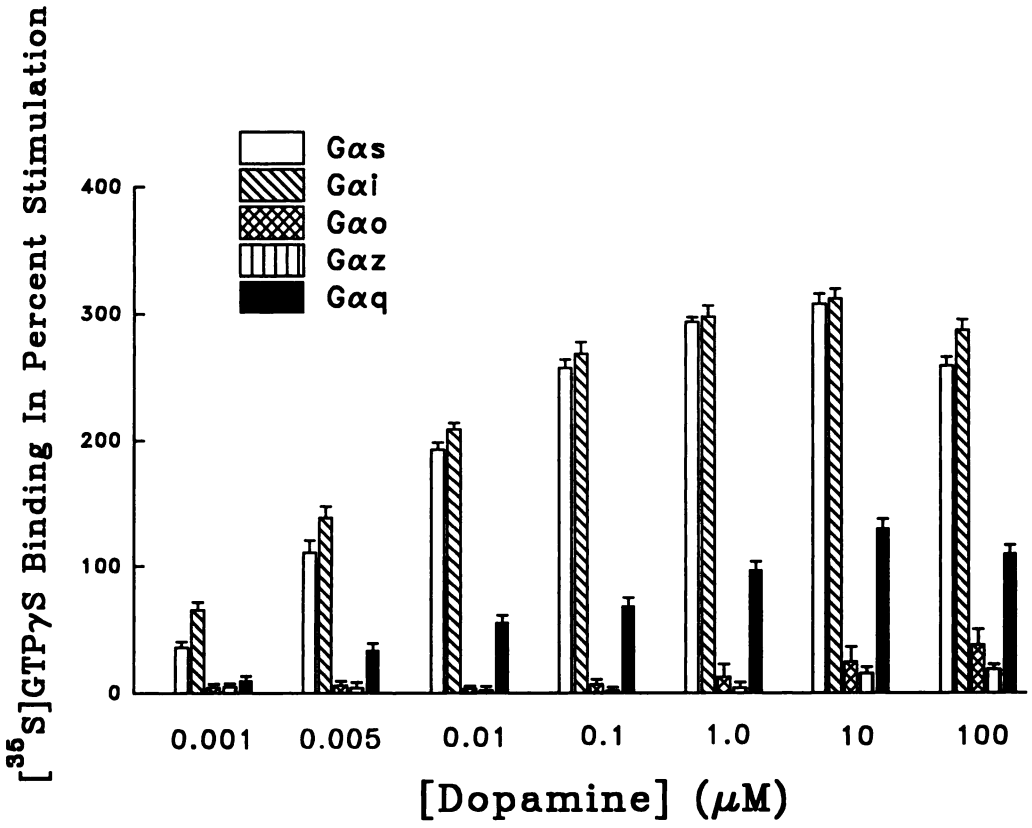

Fig. 2. Concentration-response relationship for dopamine-stimulated [^{35}S]GTPγS binding to G_α proteins in rat brain striatal membranes. Membranes prepared from rat brain striata were incubated with increasing concentrations of dopamine in the presence of 2 nM [^{35}S]GTPγS. Immunoprecipitated ^{35}S-labeled $G_{\alpha s}$, $G_{\alpha i}$, $G_{\alpha o}$, $G_{\alpha z}$, and $G_{\alpha q}$ were assessed. Basal [^{35}S]GTPγS binding to the G_α proteins (in cpm) were $G_{\alpha s}$, 355.7 ± 18.8; $G_{\alpha i}$, 402.7 ± 11.4; $G_{\alpha o}$, 429.4 ± 19.5; $G_{\alpha z}$, 250.3 ± 24.6; and $G_{\alpha q}$, 303.6 ± 11.9. The data expressed as increases in [^{35}S]GTPγS binding in the presence of dopamine compared with binding in the absence of the transmitter is depicted as the mean ± standard error obtained from five separate determinations. Statistical comparison by ANOVA indicated that dopamine significantly increased [^{35}S]GTPγS binding to G_s, G_i, and G_q in striatum ($p < 0.01$).

