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ACCELERATED COMMUNICATION

D_1-like Dopaminergic Activation of Phosphoinositide Hydrolysis Is Independent of D_{1A} Dopamine Receptors: Evidence from D_{1A} Knockout Mice

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SUMMARY

Accumulated evidence suggests that dopamine and dopamine D_1 agonists can activate phospholipase C in both brain and peripheral tissue. The receptor that mediates the hydrolysis of phosphoinositides has not been identified. The cloned dopamine D_{1A} receptor that is generally thought to be linked to adenylyl cyclase, has also been proposed to couple to phospholipase C. However, a number of studies have suggested that this signaling pathway is mediated via a distinct D_1-like dopamine receptor. We tested whether the D_{1A} site plays a role in stimulating phosphoinositide hydrolysis by using the dopamine D_{1A}-deficient mutant mice as a test model. Results show that although D_1 dopamine receptor-mediated production of cAMP is completely absent in membranes of D_{1A}-deficient mice, D_1 receptor-mediated accumulation of inositol phosphate is identical in tissues of mutant and wild-type animals. Furthermore, the coupling of [^{3}H]SCH23390 binding sites in striatal or frontal cortex membranes to $G_{\alpha s}$ is markedly reduced, although coupling of [^{3}H]SCH23390 binding sites to $G_{\alpha q}$ was unaltered in tissue taken from D_{1A} mutant mice compared with control animals. These results clearly demonstrate that dopaminergic stimulation of inositol phosphate formation is mediated by a D_1 dopamine receptor subtype that is distinct from the D_{1A} receptor that activates adenylyl cyclase.

Brain dopamine receptors that couple to stimulation of adenylyl cyclase have been classified as members of the D_1 dopamine receptor family, which includes the cloned D_{1A} and D_{1B} dopamine receptor subtypes (1, 2). Diverse neurochemical, electrophysiological, and behavioral observations have, however, suggested that other transduction systems for dopamine D_1 receptors exist in both the central and peripheral nervous systems (3–8). In a series of investigations, we demonstrated a D_1 dopaminergically mediated stimulation of IP formation in rat brain regions that does not parallel the distribution of the dopamine D_1/cyclase receptor activity (9, 10). Furthermore, the mRNA coding for the phosphatidylinositol-linked receptor site was found to differ markedly in size from that for the classic D_{1A} dopamine receptor (11). Also, the stimulation of phosphoinositide metabolism by the D_1-like dopamine receptor seems to be distinct from the classic D_1 receptor that is coupled to stimulation of adenylyl cyclase in terms of both receptor and the transducing G protein (12). Although coupling of striatal D_1-like dopamine receptors to IP formation was demonstrated to be mediated by G_q, the coupling of the D_{1A} receptor to cAMP formation was shown to occur via G_s (12). In the current study, we sought further evidence to test whether the two actions of dopamine are transduced by distinct molecular entities. The experiments were performed in tissues derived from homozygous D_{1A}-deficient mutant mice, which were produced by homologous recombination (13).

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ABBREVIATIONS: IP, inositol phosphate; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; SDS, sodium dodecyl sulfate; TBS, Tween 20-containing phosphate-buffered saline.

Experimental Procedures

Animals. Homologous recombination was used to generate mutant mice lacking functional D_{1A} dopamine receptors, as previously described (13). Homozygous mice matched for sex (seven females and one male) and age (9.0 ± 0.9 months) with wild-type animals (age, 9.3 ± 0.8 months) were singly housed with free access to food and water under standard conditions of humidity (60%), room temperature (22°), and 12-hr light/dark cycle for ≥5 days after arrival at the animal facility and before the experiments.

Daily experiments were performed on one D_{1A} mutant and one control wild-type animal. Animals were decapitated; brains rapidly removed; and several brain regions, including frontal cortex, temporoparietal cortex, and striatum, were quickly dissected onto an ice-cooled glass surface. Left frontal cortex and striatum were used for the immunoprecipitation experiments; right frontal cortex and striatum were used for the adenylyl cyclase assay; and IP formation was performed on the temporoparietal cortical area.

