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Selective Inhibition of Adenylyl Cyclase Type V by the Dopamine D_3 Receptor

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SUMMARY

Despite a great deal of research, the second messenger coupling of the dopamine D_3 receptor has not yet been clearly established. The closely related D_2 and D_4 receptors have been shown to inhibit adenylyl cyclase activity in a variety of cell types, but the D_3 receptor has little or no effect on this second messenger system. We now demonstrate that when the D_3 receptor and adenylyl cyclase type V are coexpressed in 293 cells, the agonist quinpirole causes 70% inhibition of forskolin-stimulated cAMP levels. This effect seems to be selective for this adenylyl cyclase isoform because the D_3 receptor does not inhibit adenylyl cyclase types I or VI and only weakly stimulates adenylyl cyclase type II. In contrast, the D_2 receptor inhibits cAMP accumulation in 293 cells in the absence of cotransfected adenylyl cyclases and stimulates adenylyl cyclase type II to a greater extent than the D_3 receptor. The inhibition of adenylyl cyclase type V by the D_3 receptor is sensitive to pertussis toxin, suggesting the involvement of G proteins of the G_i family. Guanosine-5′-*O*-(3-thio)triphosphate binding studies indicate that the D_3 receptor weakly activates all three $G_{i\alpha}$ subunits, whereas the D_2 receptor activates these G proteins to a substantially greater extent. However, despite its relative inability to promote G protein activation, the D_3 receptor is capable of substantial and consistent inhibition of adenylyl cyclase type V. The robust second messenger coupling of the D_3 receptor in a heterologous system with defined components provides a system for further studies of the function of this receptor and should facilitate the development and characterization of new D_3 receptor ligands.

Dopamine is an important neurotransmitter that is involved in the control of locomotor activity, emotion and affect, and neuroendocrine secretion. The actions of dopamine are mediated by a family of receptors that are part of the large superfamily of G protein-coupled receptors. On the basis of their structure, pharmacology, and signaling properties (for reviews, see Refs. 1 and 2), the dopamine receptors are divided into two subgroups: D_1-and D_2-like receptors. The dopamine D_3 receptor has been of particular interest because of its high affinity for the neuroleptic drugs used to treat schizophrenia and its localization within limbic areas of the brain, which are presumed to be involved in the control of emotion and affect.

The structure of the D_3 receptor is typical of G protein-coupled receptors that are coupled to G_i and mediate the inhibition of AC. This receptor has a large third intracellular loop and a short carboxyl-terminal tail, ending at the putatively palmitoylated cysteine residue. In addition, the D_3 receptor is highly homologous to the D_2 receptor, which inhibits AC (1, 2). Thus, after its cloning, it came as a surprise that the D_3 receptor did not inhibit AC in transfected cells (3). Since that time, the D_3 receptor has been shown to inhibit cAMP accumulation in some cases (4–7) as well as modulate other second messenger pathways that are affected by the D_2 receptor, such as ion channel activity and stimulation of Na^+/H^+ exchange (4, 8). However, the modulation of these signaling pathways by the D_3 receptor is quite weak, especially in comparison with the effects of the D_2 receptor on the same pathways. In addition, the modulation of agonist binding to the D_3 receptor by guanine nucleotides, an indicator of receptor/G protein coupling, often is not observed (3, 5, 7, 9, 10). Thus, to date it has seemed that the D_3 receptor does not modulate any second messenger system to a substantial extent.

The D_3 receptor is expressed only in the central nervous system, with a very restricted distribution and generally low levels of expression in the brain. This, coupled with a lack of agonists and antagonists that are highly selective between

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ABBREVIATIONS: AC, adenylyl cyclase; ACI, adenylyl cyclase type I; ACII, adenylyl cyclase type II; ACV, adenylyl cyclase type V; ACVI, adenylyl cyclase type VI; FBS, fetal bovine serum; GTPγS, guanosine-5′-*O*-(3-thio)triphosphate; PTX, pertussis toxin; PBS, phosphate-buffered saline; IP, immunoprecipitation; PGE_1, prostaglandin E_1; EGF, epidermal growth factor.

the D_2 and D_3 receptors, has made it difficult to examine the biochemical and physiological functions of the D_3 receptor. As a result, D_3 receptor signaling has been studied almost exclusively by its expression in heterologous cell lines that usually are not neuronal. This raises the possibility that the lack of signaling by the D_3 receptor could be the result of the absence of essential components of a signal transduction pathway in the cultured cells that have been used to study this receptor.