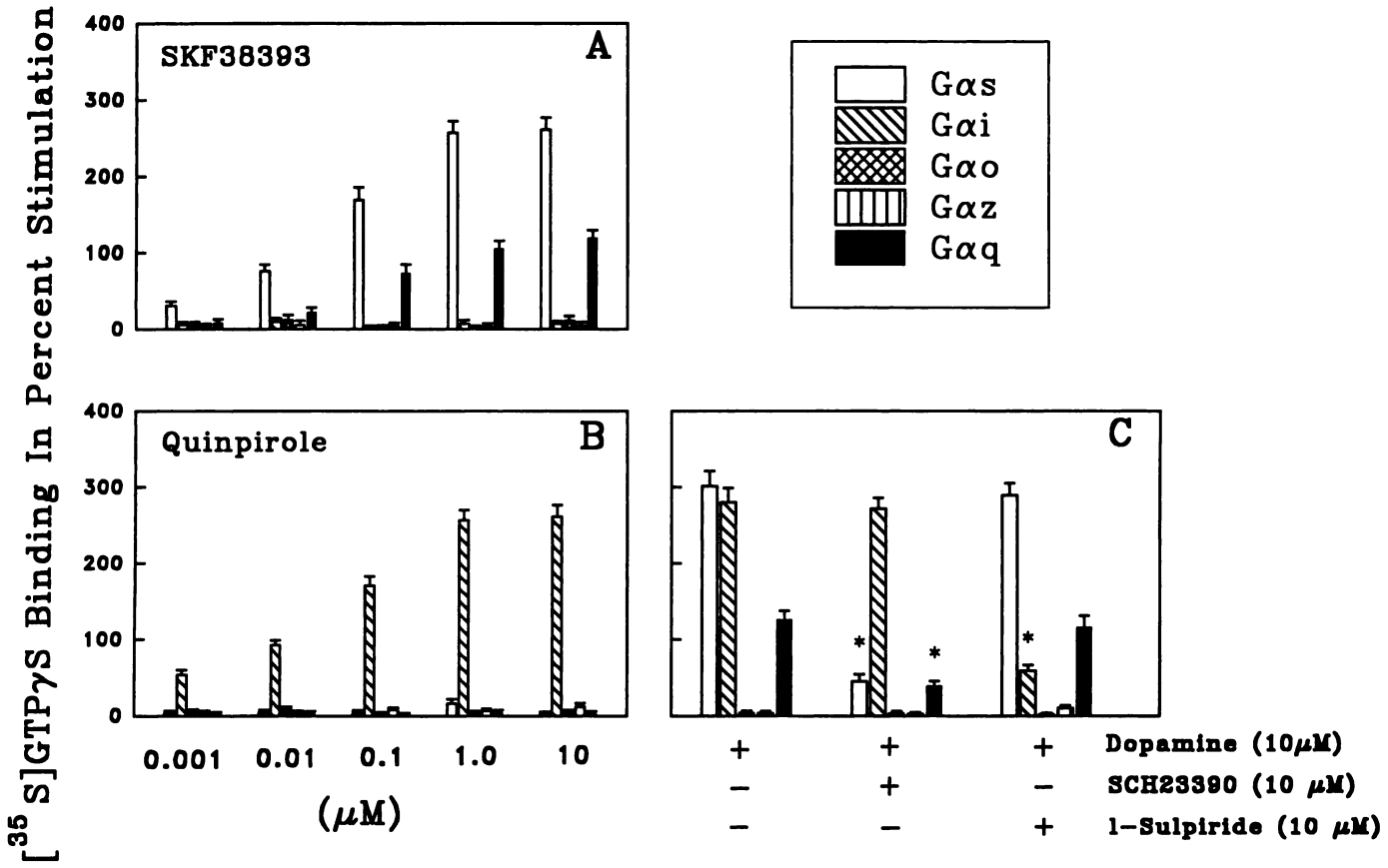

Fig. 3. Pharmacological characterization of dopamine receptor-stimulated [^{35}S]GTPγS binding to G_α proteins in rat striatal membranes. Membranes were incubated with dopamine or with dopaminergic agonists in the presence of 2 nM [^{35}S]GTPγS, and ^{35}S-labeled G_α proteins were assessed by immunoprecipitation. Incubation with 0.001–10 μM of the D_1 and D_2 receptor agonists SKF38393 (A) and quinpirole (B), respectively, increased [^{35}S]GTPγS binding to G_s/G_q and G_i in a concentration-dependent manner ($p < 0.01$, ANOVA). Pretreatment with 10 μM of the dopamine D_1 and D_2 receptor antagonists SCH23390 and l-sulpiride (C) resulted in blockade of the dopamine-induced activation of the G_s/G_q and G_i responses, respectively (*, $p < 0.01$, Dunnett's test). Neither SCH23390 nor l-sulpiride affected basal [^{35}S]GTPγS binding. Data are expressed as mean ± standard error percent increase in [^{35}S]GTPγS binding produced by the indicated drugs compared with basal binding obtained from four determinations.

receptors, respectively. This specificity is indicated by the activation profiles of the specific dopamine D_1 and D_2 receptor agonists SKF38393 and quinpirole and by the blockade observed with the selective antagonists SCH23390 and l-sulpiride. These findings are in accord with our previous observations regarding the coupling of D_1 and D_2 dopamine receptors to G_s and G_i proteins, respectively (23, 30), and with the well-characterized roles of these proteins in the regulation of adenylyl cyclase by D_1 and D_2 dopamine receptors (10, 11, 31–35). The present data also raise