Differential Dependence of the D1 and D5 Dopamine Receptors on the G Protein γ7 Subunit for Activation of Adenylyl cyclase∗

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The D1 dopamine receptor, G protein γ7 subunit, and adenylyl cyclase are selectively expressed in the striatum, suggesting their potential interaction in a common signaling pathway. To evaluate this possibility, a ribozyme strategy was used to suppress the expression of the G protein γ7 subunit in HEK 293 cells stably expressing the human D1 dopamine receptor. Prior in vitro analysis revealed that the γ7 ribozyme possessed cleavage activity directed exclusively toward the γ7 RNA transcript (Wang, Q., Mullah, B., Hansen, C., Asundi, J., and Robishaw, J. D. (1997) J. Biol. Chem. 272, 26040–26048). In vivo analysis of cells transfected with the γ7 ribozyme showed a specific reduction in the expression of the γ7 protein. Coincident with the loss of the γ7 protein, there was a noticeable reduction in the expression of the β1 protein, confirming their interaction in these cells. Finally, functional analysis of ribozyme-mediated suppression of the β1 and γ7 proteins revealed a significant attenuation of SKF81297-stimulated adenylyl cyclase activity in D1 dopamine receptor-expressing cells. By contrast, ribozyme-mediated suppression of the β1 and γ7 proteins showed no reduction of SKF81297-stimulated adenylyl cyclase activity in D2 dopamine receptor-expressing cells. Taken together, these data indicate that the structurally related D1 and D2 dopamine receptor subtypes utilize G proteins composed of distinct βγ subunits to stimulate adenylyl cyclase in HEK 293 cells. Underscoring the physiological relevance of these findings, single cell reverse transcriptase-polymerase chain reaction analysis revealed that the D1 dopamine receptor and the G protein γ7 subunit are coordinately expressed in substance P containing neurons in rat striatum, suggesting that the G protein γ7 subunit may be a new target for drugs to selectively alter dopaminergic signaling within the brain.

Dopaminergic signaling in brain is mediated by five receptor subtypes that can be grouped into D1-like (D1 and D3) and D2-like (D2a, D2b, and D3) classes based on pharmacological and physiological criteria (1). Studies suggest that imbalances between these two opposing classes lead to deficiencies in movement and cognitive performance (2, 3). In particular, alterations in the D1-like dopamine receptors are implicated in a variety of neurologic and psychiatric disorders, such as Parkinson’s disease, Tourette’s syndrome, and schizophrenia. Thus, achieving a better understanding of the D1-like dopamine receptors and the signaling pathways they activate may suggest more selective therapeutic targets in these diseases.

The D1 and D2 dopamine receptors stimulate adenylyl cyclase activity through their coupling to heterotrimeric G proteins (1, 4–6). Because the function of these heterotrimeric G proteins was originally ascribed to the α subunit, most research has focused on determining its identity. Of the several α subunits identified to date, reconstitution studies have shown that coupling of D1 dopamine receptors to adenylyl cyclase can be mediated only by the αs and αolf subunits of the Gα subclass (4, 5, 7–9). Although sharing 88% amino acid homology, the αs and αolf subunits show very divergent expression patterns, ranging from the ubiquitous expression of the αs subunit to the olfactory and neuron-specific expression of the αolf subunit (10). Immunoprecipitation studies (11) have confirmed the interaction between the αs subunit and the D1 dopamine receptors in cells, whereas in situ hybridization (12, 13) and gene targeting (14) studies have suggested a possible interaction between the αolf subunit and the D1 dopamine receptor in striatum.