IP formation in cerebral cortex slices. The experimental procedures have been previously described in detail (9). Briefly, the cerebral cortices were chopped into 350 × 350-μm slices. The resulting slices were weighed and transferred into a 25-ml screw-capped polypropylene tube containing HEPES bicarbonate buffer at 35°, which was composed of 122 mM NaCl, 1.2 mM $MgCl_2$, 4.9 mM KCl, 1.2 mM KH_2PO_4, 3.6 mM $NaHCO_3$, 30 mM HEPES, and 10 mM glucose and bubbled with 95% oxygen/5% carbon dioxide, pH 7.4. The slices were washed twice, resuspended in 5 ml of buffer, and incubated at 37° for 30 min. Then, the slices were resuspended in fresh buffer containing 1.3 mM $CaCl_2$ and labeled with 10 μl of 66.67 μM 2-[^{3}H] inositol/ml (15 Ci/mmol, American Radiolabeled Chemicals, St. Louis, MO) at 37° for 60 min before being washed twice with 2 volumes of fresh buffer. The slices were finally suspended in fresh calcium containing buffer (3 ml/80 mg of fresh tissue).

The reaction mixture routinely included 7.5 mM lithium chloride, 50 μM pargyline, and different concentrations of dopamine or SKF38393 (1–500 μM); 250 μM of SKF38393 was used in testing antagonists. The reactions were initiated by the addition of 50 μl of prelabeled and well-mixed slices (150 μg of protein) at a final volume of 250 μl. The reaction was carried out at 37° for 60 min with continuous shaking and stopped by mixing the reaction with 1.5 ml of chloroform/methanol/1 M HCl (100:200:1). The slices were allowed to stand at room temperature for 45 min before an additional 0.5 ml of chloroform and 0.75 ml of water were added. The tubes were vortexed vigorously for 15 sec and centrifuged at 800 × *g* for 10 min, and a 1.0-ml aliquot of the top aqueous phase was transferred to a polypropylene tube. The solution was neutralized with 30 μl of 1 N NaOH, and the IPs were fractionated on a Dowex anion exchange column.

Adenylyl cyclase assay. Striatum and frontal cortex were homogenized using a Teflon/glass homogenizer in 10 volumes (w/v) of prechilled buffer containing 10 mM imidazole, 2 mM EGTA, and 10% sucrose, pH 7.3. The homogenate was centrifuged at 1,000 × *g* for 10 min, and the supernatant was centrifuged at 27,000 × *g* for 20 min. The pellet was washed twice with 10 mM cold imidazole and suspended in 10 mM imidazole buffer, pH 7.3. Membrane protein was determined according to the method of Bradford (14). The adenylyl cyclase assay was performed by a modification of the method described by Salomon (15). The reaction mixture included 0.5 mM $MgCl_2$, 0.5 mM 3-isobutyl-1-methylxanthine, 0.2 mM EGTA, 0.5 mM dithiothreitol, 10 μM pargyline, 1 μM GTP, 0.1 mM ATP, 2 mM phosphocreatine, 5 units of creatine phosphokinase, and 1 μCi of [α-^{32}P]ATP (~2.2 × 10^6 cpm) in 10 mM imidazole buffer, pH 7.3, with or without dopamine or SKF38393. After preincubation at 30° for 5