ACV was cloned independently by three groups, from canine heart (11), rat liver (12), and rat striatum (13). This AC is stimulated by $G_{s\alpha}$, inhibited by $G_{i\alpha}$ and Ca^{2+}, and unaffected by $\beta\gamma$ subunits (for reviews, see Refs. 14 and 15). In addition, ACV is stimulated by protein kinase C phosphorylation and inhibited by protein kinase A phosphorylation (16, 17). This AC isoform seems to be fairly widely expressed in the periphery (11, 12); however, Northern blotting of brain structures indicated that ACV is only expressed in the striatum. Furthermore, *in situ* hybridization in the rat brain demonstrated that it is expressed in the nucleus accumbens and olfactory tubercle, in addition to the striatum (13). More detailed *in situ* hybridization studies revealed that ACV is expressed in other brain regions at lower levels, including the islands of Calleja, and most strikingly, that its distribution is limited to dopaminergically innervated regions (18).

The correlation of ACV localization with regions of expression of dopamine receptors led us to hypothesize that this effector could be involved in the signal transduction of the dopamine receptors. In particular, its expression in brain areas in which the D_3 receptor is present, such as the nucleus accumbens, olfactory tubercle, and islands of Calleja, suggested that the D_3 receptor might regulate ACV activity. We coexpressed the D_3 receptor with various AC isoforms in human embryonic kidney 293 cells to examine the ability of this receptor to inhibit specific AC subtypes.

Experimental Procedures

Materials. ACV (canine) cDNA was the gift of Dr. Y. Ishikawa (Columbia University, New York, NY). ACVI (rat) cDNA was the gift of Dr. R. Premont (Duke University Medical Center, Durham, NC), and D_3 receptor (human) cDNA was from Dr. B. Sahagan (DuPont-Merck Pharmaceutical, Wilmington, DE). $G_{i\alpha}$ subunit cDNAs were the gift of Dr. P. Casey (Duke University Medical Center). Antiserum 116 was the gift of Dr. D. Manning (University of Pennsylvania, Philadelphia, PA), and antiserum EC/2 was from DuPont-New England Nuclear (Boston, MA). Cell culture reagents were from Cellgro (Herndon, VA) except for FBS, which was from Atlanta Biologicals (Norcross, GA). Quinpirole was obtained from Research Biochemicals (Natick, MA), and other chemicals were from Sigma Chemical (St. Louis, MO). [^{3}H]Adenine, [^{14}C]cAMP, and [^{35}S]GTPγS (1250 Ci/mmol) were from DuPont-New England Nuclear. [^{3}H]Spiperone (95–105 Ci/mmol) was from Amersham (Arlington Heights, IL), and PTX was from Calbiochem (La Jolla, CA).

Cell culture. Human embryonic kidney 293 cells (CRL 1573; American Type Culture Collection; Rockville, MD) were grown in minimum essential medium containing 10% FBS and 50 μg/ml gentamicin in a humidified atmosphere of 5% CO_2/95% air at 37°. Cells were transfected by the calcium phosphate method (19). The day before transfection, cells were plated at a density of 2.5×10^6 cells/100-mm dish. On the morning after transfection, the cells were shocked with 15% glycerol in PBS and then supplied with fresh medium. In the afternoon of the same day, cells were plated onto 12-well dishes or 150-mm dishes. Cells were used for assays ~60 hr after transfection. Receptor expression was monitored by saturation binding with [^{3}H]spiperone as previously described (20).

Whole-cell cAMP assay. Transfected cells plated onto 12-well dishes were labeled with media containing 5% FBS, gentamicin, and 1 μCi/ml [^{3}H]adenine overnight before the assay. On the day of the assay, the labeling media was aspirated, and the cells were gently washed with PBS. The assay was carried out as previously described (20). Conversion of [^{3}H]ATP to [^{3}H]cAMP was assessed by sequential chromatography over Dowex and alumina columns as described by Salomon (21). For experiments including treatment with PTX, 100 ng/ml PTX was added with the labeling media.

GTPγS binding assay. Transiently transfected 293 cells were plated onto 150-mm dishes 24 hr after transfection. On the day of the assay, the cells were washed twice with cold PBS and detached from the plate. Cells were collected with a brief spin and resuspended in lysis buffer (10 mM Tris·HCl, pH 7.4, 5 mM EDTA). The [^{35}S]GTPγS binding assay was carried out essentially as described by Barr *et al.* (22). Briefly, membranes were homogenized with a Teflon pestle, centrifuged at 40,000 $\times$ *g*, and resuspended in Tris/$MgCl_2$/EDTA buffer (50 mM Tris·HCl, pH 7.4, 4.8 mM $MgCl_2$, 2 mM EDTA, 100 mM NaCl). Assays contained 20 μg of membranes in Tris/$MgCl_2$/EDTA buffer plus 1 μM GDP, 30 nM [^{35}S]GTPγS, and the indicated drugs in a volume of 62 μl. The reaction was carried out at 30° for 5 min and terminated by the addition of IP buffer (50 mM Tris·HCl, pH 7.4, 20 mM $MgCl_2$, 150 mM NaCl, 0.5% Nonidet P-40, 1 μg/ml aprotinin, 100 μM GDP, 100 μM GTP). Membranes were solubilized for 30 min at 4°. Samples were precleared for 20 min at 4° with 20% protein A-Sepharose (Pharmacia, Piscataway, NJ) in IP buffer containing 2% bovine serum albumin. Supernatants were then transferred to tubes containing antibody, and 100 μl of protein A-Sepharose was added. $G_{i1\alpha}$ and $G_{i2\alpha}$ were immunoprecipitated with antiserum 116, and $G_{i3\alpha}$ was immunoprecipitated with antiserum EC/2. After 1 hr of incubation at 4°, the beads were washed three times with IP buffer and once with wash buffer (50 mM Tris·HCl, pH 7.4, 20 mM $MgCl_2$, 150 mM NaCl, 100 μM GDP, 100 μM GTP). The immune complexes were eluted from the beads with 0.5% sodium dodecyl sulfate at 95° for 5 min, and the eluate, containing immunoprecipitated [^{35}S]GTPγS, was counted in a liquid scintillation counter.