the possibility that D_1 dopamine receptor-mediated generation of inositol phosphates occurs via the coupling of dopamine D_1-like receptors to a PTX-insensitive G_q protein. This possibility is in agreement with the emerging role of G_q in mediating the activation of other neurotransmitter-stimulated PLC systems (18–20) and is also in concert with the previous demonstration that dopamine-stimulated phosphoinositide metabolism is resistant to PTX-catalyzed ADP-ribosylation (36). The finding of coupling of D_1-like receptors to G_s and G_q in striatum is consistent with data that indicate that this brain area is rich in D_1 dopamine receptors (37–39). In addition, the presence of D_1 dopamine receptors that stimulate both adenylyl cyclase (10, 30, 40) and phosphoinositide hydrolysis (12, 13) agree well with the notion that striatal D_1-like do-

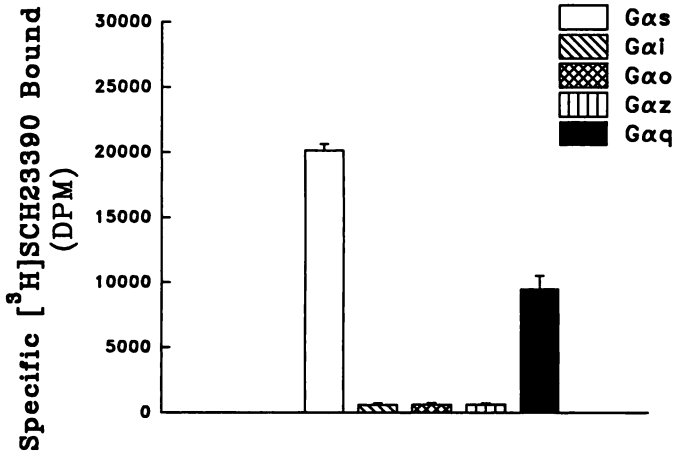

Fig. 4. Dopamine D_1 receptors couple to G_s and G_q proteins in striatum. Striatal membranes were solubilized and subjected to immunoprecipitation with anti-peptide antisera raised against $G_{\alpha s}$, $G_{\alpha i}$, $G_{\alpha o}$, $G_{\alpha z}$, or $G_{\alpha q/11}$. The immunocomplexes were incubated with 1 nM [^{3}H]SCH23390 and 1 μM mesulergine with or without 1 μM *cis*-(*Z*)-flupenthixol for 30 min at 30°. Each bar represents the mean ± standard error counts obtained from five determinations, each performed in duplicate. Bound [^{3}H]SCH23390 was assessed by counting the radioactivity collected on GF/F filters. Specific [^{3}H]SCH23390 binding was observed to be associated only with $G_{\alpha s}$ and $G_{\alpha q}$ proteins.