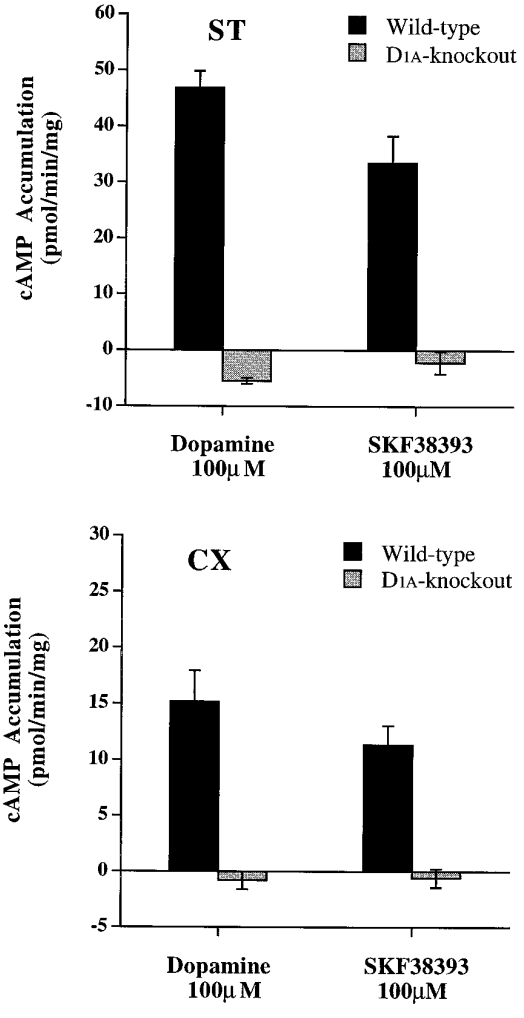

Fig. 1. Dopamine- and SKF38393-activated cAMP accumulations in striatal (*ST*) and cortical (*CX*) membranes of D_{1A} knockout mice. Membrane protein (50 μg) was exposed to either 100 μM dopamine or SKF38393 in 10 mM imidazole buffer, pH 7.3, containing 1 μCi of [α-^{32}P]ATP (2.2 × 10^6 cpm), 0.5 mM $MgCl_2$, 0.5 mM 3-isobutyl-1-methylxanthine, 1 μM GTP, and 0.1 mM ATP at 30° for 10 min. Values represent the rate of accumulated cAMP in stimulated tissue above basal activity. Data are mean ± standard error from seven animals.

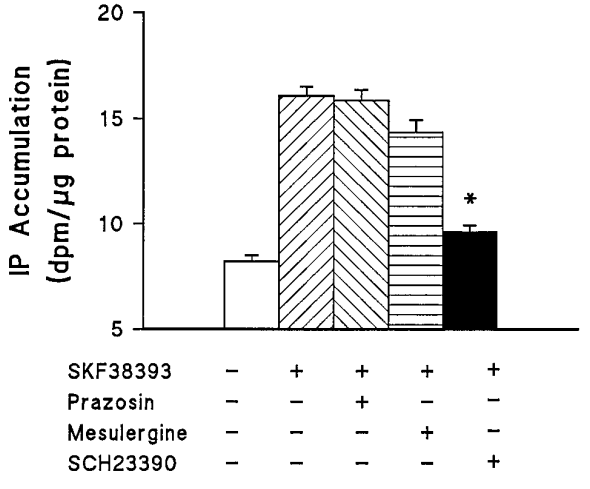

Fig. 2. The effect of receptor antagonists on SKF38393-stimulated IP accumulation. Cortical slices were prelabeled with ^{3}H-inositol and incubated with 10 mM LiCl in the presence of buffer alone or with 50 μM SCH23390, mesulergine, or prazosin for 10 min before the addition of 250 μM SKF38393. The reaction proceeded for 60 min, and accumulated IPs were determined. SCH23390 significantly inhibited the SKF38393-induced IP accumulation ($p < 0.01$; four animals).