Data analysis. Data are presented as mean $\pm$ standard error unless indicated otherwise. For cAMP assays, cAMP accumulation in the presence of 1 μM forskolin was normalized to 100%, and cAMP levels in the presence of forskolin plus quinpirole were expressed as a percentage of the cAMP accumulation stimulated by forskolin. For [^{35}S]GTPγS binding experiments, binding of [^{35}S]GTPγS in the absence of agonist was normalized to 1, and binding in the presence of agonist was expressed as fold-increase of this value. Dose-response curves were analyzed with the nonlinear curve-fitting program Prism (GraphPAD Software, San Diego, CA). Statistical analyses were performed using Student's *t* test; $p < 0.05$ was considered statistically significant.

Results

We tested the ability of the D_3 and D_2 receptors to inhibit cAMP accumulation in 293 cells. In cells transiently transfected with the human D_3 receptor, the agonist quinpirole caused no inhibition of forskolin-stimulated cAMP levels. In contrast, when the D_3 receptor was cotransfected with canine ACV, quinpirole caused a 71 $\pm$ 7% inhibition of forskolin-stimulated cAMP (Fig. 1). In comparison, when the human D_2 receptor was transfected alone, agonist evoked a 44 $\pm$ 9% inhibition of forskolin-stimulated cAMP accumulation, whereas when the D_2 receptor and ACV were cotransfected, this inhibition increased to 87 $\pm$ 4% (Fig. 1). Thus, the extent of inhibition of ACV by D_2 and D_3 receptors was not significantly different ($p > 0.05$), and inhibition of ACV by the D_3

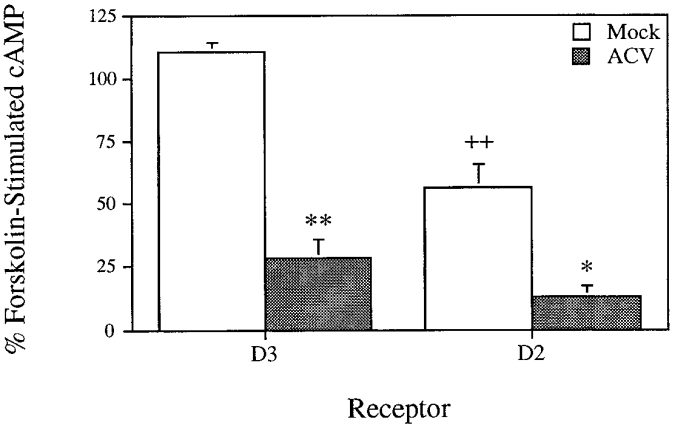

Fig. 1. Inhibition of ACV by D_3 and D_2 receptors. 293 cells were transfected with receptor alone (*Mock*) or receptor and ACV. cAMP accumulation in the presence of 1 μM forskolin plus 10 μM quinpirole was normalized as described in Experimental Procedures. Forskolin-stimulated cAMP percent conversion was as follows. D_3 receptor, 0.73 ± 0.29; D_3 receptor plus ACV, 5.4 ± 1.6; D_2 receptor, 0.64 ± 0.17; and D_2 receptor plus ACV, 4.9 ± 1.5. Data are mean ± standard error for three independent experiments. Significant decrease compared with *Mock*, *, $p < 0.01$ and **, $p < 0.0005$. Significant decrease compared with forskolin-stimulated cAMP, ++, $p < 0.05$.

receptor was more effective than inhibition of endogenous 293 cell ACs by the D_2 receptor. Inhibition of ACV by both the D_3 and D_2 receptors was also observed in COS-7 cells. The D_3 receptor caused 53 ± 9% inhibition of ACV and the D_2 receptor caused 70 ± 12% inhibition of ACV in COS-7 cells.

In all experiments, we attempted to match the levels of receptor expression between the conditions being compared. For example, in the experiments presented in Fig. 1, receptor expression was as follows: D_2 receptor, 5.2 ± 0.9 pmol/mg of protein; D_2 receptor plus ACV, 6.1 ± 0.9 pmol/mg of protein; D_3 receptor, 6.6 ± 1.9 pmol/mg of protein; and D_3 receptor plus ACV, 6.6 ± 1.9 pmol/mg of protein. Receptor expression among the different conditions was not significantly different as determined by analysis of variance. Receptor expression levels varied between 1 and 4 pmol/mg of protein in the remaining experiments and were never significantly different between the conditions being compared.