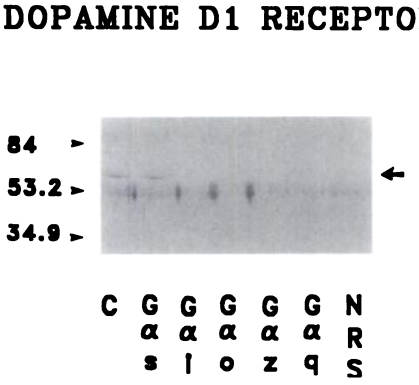

Fig. 5. G_s- but not G_q-coupled striatal dopamine D_1 receptors can be recognized by anti-human dopamine D_1 receptor antibody. Striatal membranes were solubilized and subjected to immunoprecipitation with anti-peptide antisera raised against $G_{\alpha s}$, $G_{\alpha i}$, $G_{\alpha o}$, $G_{\alpha z}$, or $G_{\alpha q/11}$. The immunocomplexes were then separated on 10% SDS-PAGE and transferred onto nitrocellulose membrane. After overnight blocking with 10% nonfat dry milk containing 1% TBS at 4°, the membrane was washed and subsequently incubated with anti-human dopamine D_1 receptor antibody (1:1000) at room temperature for 2 hr followed by 1-hr incubation with horseradish peroxidase-conjugated anti-mouse IgG. The signal was visualized using enhanced chemiluminescence (Amersham). A single 60-kDa protein was observed in 25 μg of striatal membranes (*C*) and in the anti-$G_{\alpha s}$ precipitant ($G_{\alpha s}$).

pamine receptors may be linked to two different G proteins. The present results, in addition to directly showing that D_1-like dopamine sites are coupled to $G_{\alpha s}$ and $G_{\alpha q}$, suggest that these G proteins may couple to at least two independent D_1-like sites. Although the D_1 dopamine receptor that was identified by a selective receptor antibody under basal conditions couples to $G_{\alpha s}$, other [^{3}H]SCH23390-labeled sites are associated with $G_{\alpha q}$ proteins. This heterotrimeric membrane protein may couple a D_1-like dopamine receptor to PLC and thus stimulates the generation of the second messengers inositol phosphates and diacylglycerol. The possibility that dopamine-stimulated cAMP and inositol phosphate formation are mediated via two distinct sites was previously suggested by a differential order of potencies of a series of benzazepine-derived D_1 dopamine receptor agonists in stimulating these two effector systems (17). This possibility is also generally supported by other findings that report that D_1 dopamine receptor-mediated behaviors (3) and other neurochemical effects are not mediated via cAMP (41–43). The present data therefore raise the possibility that some of the D_1-mediated effects may be transduced via G_q. The suggestion that D_1 dopamine receptor-mediated activation of G_q protein and inositol phosphate formation are mediated via the same site is tempered by the large discrepancy in apparent EC_{50} values for these two effects. This discrepancy may, however, be related to the very different assay conditions under which these receptor-stimulated effects were obtained. Although receptor coupling to G_q proteins was examined in membrane preparations, inositol phosphate accumulation was investigated in brain slices. In addition, the differences may be related to the different durations of incubation with agonists that are inherent to the two experimental systems.