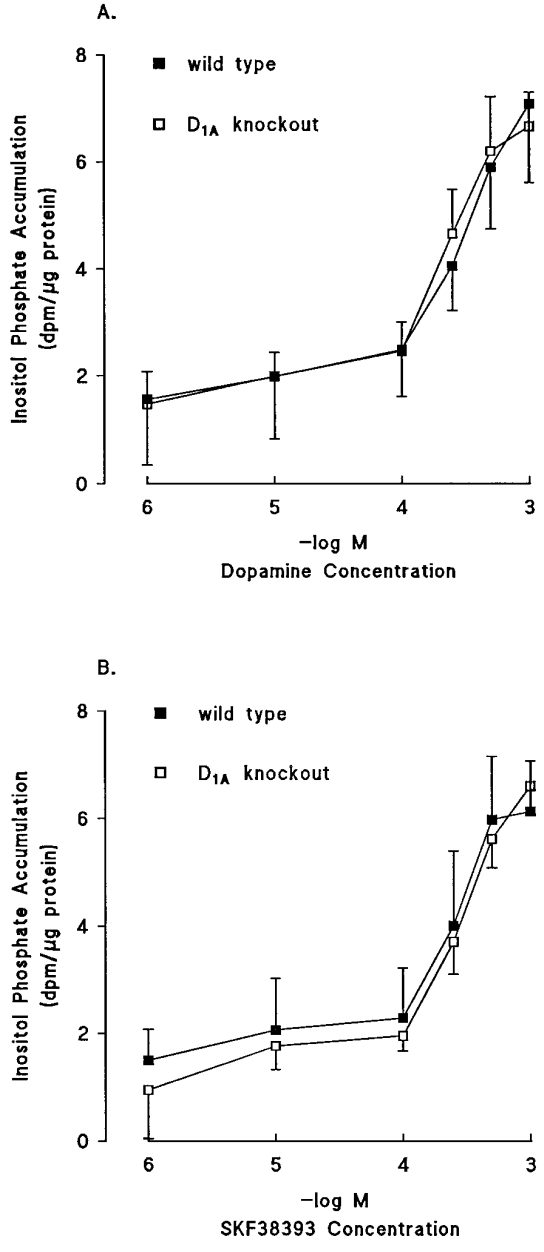

Fig. 3. Dopamine- and SKF38393-activated IP formation in cortical slices of D_{1A} knockout mice. Drug-induced IP accumulation was calculated as net change from basal accumulation. Basal accumulation across all groups was 7.21 ± 0.39 dpm/μg of protein (28 animals). *Points*, mean ± standard error of seven experiments performed in triplicate. The accumulation of IP in responses to increasing concentrations of (A) dopamine or (B) SKF38393 were not different in D_{1A} knockout than in wild-type mice.

min, the reaction was started by the addition of 50 μg of membrane protein. The reaction was terminated 10 min later by the addition of 300 μl of a solution containing 2% SDS, 25 mM ATP, and 1.3 mM cAMP. Formed [^{32}P]cAMP was separated by Dowex and alumina columns. [^{3}H]cAMP was included in each reaction for estimation of column recovery (typically ~70-80%).

Coprecipitation of [^{3}H]SCH23390-bound receptor with discrete G_{α} proteins. Determination of the linkage between receptor and G proteins was carried out as previously described (12). 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 with the use of a glass/glass homogenizer. The homogenate was centrifuged at 750 × *g* for 5 min, and the supernatant was centrifuged for 10 min at 48,200 × *g*. Membranes were washed and resuspended in 100 mM Tris·HCl immunoprecipitation buffer, pH 7.5, containing 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. The concentration of membrane proteins was determined (16), and 200 μg of membrane proteins was solubilized in 1 ml of immunoprecipitation buffer with 0.2% cholate and 0.5% digitonin. Solubilized tissues were precleared by incubation with normal rabbit serum (1:100 dilution) at 4° for 60 min followed by an additional 30 min with 100 μl of a 10% suspension of protein A-bearing *Staphylococcus aureus* cells (Pansorbin cells, Calbiochem, San Diego, CA). The suspension was centrifuged at 4°, and the supernatant was combined with antisera (1:1000 dilution) raised against specific peptides of G_{α} proteins (New England Nuclear Research Products, Boston, MA) for 3 hr at 4° followed by an additional 30-min incubation with 100 μl of Pansorbin. The specificity of antisera used was previously defined (17). The mixture was centrifuged and washed, and the pellet was suspended and incubated for 30 min at 30° in 500 μl of 50 mM Tris·HCl binding buffer, pH 7.5, which included 5 mM $MgCl_2$, 1 μM mesulergine, and 1 nM [^{3}H]SCH23390. 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 buffer and immediately vacuum filtered over Whatman GF/F filters. The amount of radioactivity on the filter was assessed by liquid scintillation counting, and specific [^{3}H]SCH23390 binding was determined.

Immunoblot analysis. Twenty-five micrograms of membrane proteins was solubilized in sample preparation buffer, and proteins were separated by SDS-polyacrylamide gel electrophoresis (12%) according to the method of Laemmli (18). 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 0.1% TBS to block nonspecific sites, washed with 0.1% TBS, and incubated for 2 hr with antisera directed against $G_{\alpha s}$, $G_{\alpha i1/2}$, $G_{\alpha o}$, or $G_{\alpha q}$ (New England Nuclear Research Products) at 1:2,000 dilution or with affinity-purified G_{β} protein antibody at 0.25 μg/ml (Santa Cruz Biochemicals, Santa Cruz, CA) in 0.1% TBS. The unbound antibody was washed out with 0.1% TBS. After a 60-min incubation with horseradish perioxidase-conjugated anti-rabbit IgG (Amersham, Arlington Heights, IL) (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 with the enhanced chemiluminescence Western blot detection system (Amersham/Searle, Des Plaines, IL) and visualized by a 2-min exposure to film.