To determine the specificity of the inhibition of ACV by the D_3 receptor, both the D_3 and D_2 receptors were tested for their ability to affect the activity of other AC isoforms. ACs are divided into three major subgroups. Members of each of the subfamilies were tested for modulation of their activity by the D_3 and D_2 receptors. ACI, which is a member of the Ca^{2+}/calmodulin-stimulated subgroup, and ACII, which is the prototypical $\beta\gamma$-stimulated isoform, were tested. ACVI, which belongs to the same subfamily as ACV, was also tested.

The D_3 receptor was unable to inhibit forskolin-stimulated ACI (Fig. 2A). Interestingly, the D_2 receptor caused only a 13 ± 2% inhibition of cAMP in the presence of ACI, whereas when the D_2 receptor was expressed alone, it inhibited cAMP accumulation by 37 ± 11% (Fig. 2B). This difference is significant ($p < 0.05$), suggesting that the D_2 receptor does not inhibit ACI activity as effectively as it inhibits other AC isoforms present in 293 cells. Similar results were obtained when PGE_1, an endogenous receptor that activates $G_{s\alpha}$, was used to stimulate cAMP formation (data not shown). In these experiments, the forskolin-stimulated cAMP accumulation

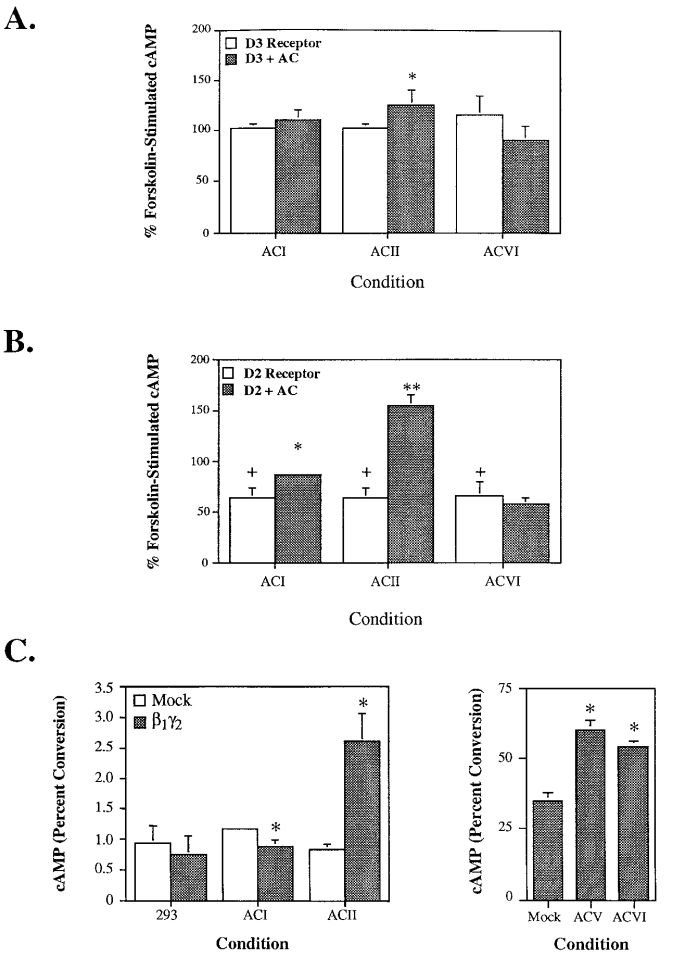

Fig. 2. Effect of D_3 and D_2 receptors on cAMP accumulation in 293 cells. A, The D_3 receptor was transfected in 293 cells with or without the indicated AC isoforms. B, Transfection of the D_2 receptor in the presence or absence of the indicated AC isoforms. Data are the mean ± standard error of three to five experiments. cAMP accumulation in the presence of 1 μM forskolin plus 10 μM quinpirole was normalized as described in Experimental Procedures. Forskolin-stimulated cAMP (percent conversion) for each condition was as follows: D_3 receptor, 0.74 ± 0.36; D_3 receptor plus ACI, 0.95 ± 0.31; D_3 receptor plus ACII, 0.58 ± 0.25; and D_3 receptor plus ACVI, 0.89 ± 0.28 (A); and D_2 receptor, 0.69 ± 0.09; D_2 receptor plus ACI, 0.79 ± 0.28, D_2 receptor plus ACII, 0.58 ± 0.19; and D_2 receptor plus ACVI, 0.82 ± 0.22 (B). *, $p < 0.05$; **, $p < 0.01$, significantly different from cAMP accumulation in the presence of receptor alone. +, $p < 0.05$, significant decrease compared with forskolin-stimulated cAMP. C, Confirmation of expression of transfected ACs. *Left*, effect of the absence (*Mock*) or presence of transfected $\beta_1\gamma_2$ subunits on 1 μM forskolin-stimulated cAMP accumulation. *, $p < 0.05$, significantly different from mock transfected cells. *Right*, stimulation of cAMP formation by the simultaneous addition of 1 μM forskolin and 1 μM PGE_1 in mock transfected 293 cells or cells expressing ACV or ACVI. *, $p < 0.05$, significantly different from mock transfected cells.