By contrast, little effort has focused on determining the identity of the βγ subunits of the G protein despite mounting evidence for their importance in receptor recognition (15, 16). Of particular interest, reconstitution studies (17–21) and reverse genetic approaches (22, 23) have shown that the nature of the γ subunit is an important determinant of its interaction with receptor. Consistent with such a role, 12 γ subunit genes have been identified that show extensive structural diversity (24). Recently, we used a ribozyme approach to begin to elucidate their functions (23, 25, 26). This approach identified the γ7 subunit as a specific component of the G protein that couples the β-adrenergic receptor, but not the prostaglandin E2 receptor, to stimulation of adenylyl cyclase in HEK 293 cells (23). Although expressed in a variety of tissues and cell types (27, 28), the γ7 subunit expression is most abundant in medium spiny neurons in striatum (13). This pattern of expression is shared by the D1 dopamine receptor and adenylyl cyclase (4, 12, 29–31), raising the possibility that all of these components may be involved in the same signaling pathway. In the present study, we used the ribozyme approach to identify a role for the
γ-subunit as a specific component of the G protein that couples the D₁ dopamine receptor to activation of adenylate cyclase.

**MATERIALS AND METHODS**

**Single Cell Reverse Transcriptase-Polymerase Chain Reaction Analysis**—Following dissociation and plating at low density, single neotria- nal neurons from 4-week-old rats, cells were aspirated into electrodes by applying negative pressure. The electrode contents (5–5 μl) were ejected into a thin walled PCL tube (MJ Research, Watertown, MA) containing diethylpyrocarbonate-treated water, RNAin (Promega, Madison, WI), dithiothreitol, and oligo(dT), as described previously (31). First strand cDNA was generated from mRNA using SuperScript II reverse transcriptase (Life Technologies, Inc.). After reverse transcription, mRNA was analyzed by the addition of 1 μl of each of the two tubes together in the presence of 20 mM MgCl₂. In the reaction, the γ₁ RNA transcript was present at 50 nM, whereas the γ₂ ribosome was present at a 20–100-fold higher concentration. Following incubation at 42°C for 1 h, the reaction was stopped by the addition of 1 volume of stop solution consisting of 95% formamide and 60 mM EDTA, pH 8.0. The resulting cleavage products were analyzed on a 5% polyacrylamide gel containing 7 M urea.

**Cellular Analysis of G protein γ subunit**—For immunoblot analysis, HEK cells stably expressing the D₁ or D₅ dopamine receptor were transfected with either 60–80% of the tandem repeat construct (25) or mock transfected. At 72 h post-transfection, the cells were harvested. The membrane fractions were prepared from control and ribozyme-treated cells and then extracted overnight in the cold room with 1% sodium cholate (23, 25). The concentrations of the cholate-solubilized proteins were determined by Amido Black assay (37).

**RESULTS**

**Characterization of HEK 293 Cell Lines Stably Expressing the D₁ or D₅ Dopamine Receptor**—HEK 293 cells are a useful model for studying D₁ or D₅ dopamine receptors that are linked to the adenylate cyclase (11). Accordingly, we generated clonal cell lines stably expressing one or the other dopamine receptor subtype. Expression was confirmed by radioligand binding and functional coupling to adenylate cyclase. As shown in Fig. 1A, saturation binding studies performed on intact cells stably expressing the D₁ dopamine receptor revealed that the antagonist [³²P]CTP (10 μCi/ml, 3000 Ci/mmol; PerkinElmer Life Sciences) in the reaction. To perform the cleavage with the D₁ ribozyme (RZm1) instead of the wild type or mutant ribozyme (RZm2) retained catalytic activity but scrambled the flanking arms. The cleavage activities of the wild type and mutant γ₁ ribosomes were confirmed in a cell-free system, as described previously (23).

**To demonstrate functional coupling with adenylate cyclase,
control and receptor-expressing cell lines were treated with the selective D₁ dopamine receptor agonist (SKF81297). As shown in Fig. 1C, control cells showed no elevation of cAMP accumulation in response to SKF81297. By contrast, the D₁ and D₅ dopamine receptor-expressing cells displayed comparably large increases in cAMP accumulation following exposure to 1 μM SKF81297 for 5 min. Taken together, these results show that both receptor subtypes were similarly expressed in the plasma