Materials. For these experiments, dopamine HCl, pargyline HCl, soybean trypsin inhibitor, and the buffer reagents were purchased from Sigma Chemical (St. Louis, MO). The chemicals used for IP isolation and determination were purchased from Fisher Scientific (Pittsburgh, PA). Mesulergine HCl [*N'*-[(8α)-1,6-dimethylergolin-8-yl]*N*,N-dimethylsulfamide HCl], *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], and SKF38393 HCl [1-phenyl-2,3,4,5-tetrahydro-(1*H*)-3-benzazepine-7,8-diol hydrochloride] were purchased from Research Biochemicals (Natick, MA). Normal rabbit serum and Pansorbin were purchased from Calbiochem. Prazosin HCl and SCH23390 hemimaleate (8-chloro-2,3,4,5-tetrahydro-3-methyl-5-phenyl-1*H*-3-benzazepin hemimaleate) were generously supplied by Pfizer (New York, NY) and Schering (Bloomfield, NJ), respectively. SCH23390 [*N-methyl*-^{3}H](71.3 Ci/mmol) and antisera for $G_{\alpha s}$ (RM/1), $G_{\alpha i}$(1, 2) (AS/7), $G_{\alpha o}$ (GC/2), and $G_{\alpha q}$ (QL) were purchased from DuPont-New England Nuclear (Boston, MA).

TABLE 1
Forskolin-stimulated cAMP accumulations in tissue from D_{1A}-deficient mutant mice

	Striatum		Cortex	
	Wild-type miceD_{1A}	knockout mice	Wild-type miceD_{1A}	knockout mice
Basal	136 ± 13[a]	139 ± 12	59 ± 8	55 ± 8
Forskolin (10 μM)	847 ± 16[b]	796 ± 31[b]	424 ± 36[b]	460 ± 14[b]

[a] Values represent cAMP accumulations in pmol/min/mg of striatal or cortical membranes measured in the presence of 1 μM GTP. Each value is mean ± standard error of seven individual experiments.
[b] $p < 0.01$ compared with the respective basal activity.

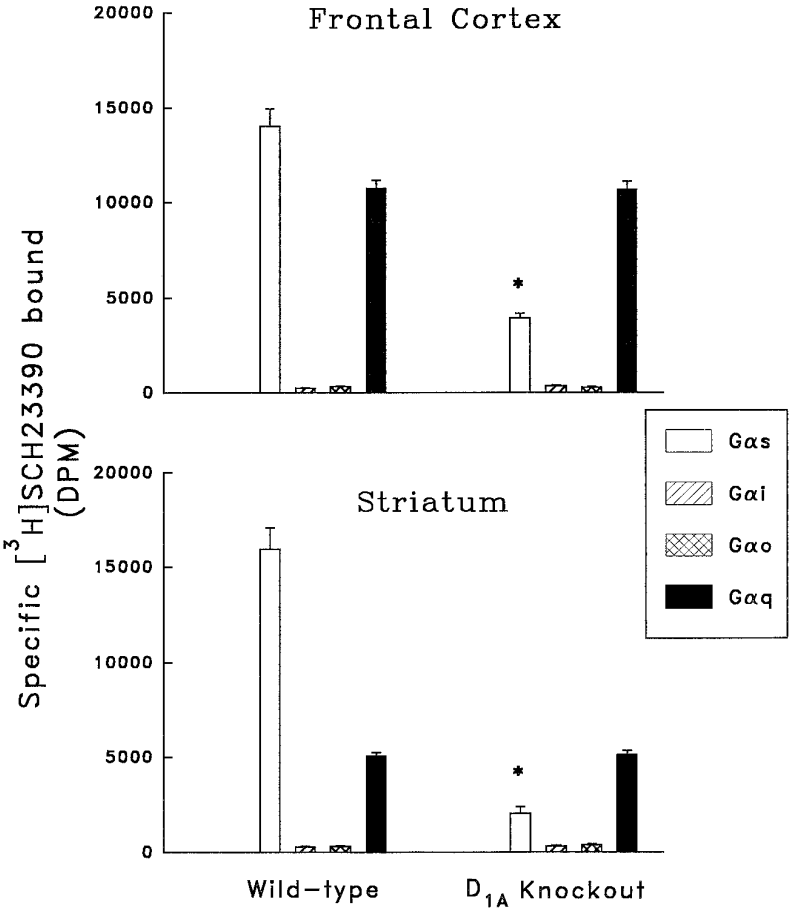

Fig. 4. Copurification of [^{3}H]SCH23390 binding sites with $G_{\alpha s}$ or $G_{\alpha q}$ in D_{1A}-deficient mutant mice tissue. Frontal cortical or striatal membranes obtained from D_{1A} knockout or wild-type mice were solubilized and subjected to immunoprecipitation with the indicated anti-G_α antisera. 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°. Bound [^{3}H]SCH23390 was assessed by counting the radioactivity collected on GF/F filters. *Bar*, mean ± standard error obtained from seven determinations, each performed in duplicate. Significant reduction in specific [^{3}H]SCH23390 binding was observed to be associated only with $G_{\alpha s}$ in the membranes of D_{1A} knockout mice ($p < 0.05$), whereas [^{3}H]SCH23390 binding sites coupled with $G_{\alpha q}$ remained unchanged.