was not greater in cells transfected with ACI than in 293 cells not expressing this isoform (see legend to Fig. 2). ACI has been shown to be inhibited by G protein $\beta\gamma$ subunits (23). In these experiments, expression of ACI was confirmed by a decrease in forskolin-stimulated cAMP when $\beta1\gamma2$ subunits were cotransfected with ACI, an effect that was not observed when 293 cells were transfected with $\beta1\gamma2$ alone (Fig. 2C).

Stimulation of the ACII isoform by $\beta\gamma$ subunits has been shown to depend on simultaneous receptor-mediated activation of $G_{s\alpha}$ (23, 24). We observed that this stimulation by $\beta\gamma$

subunits is also evident when forskolin is used to activate ACII in place of activation of $G_{s\alpha}$ by hormone. When the D_3 receptor and ACII were coexpressed, forskolin plus the agonist quinpirole significantly stimulated cAMP accumulation to 125 ± 15% of forskolin-stimulated cAMP levels (Fig. 2A). This suggests that the D_3 receptor is capable of providing $\beta\gamma$ subunits required for stimulation of ACII. In comparison, in the presence of forskolin and quinpirole, the D_2 receptor stimulated ACII to a greater extent (155 ± 11%) than the D_3 receptor (Fig. 2B). Similar results were obtained when ACII was activated with PGE_1 (data not shown). Similar to the case for ACI, we did not observe increases in forskolin-stimulated cAMP when ACII was transfected in 293 cells (see legend to Fig. 2). The expression of this isoform was confirmed by an increase in forskolin-stimulated cAMP levels in cells transfected with ACII and $\beta1\gamma2$, whereas this was not observed in cells transfected with $\beta1\gamma2$ only (Fig. 2C).

The D_3 receptor was unable to significantly inhibit ACVI (Fig. 2A), despite its similarity to ACV. Likewise, the D_2 receptor caused no greater inhibition of cAMP accumulation in the presence of ACVI than when the receptor was expressed alone (Fig. 2B). As was the case for ACI and ACII, ACVI does not seem to be substantially stimulated by forskolin in 293 cells (see legend to Fig. 2). However, ACV and ACVI have been observed to be synergistically activated by forskolin and $G_{s\alpha}$ (25). We found that in cells transfected with ACVI, cAMP levels generated by the simultaneous addition of forskolin and PGE_1 were equivalent to those generated in the presence of ACV, whereas a significantly lower amount of cAMP is generated by forskolin and PGE_1 in untransfected 293 cells (Fig. 2C). Thus, the expression levels of ACV and ACVI are presumed to be similar.

The coexpression of the D_3 receptor with ACV was also found to affect the potency of quinpirole to inhibit forskolin-stimulated cAMP levels (Fig. 3). In many experiments, no inhibition of cAMP accumulation by the D_3 receptor was observed (e.g., Fig. 1). However, in some cases, a slight inhibition of forskolin-stimulated cAMP levels occurred. In these experiments, the IC_{50} value of quinpirole was 0.56 ± 0.18 nM. When the D_3 receptor and ACV were coexpressed, the IC_{50} value of quinpirole decreased significantly to 0.26 ± 0.04 nM ($p < 0.05$). Fig. 3 shows one experiment selected from the occasional experiments in which the D_3 receptor inhibited cAMP accumulation in the absence of ACV. The IC_{50} value of quinpirole at the D_2 receptor was not changed by the coexpression of ACV. The increased potency for inhibition of ACV by the D_3 receptor could reflect a situation in which ACV is inhibited more strongly by G proteins activated by this receptor than are other AC subtypes.

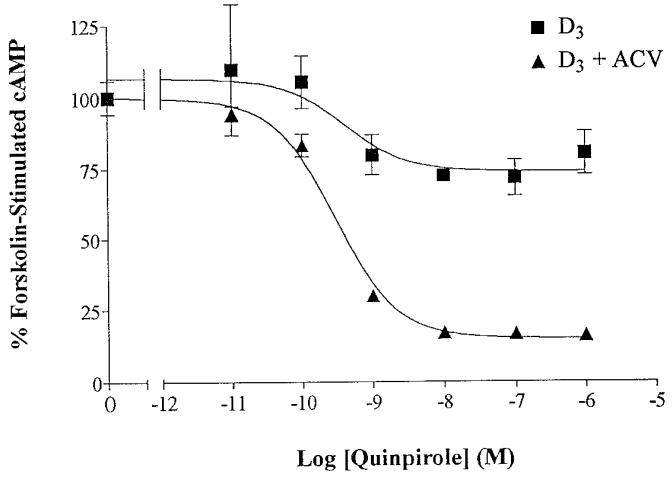

Fig. 3. Dose-response curves for the inhibition of cAMP accumulation by the D_3 receptor in 293 cells. The D_3 receptor was expressed in the absence or presence of ACV (representative of four independent experiments). The experiment was performed in triplicate. *Error bars*, standard deviation. Forskolin-stimulated cAMP (percent conversion): D_3 receptor, 1.5 ± 0.5; D_3 receptor plus ACV, 3.9 ± 2.3. Data were normalized as described in Experimental Procedures.