G proteins dictate the specificity and functions of downstream intracellular effectors. Thus, identification of the interaction of a particular dopamine receptor with its G protein is an important step in unraveling the signal transduction cascades by which specific dopamine receptor subtypes exert their effects. The finding presented here lend support to the suggested heterogeneity of the signaling pathways for the dopamine receptors. The results indicate that in addition to the classic D_1 dopamine receptor/G_s/adenylyl cyclase cascade, dopamine D_1-like receptors couple to G_q protein, and this coupling may in turn modulate dopamine-stimulated phosphoinositide hydrolysis. Furthermore, the dissimilarity in pharmacological characteristics of these D_1-like dopamine receptor systems (17) and the fact that the D_1 dopamine receptor identified by a selective D_1 dopamine receptor antibody coprecipitated with $G_{\alpha s}$ but not with $G_{\alpha q}$ may suggest that the D_1 dopamine sites that activate adenylyl cyclase and those linked to G_q that may modulate PLC activity are distinct molecular entities.

References

1. Mailman, R. B., D. W. Schulz, C. D. Kilts, M. H. Lewis, H. Rollema, and S. Wyrick. Multiple forms of the D_1 dopamine receptor: its linkage to adenylate cyclase and psychopharmacological effects. *Psychopharmacol. Bull.* **22**:593–598 (1986).
2. Clark, D., and F. J. White. D1 dopamine receptor: the search for a function: a critical evaluation of the D1/D2 dopamine receptor classification and its functional implications. *Synapse* **1**:347–388 (1987).
3. Waddington, J. L., K. O'Boyle, and A. M. Murray. Stimulation of dopamine sensitive adenylate cyclase and inducing of grooming behavior by new selective D-1 agonists. *Neurochem. Int.* **13**:S188 (1993)
4. De Keyser, J., H. Wlraevens, G. Ebinger, and G. Vauquelin. In human brain two subtypes of D_1 dopamine receptors can be distinguished on the basis of differences in guanine nucleotide effect on agonist binding. *J. Neurochem.* **53**:1096–1102 (1989).
5. Anderson, P. H., and J. A. Jansen. Dopamine receptor agonists: selectivity and dopamine D1 receptor efficacy. *Eur. J. Pharmacol.* **188**:335–347 (1990).
6. Gilman, A. G. G proteins: transducers of receptor-generated signals. *Annu. Rev. Biochem.* **56**:615–649 (1987).
7. Birnbaumer, L. G. Proteins in signal transduction. *Annu. Rev. Pharmacol. Toxicol.* **30**:675–705 (1990).
8. Tang, W. J., and A. G. Gilman. Type-specific regulation of adenylyl cyclase by G protein $\beta\gamma$ subunits. *Science (Washington D. C.)* **254**:1500–1503 (1991).
9. Smrcka, A. V., and P. C. Sternweis. Regulation of purified subtypes of phosphoinositol-specific phospholipase Cβ by G protein α and $\beta\gamma$ subunits. *J. Biol. Chem.* **268**:9667–9674 (1993).
10. Kebabian, J. K., G. L. Petzold, and P. Greengard. Dopamine-sensitive adenylate cyclase in caudate nucleus of rat brain, and its similarity to the "dopamine receptor." *Proc. Natl. Acad. Sci. USA* **69**:2145–2149 (1972).
11. Monsma, F. J., L. C. Mahan, L. D. McVittie, C. R. Gerfen, and D. R. Sibley. Molecular cloning and expression of a D_1 dopamine receptor linked to adenylyl cyclase activation. *Proc. Natl. Acad. Sci. USA* **87**:6723–6727 (1990).
12. Undie, A. S., and E. Friedman. Stimulation of a dopamine D_1 receptor enhances inositol phosphate formation in rat brain. *J. Pharmacol. Exp. Ther.* **253**:987–992 (1990).
13. Undie, A. S., and E. Friedman. Selective dopaminergic mechanism of dopamine and SKF38393 stimulation of inositol phosphate formation in rat brain. *Eur J. Pharmacol.* **226**:297–302 (1992).
14. Arias-Montano, J.-A., J. Aceves, and J. M. Young. Carbachol-induced phosphoinositide metabolism in slices of rat substantia nigra pars reticulata. *Mol. Brain Res.* **19**:233–236 (1993).
15. Martin, L. P., and B. L. Waszczak. D_1 receptor stimulation enhances phosphoinositol (PI) hydrolysis in slices from rat substantia nigra (SN). *Soc. Neurosci. Abst.* **19**:82 (1993).
16. Wallace, M. A., and E. Claro. Transmembrane signaling through phospholipase C in human cortical membranes. *Neurochem. Int.* **18**:139–145 (1993)
17. Undie, A. S., J. Weinstock, H. M. Sarau, and E. Friedman. Evidence for a distinct D_1-like dopamine receptor that couples to activation of phosphoinositide metabolism in brain. *J. Neurochem.* **62**:2045–2048 (1994).
18. Berstein, G., J. L. Blank, D.-Y. Jhon, J. H. Exton, S. G. Rhee, and E. M. Ross. Phospholipase C-β1 is a GTPase-activating protein for $G_{q/11}$, its physiologic regulator. *Cell* **70**:411–418 (1992).
19. Berstein, G., J. L. Blank, A. V. Smrcka, T. Higashijima, P. C. Sternweis, J. H. Exton, and E. M. Ross. Reconstitution of agonist-stimulated phosphatidylinositol 4:5-bisphosphate hydrolysis using purified m1 muscarinic receptor, $G_{q,11}$, and phospholipase C-beta1. *J. Biol. Chem.* **267**:8081–8088 (1992).
20. Jope, R. S., L. Song, and R. Powers. Agonist-induced, GTP-dependent phosphoinositide hydrolysis in postmortem human brain membranes. *J. Neurochem.* **62**:180–186 (1994).