**Fig. 1.** Characterization of HEK 293 cells expressing the D₁ or D₅ dopamine receptor subtype. A, cell surface expression of the D₁ dopamine receptor. B, cell surface expression of the D₅ dopamine receptor. C, agonist-stimulated adenylylcyclase activity in cells expressing the D₁ or D₅ dopamine receptor. Wild type cells (HEK 293) and cells expressing either the D₁ dopamine receptor (D₁ Receptor) or HEK 293 or the D₅ dopamine receptor (D₅ Receptor) were stimulated for 5 min with no (Ro) or 1 μM SKF81297 (SKF) in the presence of 100 μM Ro 20-1724 and then extracted for measurement of cAMP accumulation. The data shown are the means ± S.E. from three to five experiments.
membrane, where they functionally couple with $G_\alpha$ and adenyllylcyclase.

**Effect of $\gamma_7$ Ribozyme on Cleavage of $\gamma_7$ RNA in Vitro**—To compare the involvement of the G protein $\gamma_7$ subunit in the dopaminergic receptor signaling pathways, a ribozyme was designed to target the GUC sequence at positions 11001 to 11005 in relation to the translational start site of the $\gamma_7$ mRNA. The $\gamma_7$ ribozyme was chemically synthesized as a DNA-RNA chimera (Fig. 2A). The flanking sequences, which confer the specific binding to the $\gamma_7$ mRNA, were composed of deoxyribonucleotides (in capital letters), whereas the catalytic core region, which confers the cleavage activity, was composed of ribonucleotides (in lowercase letters). Two phosphorothioate linkages (\*S) were added to the 3'-end to enhance the stability of the ribozyme. The first mutant ribozyme (RZm1) contains scrambled flanking regions as well as two nucleotide substitutions in the catalytic core region (underlined). The second mutant ribozyme (RZm2) contains the scrambled flanking regions only.

**B**, cleavage activities of the wild type and mutant ribozymes. The radiolabeled $\gamma_7$ RNA transcript was incubated with either wild type ribozyme (RZ) or mutant ribozyme (RZm1) at the indicated ribozyme to template ratios. The $\gamma_7$ RNA transcript and its cleavage products were resolved by electrophoresis and visualized by autoradiography.

To assess the cleavage activity in vitro, the wild type or mutant $\gamma_7$ ribozyme (RZ or RZm1, respectively) was incubated with $\gamma_7$ RNA transcript at varying ribozyme to template ratios for 1 h. Addition of the mutant $\gamma_7$ ribozyme did not result in cleavage of the $\gamma_7$ RNA transcript even at a 100-fold excess of ribozyme to template (Fig. 2B). However, addition of the wild type $\gamma_7$ ribozyme readily induced cleavage of the $\gamma_7$ RNA transcript, yielding products of the expected sizes (indicated by arrows). Taken together, these results confirmed that the wild type $\gamma_7$ ribozyme showed the expected cleavage activity toward the $\gamma_7$ RNA transcript, whereas the mutant $\gamma_7$ ribozyme (RZm1) does not.

**Effect of $\gamma_7$ Ribozyme on the G Protein $\gamma_7$ Protein Level**—Having previously established the ability of the wild type $\gamma_7$ ribozyme to specifically suppress the level of the $\gamma_7$ mRNA in intact cells (23), the present study confirmed that mRNA suppression was paralleled by a reduction in the amount of $\gamma_7$ protein. For this purpose, D1 and D5 dopamine receptor-expressing cells were transfected with either no ribozyme (CON), wild type $\gamma_7$ ribozyme (RZ), or mutant $\gamma_7$ ribozymes (RZm1/2).
Fig. 3. Ribozyme suppression of the γ7 protein and concomitant reduction of the β1 protein. A, representative immunoblot of ribozyme suppression of the γ7 protein and concomitant reduction of the β1 protein. D1 dopamine receptor-expressing cells were transfected with no (CON), wild type γ7 ribozyme (RZ), or mutant γ7 ribozymes (RZm1 or RZm2). At 72 h post-transfection, the membrane proteins were extracted with 1% cholate, and equal amounts of membrane proteins (180 µg/lane) were resolved on 15% polyacrylamide-SDS gels, transferred to nitrocellulose, and immunoblotted. Following transfer, the nitrocellulose blots were cut along the 30-kDa marker; the bottom halves were probed with the γ7-specific antibody (A-67), and the top halves were probed with either the β1 antibody (B-69) or the G protein αs-specific antibody (584). The αs-specific antibody recognizes two alternatively spliced proteins of 45 and 52 kDa. B, quantitation of ribozyme suppression. The intensities of the bands were determined by PhosphorImager analysis. The relative amounts of proteins were expressed as percentages of the control levels. In the case of the αs protein, the sum of the two alternatively spliced species is plotted. The data shown are the means ± S.E. obtained from at least three separate experiments (n = 4–12).