The involvement of G proteins in the inhibition of ACV by the D_3 receptor was examined. To test for the involvement of members of the G_i class of G proteins, cells were treated overnight before cAMP assay with 100 ng/ml PTX. Treatment with PTX prevented the inhibition of forskolin-stimulated ACV by the D_3 receptor (Table 1). Inhibition of ACV by the D_2 receptor was also sensitive to PTX (data not shown). This suggests that inhibition of ACV by the receptors is mediated through G_i or G_o. Interestingly, PTX treatment decreased the maximal forskolin-stimulated cAMP level that could be obtained by approximately half (Table 1). This effect was observed only when ACV was expressed in 293 cells, not when ACI or ACII were overexpressed or in the absence of transfected ACs (data not shown).

Possible explanations for the apparent selectivity of the inhibition of ACV by the D_3 receptor are that this receptor activates a G protein that specifically inhibits ACV or activates a G protein that inhibits ACV to a greater extent than other ACs. To test this hypothesis, we evaluated the ability of the D_3 and D_2 receptors to activate $G_{i\alpha}$ subunits by using an assay consisting of [^{35}S]GTPγS binding, followed by immunoprecipitation of a $G_{i\alpha}$ subunit. We chose to test $G_{i\alpha}$ subunits because of the PTX sensitivity of the inhibition of ACV by the D_3 receptor. To produce a detectable signal, individual $G_{i\alpha}$ subunits were coexpressed with each receptor and ACV. No receptor-dependent [^{35}S]GTPγS binding was observed in the absence of cotransfected $G_{i\alpha}$ subunits (data not shown).

These experiments indicated that the D_3 receptor weakly activated all three $G_{i\alpha}$ subunits (Fig. 4). Incubation with quinpirole caused a 1.2–1.7-fold increase in [^{35}S]GTPγS binding over that observed in the absence of agonist. In contrast, the D_2 receptor stimulated the activation of $G_{i1\alpha}$ and $G_{i2\alpha}$ subunits to a greater extent than the D_3 receptor. [^{35}S]GTPγS binding was increased ~3-fold by the D_2 receptor when quinpirole was included in the incubation. The activation of $G_{i3\alpha}$ by the D_2 receptor was more variable and was not significant but seemed to be greater than the activation of $G_{i3\alpha}$ by the D_3 receptor. The overall profile of activation of $G_{i\alpha}$ subunits by the D_3 and D_2 receptors is quite similar and

TABLE 1

cAMP accumulation in 293 cells expressing the D_3 receptor and ACV in the presence or absence of PTX

Cells were labeled overnight in the absence or presence of 100 ng/ml PTX. cAMP accumulation was assayed as described in Experimental Procedures in the presence of 1 μM forskolin with or without 10 μM quinpirole. cAMP accumulation is expressed as percent conversion from [^{3}H]ATP to [^{3}H]cAMP.

Values are average ± standard error of four experiments.

	Forskolin	Forskolin + quinpirole
−PTX	5.56 ± 1.26	1.81 ± 0.38[a]
+PTX	2.45 ± 0.56	2.51 ± 0.63

[a] Significantly less than forskolin-treated cAMP ($p < 0.05$).