21. Gill, D. M., and M. J. Woolkalis. Cholera toxin-catalyzed [^{32}P]ADP-ribosylation of proteins. *Methods Enzymol.* **195:**267–280 (1991).
22. Lowry O. H., N. J. Rosenbrough, A. L. Farr, and R. J. Randall. Protein measurement with the folin phenol reagent. *J. Biol. Chem.* **193:**265–275 (1951).
23. Friedman, E., P. Butkerait, and H.-Y. Wang. Analysis of receptor-stimulated and basal guanine nucleotide binding to membrane G proteins by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. *Anal. Biochem.* **241:**171–178 (1993).
24. Carlson, K. E., L. F. Brass, and D. R. Manning. Thrombin and phorbol esters cause the selective phosphorylation of a G protein other than G_i in human platelets. *J. Biol. Chem.* **264:**13298–13305 (1989).
25. Spiegel, A. M. Immunologic probes for heterotrimeric GTP-binding proteins, in *G Proteins* (R. Iyengar and L. Birnbaumer, eds.). Academic Press, San Diego, 115–143 (1990).
26. Wang, H. Y., K. Eberle-Wang, K. J. Simansky, and E. Friedman. Serotonin-induced muscle contraction in rat stomach fundus is mediated by a Gαz-like guanine nucleotide binding protein. *J. Pharmacol. Exp. Ther.* **267:**1002–1011 (1993).
27. Laemmli, U. K. Cleavage of structural protein during assembly of the head of bacteriophage T4. *Nature (Lond.)* **227:**680–685 (1970).
28. Matesic, D. F., Manning D. R., Wolfe B. B., and Luthin G. R. Pharmacological and biochemical characterization of complexes of muscarinic acetylcholine receptor and guanine nucleotide-binding protein. *J. Biol. Chem.* **264:**21638–21645 (1989).
29. Levey, A. I., Hersch, S. M., Rye, D. B., Sunahara, R. K., Niznik, H. B., Kitt, C. A., Price, D. L., Maggio, R., Brann, M. R., and Ciliax, B. J. Localization of D_1 and D_2 dopamine receptors in brain with subtype-specific antibodies. *Proc. Natl. Acad. Sci. USA* **90:**8861–8865 (1993).
30. Wang, H. Y., S. Runyan, E. Yadin, and E. Friedman. Prenatal exposure to cocaine selectively affects D_1 dopamine receptor-mediated activation of striatal G_s proteins. *J. Pharmacol. Exp. Ther.* **273:**492–498 (1995).
31. Kebabian, J. K., and D. B. Caine. Multiple receptors for dopamine. *Nature (Lond.)* **277:**93–96 (1979).
32. Stoof, J. C., and J. W. Kebabian. Opposing roles for D-1 and D-2 dopamine receptors in efflux of cyclic AMP from rat neostriatum. *Nature (Lond.)* **294:**366–368 (1981).
33. Onali, P., M. C. Oloanas, and G. L. Gessa. Selective blockade of dopamine D-1 receptors by SCH23390 discloses striatal dopamine D-2 receptors mediating the inhibition of adenylate cyclase in rats. *Eur. J. Pharmacol.* **99:**127–128 (1984).