Results

Dopamine- or SKF38393-activated cAMP production in striatal and cortical membranes is absent in D_{1A}-deficient mice. Incubation of striatal and frontal cerebrocortical membranes obtained from wild-type mice with dopamine or with the D_1-selective agonist SKF38393 resulted in concentration-dependent elevations in cAMP production. The maximal responses for both dopamine and SKF38393 were achieved at 100 μM in both brain areas. The results summarized in Fig. 1 indicate that adenylyl cyclase activity in response to dopamine or SKF38393 was completely absent in both brain regions of D_{1A}-deficient mice. In contrast, direct enzyme stimulation with forskolin was unchanged in brain membranes obtained from D_{1A} mutant mice (Table 1), suggesting that the mutation does not affect the activity of adenylyl cyclase *per se*.

Dopamine- or SKF38393-activated IP formation is not altered in cortical slices of D_{1A} gene-deficient mice. Incubation with the D_1 dopamine receptor agonist SKF38393 of frontal cerebrocortical slices obtained from control mice increased the formation of IPs. This dopaminergic effect was inhibited by the D_1-selective antagonist SCH23390 but not by the α_1-adrenergic antagonist prazosin or by the 5-hydroxytryptamine$_{2C/A}$ serotonin receptor antagonist mesulergine (Fig. 2). In contrast to the absence of D_1 receptor-mediated cAMP responses in D_{1A}-deficient mice, the concentration-response curves for dopamine-induced (Fig. 3A) or SKF38393-induced (Fig. 3B) elevations in IP were identical in D_{1A}-deficient and wild-type mice, suggesting that D_1 dopaminergic stimulations of cAMP and IP formations are mediated by structurally distinct dopamine receptors.

Coprecipitation of D_1 dopamine receptors with $G_{\alpha q}$ and $G_{\alpha s}$ in striatal and cortical membranes. The results summarized in Fig. 4 demonstrate that $G_{\alpha s}$ and $G_{\alpha q}$ antisera coimmunoprecipitated specific D_1 dopamine receptor binding sites labeled by the selective D_1 receptor ligand [^{3}H]SCH23390

in striatal or frontal cortex membranes of wild-type mice; antisera recognizing $G_{\alpha i}$ and $G_{\alpha o}$ proteins or normal rabbit serum did not immunoprecipitate [^{3}H]SCH23390 binding sites. Fig. 4 also illustrates that coupling of D_1 dopamine receptors to $G_{\alpha s}$ is reduced by 75–82%, whereas the association of [^{3}H]SCH23390 binding sites with $G_{\alpha q}$ were unaltered in tissues from D_{1A} mutant mice. The reduction in coupling of specific [^{3}H]SCH23390 binding sites to $G_{\alpha s}$ in brains of D_{1A} receptor-deficient mice does not result from reduced $G_{\alpha s}$ because similar levels of $G_{\alpha s}$ were found in membranes of wild-type and D_{1A}-deficient mice (Fig. 5). The results demonstrate that $G_{\alpha s}$-coupled D_1 dopamine sites are selectively reduced in D_{1A} receptor-deficient mice.

Discussion

The current findings clearly demonstrate that the dopamine receptor that stimulates the formation of IPs is completely independent of the D_{1A} dopamine receptor system, which is known to couple to adenylyl cyclase. In addition, the data confirm our previous conclusion that the D_{1A} dopamine receptors couple to adenylyl cyclase via G_s protein, whereas G_q protein links D_1-like dopamine receptors to the activation of phosphoinositide hydrolysis.