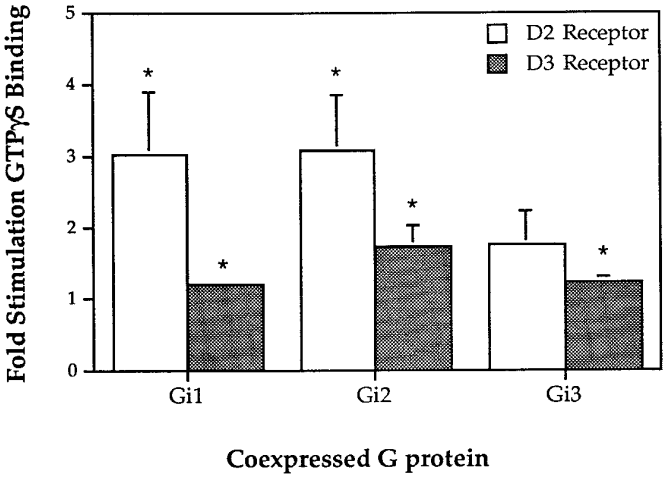

Fig. 4. Binding of GTPγS to overexpressed $G_{i\alpha}$ subunits in 293 cells. The D_2 or D_3 receptor and the indicated $G_{i\alpha}$ subtype were transiently transfected in 293 cells. Data are expressed as fold-increase in [^{35}S]GTPγS immunoprecipitated in the presence of 50 μM quinpirole compared with binding in the absence of agonist (cpm immunoprecipitated: $D_2/G_{i1\alpha}$: −quin, 934 ± 68; +quin, 2881 ± 963; $D_3/G_{i1\alpha}$: −quin, 4561 ± 1075; +quin, 5034 ± 1158; $D_2/G_{i2\alpha}$: −quin, 804 ± 163; +quin, 2313 ± 656; $D_3/G_{i2\alpha}$: −quin, 2097 ± 913; +quin, 3301 ± 921; $D_2/G_{i3\alpha}$: −quin, 944 ± 283; +quin, 1441 ± 452; $D_3/G_{i3\alpha}$: −quin, 8603 ± 1284; +quin, 9949 ± 1837). Values are mean ± standard error for three to five experiments. *, $p < 0.05$, significant increase over [^{35}S]GTPγS binding in the absence of agonist.

differs primarily in the extent of G protein activation, with activation by the D_3 receptor much weaker than that by the D_2 receptor.

Discussion

Despite the expression of the D_3 receptor in numerous cell culture lines, no second messenger pathways for this receptor have been clearly identified. When second messenger coupling has been demonstrated, the magnitude of the response is considerably weaker than that generated by the D_2 receptor. This raised the possibility that these cell lines lack a necessary component of the D_3 receptor signaling pathway. The identification of ACV as being preferentially expressed in dopaminergically innervated brain regions, and particularly its expression in the islands of Calleja and nucleus accumbens (18), in which the D_3 receptor is expressed, suggested that it might be a specific downstream target of the D_3 receptor. Strikingly, this AC subtype does not seem to be expressed in any cultured cell line, although the related ACVI is expressed in several lines (26). In this study, we used the human embryonic kidney 293 cell line. The expression of AC isoforms in these cells has been characterized by several groups using reverse transcription-polymerase chain reaction cloning. It seems that 293 cells express ACVI and ACVII (26–28) and probably also express ACI, ACII, and ACIII (27, 28).

Coexpression of the D_3 receptor and ACV in both 293 and COS-7 cells conferred on the receptor the ability to effectively and consistently inhibit forskolin-stimulated cAMP accumulation, which did not occur in the absence of ACV or the presence of other AC subtypes. Similar results were observed with the D_2 receptor, although a critical difference is that this receptor is able to strongly and consistently inhibit cAMP accumulation in the absence of transfected ACs in 293 cells.

Treatment of the cells with PTX prevented the inhibition of forskolin-stimulated ACV by the agonist quinpirole. This suggested that the D_3 receptor might be coupled to a member of the G_i family. Surprisingly, PTX treatment substantially reduced the extent to which forskolin could stimulate cAMP accumulation in cells expressing ACV but not other ACs. One property of ACV is that it is inhibited by micromolar concentrations of Ca^{2+} (14). A possible explanation for the reduction of forskolin-stimulated activity is that PTX might raise intracellular Ca^{2+} levels sufficiently to inhibit ACV activity but have little or no effect on other ACs. Testing of the effects of PTX on ACVI activity, which is also inhibited by Ca^{2+}, may shed further light on this possibility. Despite this decrease in forskolin-stimulated cAMP in PTX-treated cells, the D_3 receptor did not cause any further inhibition of cAMP accumulation in the presence of PTX.

The coexpression of ACV with the D_3 receptor increased the potency of agonist to inhibit cAMP accumulation compared with the D_3 receptor alone. This suggested that one or more G proteins activated by the D_3 receptor might inhibit ACV more effectively than other AC isoforms. When the ability of the D_3 receptor to activate specific $G_{i\alpha}$ subunits was examined more directly, we found that this receptor weakly activates all three $G_{i\alpha}$ subtypes. The D_2 receptor exhibited a very similar specificity of activation of $G_{i\alpha}$ subunits, although the activation was to a much greater extent (Fig. 4). The activation of $G_{i1\alpha}$ and $G_{i2\alpha}$ by the D_2 receptor was ~3-fold over the basal level, an amount that is consistent with that observed for other receptors by this method (22).

The expression levels of the $G_{i\alpha}$ subunits and the efficiency of the antibodies to immunoprecipitate these subunits were not examined in this study. Therefore, the extent of activation of the $G_{i\alpha}$ subunits by the D_3 receptor cannot be compared, and we cannot make a strong case that $G_{i2\alpha}$ is more activated more robustly by the D_3 receptor than $G_{i1\alpha}$ or $G_{i3\alpha}$. However, the conditions for GTPγS binding for each $G_{i\alpha}$ subtype were identical when either the D_2 or D_3 receptor was expressed. Thus, we can, for example, compare the extent of activation of $G_{i1\alpha}$ by the D_2 and D_3 receptors. In this case, it is clear that the D_2 receptor promotes greater activation of $G_{i\alpha}$ subunits than the D_3 receptor.