34. Senogles, S. E., A. M. Spiegel, E. Padrell, R. Iyengar, and M. C. Caron. Specificity of receptor-G protein interactions: discrimination of G_i subtypes by the D_2 dopamine receptor in a reconstituted system. *J. Biol. Chem.* **265:**4507–4514 (1990).
35. Senogles, S. E. The D_2 dopamine receptor isoforms signal through distinct $G_{i\alpha}$ proteins to inhibit adenylyl cyclase. *J. Biol. Chem.* **269:**23120–23127 (1994).
36. Felder, C. C., A. J. Pedro, and J. Axelord. The dopamine-1 agonist, SKF82586, stimulates phospholipase-C activity independent of adenylate cyclase. *J. Pharmacol. Exp. Ther.* **248:**171–175 (1989).
37. Boyson, S. J., P. McG$_o$nigle, and P. B. Molinoff. Quantitative autoradiographic localization of the D1 and D2 subtypes of dopamine receptors in rat brain. *J. Neurosci.* **6:**3177–3188 (1986).
38. Dawson, T. M., D. R. Gehlert, R. T. McCabe, A. Barnett, and J. K. Wamsley. D-1 dopamine receptors in rat brain: a quantitative autoradiographic analysis. *J. Neurosci.* **6:**2352–2365 (1986).
39. Dubois, A., M. Savasta, O. Curet, and B. Scatton. A autoradiographic distribution of the D1 agonist [^{3}H]SKF38393 in the rat brain and spinal cord: comparison with the distribution of D2 dopamine receptors. *Neuroscience* **19:**125–137 (1986).
40. Battaglia, G., A. B. Norman, E. Hess, and I. Creese. Functional recovery of D-1 dopamine receptor-mediated stimulation of rat striatal adenylate cyclase activity following irreversible receptor modification by *N*-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline (EEDQ):Evidence for spare receptors. *Neurosci. Lett.* **69:**290–295 (1986).
41. Laitinen, J. T. Dopamine stimulates K^+ efflux in the chick retina via D_1 receptors independently of adenylyl cyclase activation. *J. Neurochem.* **61:**1461–1469 (1993).
42. McMillian, M. K., He, X. P., Hong, J. S., Pennypacker, and K. R. Dopamine stimulates ^{3}H phorbol 12,13-dibutyrate binding in cultured striatal cells. *J. Neurochem.* **58:**1308–1312 (1992).
43. Felder, C. C., Albrecht, F. E., Campbell, T., Eisner, G. M., and Jose, P. A. cAMP independent, G protein-linked inhibition of Na^+/H^+ exchange in retinal brush border by D_1 dopamine agonists. *Am. J. Physiol.* **264(6 pt 2)F:**1032–1037 (1993).

Send reprint requests to: Eitan Friedman, Ph.D., Division of Molecular Pharmacology, Departments of Pharmacology and Psychiatry, Medical College of Pennsylvania and Hahnemann University/EPPI, 3200 Henry Avenue, Philadelphia, PA 19129.