The results of pharmacological and neurochemical investigations have previously suggested that the D_1 dopamine receptors that are coupled to phospholipase C and adenylyl cyclase are distinct receptors that are linked to their respective effector systems via different coupling proteins. Evidence demonstrating size differences for mRNAs coding for the two receptors first suggested that the D_1 dopamine receptor sites that couple to phospholipase C and adenylyl cyclase may be distinguishable molecular moieties (11). Differential order of potencies and efficacies for a series of benzazepine derivatives in activating striatal phosphoinositide hydrolysis and adenylyl cyclase (10) and the unique regional distributions of the two D_1 dopaminergic transduction systems in the rat brain (9) further support this possibility. D_1 dopamine receptors, which activate cyclase and phospholipase C, were also shown to couple to their respective effectors via G_s and G_q (12). Both of these G proteins were in turn found to interact with [^{3}H]SCH23390 binding sites. However, the sites that were coupled to G_s were identified as being the D_{1A} receptors, whereas those that were linked to G_q were not recognized by the same selective monoclonal antibody that recognizes D_{1A} receptors (12). The G_q/phosphatidylinositol-linked dopaminergic receptor site therefore seems to be a subtype of the D_1 dopamine receptor family.

The current data demonstrating that the D_{1A}-deficient mutant mice are dramatically impaired in dopamine-stimulated adenylyl cyclase without a parallel loss in dopamine-stimulated phosphoinositide metabolism directly support the conclusion that the two D_1 dopaminergic signal transduction systems are independently activated by two dopamine receptors. The discrepancy between the total absence of dopamine-mediated cyclase activation and a residual coupling of [^{3}H]SCH23390 binding sites to $G_{\alpha s}$ is probably a function of the greater sensitivity of the binding experiment in comparison to the measurement of dopamine-stimulated adenylyl cyclase. Alternatively, the residual $G_{\alpha s}$/[^{3}H]SCH23390 coupling found in mutant mice may reflect the coupling of $G_{\alpha s}$ to other members of the D_1 dopamine receptor family that are not linked to adenylyl cyclase but activate other effector systems (7, 8).

The findings presented here lend support to the suggested molecular heterogeneity of the signaling pathways for the D_1 dopamine receptors. The results indicate that in addition to the classic dopamine D_{1A} receptor/G_s/adenylyl cyclase cascade, an unidentified dopamine D_1 receptor also couples to G_q protein and that this interaction may in turn modulate dopamine-stimulated phosphoinositide hydrolysis.

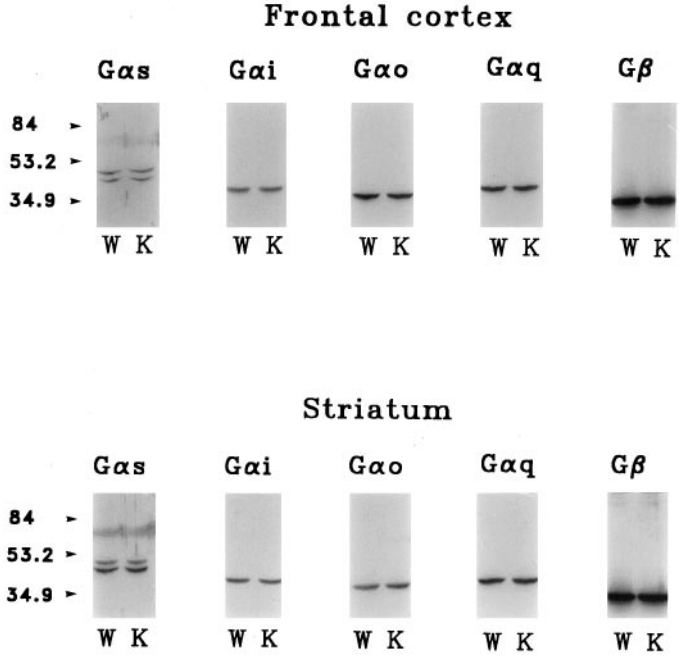

Fig. 5. Immunoblots of $G_{\alpha s}$, $G_{\alpha i1/2}$, $G_{\alpha o}$, $G_{\alpha q}$, and G_β in frontal cortical and striatal membranes of wild-type (*W*) and D_{1A} knockout (*K*) mice. Twenty-five micrograms of membrane proteins was separated by SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose. G_α proteins and G_β were immunoblotted with antibodies specific for the G protein subunits. The immunocomplexes were detected using anti-rabbit IgG and enhanced chemiluminescence. Densitometry showed no significant changes in the level of G protein subunits in brain membranes of D_{1A} knockout mice.

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