These results support our hypothesis that ACV may be more efficiently inhibited by G proteins activated by the D_3 receptor than are other ACs; apparently only a small amount of activated $G_{i\alpha}$ is required to robustly inhibit this AC. Studies using recombinant $G_{i\alpha}$ subunits have shown that the three subtypes are equivalent in their ability to inhibit ACV (29). However, recombinant $G_{i\alpha}$ subunits are clearly able to inhibit ACV to a greater extent than they inhibit forskolin- or $G_{s\alpha}$-activated ACI (29, 30). The small stimulation of forskolin-activated ACII by the D_3 receptor may also be a reflection of the weak G protein activation by this receptor, which would supply only a small amount of free $\beta\gamma$ subunits in the cell. Furthermore, the D_2 receptor activates all three $G_{i\alpha}$ subtypes much more robustly than the D_3 receptor, and this receptor can efficiently inhibit the endogenous 293 cell ACs as well as stimulate transfected ACII to a 2-fold-greater extent than does the D_3 receptor (Fig. 2).

However, the explanation that ACV is inhibited more effectively by $G_{i\alpha}$ subunits than other ACs does not fully account for the specific inhibition of ACV by the D_3 receptor. For example, ACVI is inhibited by recombinant $G_{i\alpha}$ subunits

to a similar extent and with a similar potency as ACV (29), but ACVI, which is endogenously expressed in 293 cells, does not seem to be inhibited by the D_3 or D_2 receptors (Fig. 2). This suggests that there could be differences in the actions of G proteins on ACV and ACVI or that other signaling molecules are involved in this process. One possibility is that the D_3 receptor, G proteins, and ACV are compartmentalized within the cell in such a way that preferential coupling between these components occurs, whereas ACVI, and perhaps other ACs, is excluded from this compartment. Another formal possibility is that a complex of the D_3 receptor and G_i subunits exists that exhibits increased potency for inhibition of ACV but not other ACs. Additional studies are necessary to elucidate the mechanism by which the D_3 receptor inhibits ACV in greater detail, by, for example, testing the ability of the D_3 receptor to activate other G protein subtypes.

The D_3 receptor seems to demonstrate remarkable specificity of signaling for ACV rather than other ACs. This selectivity at the level of an effector may be a general mechanism for tailoring second messenger signals in a particular cell. In cardiac myocytes, the EGF receptor has been shown to stimulate cAMP accumulation. This effect seems to require the expression of ACV and does not occur when ACI, ACII, or ACVI is expressed (31). The mechanism of stimulation of cAMP accumulation by the EGF receptor is via activation of $G_{s\alpha}$ (32), and an antibody against $G_{s\alpha}$ blocks the stimulation of ACV by the EGF receptor in 293 cells (31). This suggests that the mechanisms determining specificity of receptor/AC coupling are not yet fully understood.

In summary, in heterologous systems, the D_3 receptor does not activate G proteins robustly, as reflected by our measurements of [^{35}S]GTPγS binding, as well as by its lack of a shift in agonist affinity in the presence of GTP and its weak modulation of second messengers in most cell lines (see above). However, when it is coexpressed with ACV, as it may be *in vivo*, D_3 receptor activation results in a significant signal (i.e., inhibition of cAMP accumulation). This suggests that the D_3 receptor may generate meaningful second messenger signals *in vivo*. This also raises the possibility that specific isoforms of other dopamine receptor effectors, such as K^+ or Ca^{2+} channels, may exist that are more sensitive to the weak G protein activation generated by the D_3 receptor than those present in the cultured cells examined to date.

The physiological significance of results obtained in transfected cell lines is often difficult to interpret. In these experiments, the D_3 receptor and ACV were expressed at higher levels than those that presumably exist *in vivo*; therefore, demonstration of selective coupling of the D_3 receptor to ACV is subject to the usual caveat of results obtained with overexpressed proteins in heterologous systems. However, regardless of whether inhibition of ACV plays an important role in the function of the D_3 receptor *in vivo*, our discovery of the generation of a robust second messenger signal by this receptor in a defined environment is highly significant. First, it indicates that this receptor is capable of substantially modulating second messenger levels under some conditions, which had previously been unclear. Second, this system provides a tool for further study of the D_3 receptor. Through coexpression of the D_3 receptor and ACV, new studies on the biology of this receptor can be undertaken, such as experiments designed to examine the regulation of the activity of this receptor. In addition, this discovery provides a system for the positive identification of D_3 receptor agonists and antagonists. The development of ligands that are selective for the D_3 receptor and clearly demonstrated to be agonists or antagonists is an important and necessary step in elucidating the physiological role of this poorly understood receptor.

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