At 72 h after transfection, membrane fractions were prepared from these cells and evaluated by immunoblotting with various antibodies. Fig. 3A shows a representative immunoblot in which the bottom half of the gel was blotted with antibody (A-67) specific for the G protein γ7 subunit (23, 27), and the top half of the gel was blotted with antibodies (B-69 and 584) specific for the G protein β1 and αs subunits, respectively (41). Of particular interest, the wild type γ7 ribozyme produced a dramatic reduction in the level of the γ7 protein in both the D1 and D5 dopamine receptor-expressing cell lines. Along with loss of the γ7 subunit, there was also a significant reduction in the amount of the β1 protein, in agreement with results of our previous papers showing a functional association between these two proteins in HEK 293 cells (23, 25).

Fig. 3B shows the quantitation of these results in which the relative amounts of the γ7, β1, and αs proteins were expressed as percentages of their control levels. Based on this analysis, the level of the γ7 protein was markedly suppressed in both the D1 and D5 dopamine receptor-expressing cells transfected with the wild type γ7 ribozyme (for D1 dopamine receptor-expressing cells, 20 ± 6%, n = 7; for D5 dopamine receptor-expressing cells, 18 ± 6%, n = 4) compared with control cells. By comparison, the level of the γ7 protein was not reduced in cells transfected with the two mutant γ7 ribozymes (for D1 dopamine receptor-expressing cells, 111 ± 18%, n = 7 for RZm1 and 125 ± 23%, n = 4 for RZm2; for D5 dopamine receptor-expressing cells, 101 ± 19%, n = 4 for RZm1 and 93 ± 4%, n = 4 for RZm2). These data demonstrated that suppression of the γ7 protein was a specific consequence of the wild type γ7 ribozyme and that the effect was similar in both the D1 and D5 dopamine receptor-expressing cells. Moreover, the wild type γ7 ribozyme had no impact on the levels of other G protein subunits, such as the αs and the γ7-like subunits (data not shown), attesting to the specificity of this effect.

Next, we asked whether loss of the γ7 subunit had any effect on the expression of the associated β1 and αs subunits that comprise the Gs heterotrimer (Fig. 3B). Previously, we showed that expression of the β1 and γ7 proteins are tightly linked in HEK 293 cells (23, 25), consistent with their functional interaction to form a β1γ7 dimer. Confirming this finding, we show that the level of the β1 protein was substantially reduced in both D1 and D5 dopamine receptor-expressing cells transfected with the wild type γ7 ribozyme (for D1 dopamine receptor-expressing cells, 50 ± 5%, n = 4; for D5 dopamine receptor-expressing cells, 57 ± 8%, n = 4) compared with control cells. Moreover, the level of the β1 protein was not significantly altered in cells transfected with the two mutant γ7 ribozymes (for D1 dopamine receptor-expressing cells, 93 ± 11%, n = 4 for RZm1 and 90 ± 10%, n = 4 for RZm2; for D5 dopamine receptor-expressing cells, 117 ± 12%, n = 4 for RZm1 and 98 ± 17%, n = 4 for RZm2). By comparison, the total level of the αs proteins (sum of the 45- and 52-kDa bands) was only slightly reduced in cells transfected with the wild type γ7 ribozyme (for D1 dopamine receptor-expressing cells, 82 ± 5%, n = 4; for D5 dopamine receptor-expressing cells, 65 ± 14%, n = 4) compared with control cells. Taken together, these results indicated that ribozyme-mediated loss of the γ7 protein occurs in concert with suppression of the β1 protein and, to a lesser extent, the αs protein.

Effect of Ribozyme-mediated Loss of the G Protein γ7 Subunit on the D1 and D5 Dopamine Receptor Signaling Pathways—Receptor interaction requires the combined interaction of the G
protein α, β, and γ subunits (19, 21). Therefore, ribozyme-mediated loss of the γ subunit would be expected to compromise functional coupling to its associated receptor. Accordingly, we compared the relative dependence of the D1 and D5 dopamine receptor signaling pathways on the G protein γ subunit. For this purpose, cells were transfected with either no ribozyme (CON), wild type γ subzyme (RZ), or mutant γ subzymes (RZm1 or RZm2). At 72 h post-transfection, the cells were stimulated for 5 min with either 1 μM SKF81297 (SKF), 1 μM isoproterenol (ISO), or 1 μM prostaglandin E1 (PGE1) in the presence of 100 μM Ro 20–1724 followed by measurement of cAMP accumulation. The data shown are the means ± S.E. obtained from at least three separate experiments (n = 16–22). B, D5 dopamine receptor cells. HEK 293 cells expressing the D5 dopamine receptor were transfected with either no (CON) or wild type γ subzyme (RZ). At 72 h post-transfection, the cells were stimulated for 5 min with either 1 μM SKF81297 (SKF) or 1 μM isoproterenol (ISO) in the presence of 100 μM Ro 20–1724 followed by measurement of cAMP accumulation. The data shown are the means ± S.E. obtained from at least three separate experiments (n = 6–22). To extend these results to a more physiologic setting, we next performed single-cell reverse transcriptase-PCR analysis to localize these signaling components within neurochemically distinct neurons of the rat striatum (31). In this regard, studies have shown that neurons containing substance P (SP) and projecting to the substantia nigra express the D1 dopamine receptor (12, 29), whereas neurons containing enkephalin (ENK) and projecting to the globus pal-
lidus express the D₃ dopamine receptor (42). Fig. 5 shows the results of this analysis for single neurons representative of each type. Of the 10 neurons analyzed, three neurons resulted in amplification of PCR product for SP but not for ENK from first strand cDNA (for example, Neuron 1). Those neurons expressing SP also generated PCR products for the D₁ dopamine receptor and the G protein γ₇ subunit. By contrast, two neurons resulted in detection of PCR products for ENK but not for SP (for example, Neuron 10). Those neurons expressing ENK did not generate PCR products for the D₁ dopamine receptor or the G protein γ₇ subunit. One neuron expressing both SP and ENK resulted in amplification of PCR product for the G protein γ₇ subunit but not for the D₁ dopamine receptor, whereas the remaining four neurons did not result in amplification of any of these PCR products (data not shown). Although only a small number of neurons were sampled, this analysis indicated that a subset of neurons co-express the D₁ dopamine receptor and the G protein γ₇ subunit, consistent with their involvement in a common signaling pathway.

**DISCUSSION**

A growing body of evidence suggests that the G protein βγ dimer composition is an important determinant for receptor recognition (15–23). In the present study, a ribozyme approach is used to provide further support for this hypothesis by showing that the closely related D₁ and D₃ dopamine receptors utilize distinct Gα proteins that vary in their βγ subunit composition.

**Ribozyme Suppression of the G Protein γ₇ Subunit—**Ribozymes are powerful tools for suppressing gene expression and studying the functional consequences thereof. In the present study, ribozymes directed against the γ₇ mRNA (23) were introduced into HEK 293 cell lines stably expressing either the D₁ or the D₃ dopamine receptor. After 72 h, the level of the γ₇ protein was reduced to a similar extent in both cell lines transfected with the wild type γ₇ ribozyme (Fig. 3). Attesting to the specificity of ribozyme action, the amount of the γ₇ protein was not altered in both cell lines transfected with the mutant γ₇ ribozymes. Associated with the ribozyme-mediated loss of the γ₇ protein, the level of the β₁ protein was also reduced in both cell lines (Fig. 3). As shown previously, this occurs when there is not a sufficient amount of the γ₇ protein to dimerize with the β₁ protein to prevent its degradation (23, 25). The expression of the α₇ proteins was only slightly reduced (Fig. 3). Presumably, this reflects a lesser dependence of the α₇ proteins on the presence of the β₁γ₇ subunit complex for stabilization. Although not directly examined, this is consistent with studies showing the α subunits contain their own membrane targeting signals (43). Taken together, these results demonstrate the ability of the wild type γ₇ ribozyme to suppress the G protein γ₇, β₁, and α₇ proteins in that order.

**Dependence of the D₁ Dopamine Receptor Subtype on the G Protein γ₇ Subunit for Activation of Adenylyl cyclase Activity—**Both D₁ and D₃ dopamine receptor-expressing cell lines contained a similar number of receptors in the plasma membrane and showed comparable levels of SKF81297-stimulated cAMP accumulation (compare Fig. 1). Nevertheless, the D₃ dopamine receptor-expressing cell line transfected with the wild type γ₇ ribozyme showed no attenuation of SKF28197-induced cAMP accumulation compared with the 32% reduction observed in the D₁ dopamine receptor-expressing cell line (Fig. 4). That this difference is real is shown by the similar reduction in the levels of the γ₇, β₁, and α₇ proteins in both cell lines (Fig. 3) and by the comparable attenuation in the levels of isoproterenol-stimulated cAMP accumulation (Fig. 4). Taken together, these results are most consistent with the notion that the D₁ and D₃ dopamine receptor subtypes recruit different Gα heterotrimers to mediate this response. Furthermore, these findings suggest that the D₁ dopamine receptor interacts with a form of Gα containing the β₁ and γ₇ subunits, whereas the D₃ dopamine receptor interacts with a form of Gα containing a different combination of β and γ subunits.

**Implications for Specificity of the D₁ and D₃ Dopamine Receptor Signaling Pathways in Vivo—**The results obtained from this heterologous expression system are intriguing because they point to intrinsic differences between the two receptor subtypes in terms of their G protein coupling properties. Future structure-function mapping of the two receptor subtypes

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"J. D. Robishaw, unpublished observations."
should allow the molecular basis for this to be identified. In this regard, fluorescence energy transfer (15) and cross-linking (16) studies have identified direct interactions between receptor and the G protein βγ dimer. Although cross-linking studies have yet to identify contact site(s) between the receptor and the γ subunit, numerous reconstitution studies have underscored the importance of the carboxyl-terminal region of the γ subunit in receptor recognition, with both primary structure and type of prenyl group contributing to the specificity (17, 18, 21). Thus, these results lay the groundwork for more precisely identifying the molecular determinants specifying the differential interaction of the D1 and D2 dopamine receptors with the γ subunit and, ultimately, in designing tools to selectively disrupt their individual signaling pathways.

On the other hand, we recognize that results obtained from a heterologous expression system do not constitute proof that the D1 dopamine receptor subtype interacts with a form of Gs containing the γ subunit in a native system. However, in support of this possibility, we used reverse transcriptase-PCR analysis of individual, striatal neurons (31, 32) to show that the D1 dopamine receptor and G protein γ7 subunit are predominantly and coordinately expressed in SP containing neurons that project to the substantia nigra par reticulata. Intriguingly, the Gng7 locus encoding the G protein γ subunit has been localized to mouse chromosome 10 in the vicinity of several neurological mutations, such as jittery, Ames Waltzer, and mocha (13). Thus, on the basis of these findings, the G protein γ7 subunit should be considered as a candidate for these and other genetic disorders that map near this region in the future.

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