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Transcription mediated by a cAMP-responsive promoter element is reduced upon activation of dopamine D_2 receptors

(adenylyl cyclase pathway/G protein/membrane receptor)

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ABSTRACT **Dopaminergic D_2 receptors mediate the effect of dopamine on cellular effector systems by means of guanine nucleotide-binding proteins (G proteins). The major biochemical effect evoked by these receptors is the inhibition of adenylyl cyclase. As a consequence, the activation of D_2 receptors lowers the intracellular cAMP level. Two cDNAs, originated by alternative splicing of the same gene, have been isolated: D_2A and D_2B. They code for two proteins of 444 and 415 amino acids. These proteins display high affinity for selective D_2 dopamine ligands. D_2A differs from D_2B by an insertion of 87 nucleotides in its cDNA, which is located in a region of the protein considered important for the coupling to G proteins. To investigate functional differences between the two dopamine D_2 receptor isoforms, we transiently expressed them in cultured cells. To do so we developed an assay to study membrane receptors that are coupled to the adenylyl cyclase. Using this assay, we were able to show that the stimulation of the adenylyl cyclase induced by the activation of the β_2-adrenergic receptor is inhibited more efficiently by D_2B than D_2A. The effects elicited by the D_2 receptors are mediated by pertussis toxin-sensitive G proteins. Treatment of transfected cells with pertussis toxin abolishes the inhibitory effects in a dose- and receptor isoform-dependent manner. Our results suggest that the two dopamine receptor isoforms are differentially coupled to G proteins.**

Cells receive signals from their environment through molecules of different origin, which interact with membrane components. A well-defined class of these components is represented by membrane receptors characterized by the presence of seven putative transmembrane domains (1). These receptors regulate cell physiology by the induction of intracellular second messengers. To elicit this physiological effect, the receptors need to interact with guanine nucleotide-binding proteins (G proteins) (2). We have cloned one member of this family, the mouse dopamine D_2 receptor (3). Activation of this receptor upon ligand binding provokes a rapid decrease of the intracellular cAMP level by a mechanism that involves coupling to inhibitory G proteins (G_i proteins) (4). It has also been shown that this receptor is able to modulate other signal transduction pathways, depending on the cell type (5). The isolation of the D_2 receptor gene has revealed the presence of two isoforms generated by a mechanism of differential splicing (ref. 3 and references therein). The putative proteins encoded by these cDNAs are 444 amino acids (D_2A) and 415 amino acids (D_2B) long. These two cDNAs are identical except for the insertion of 87 nucleotides in the region corresponding to the putative third intracytoplasmic domain of the proposed G protein-coupled membrane receptor's structure (1). This region seems to be involved in the interaction of the receptors with G proteins (6).

To determine whether their differential structure could influence their ability to couple to G proteins, we decided to analyze the expression of the two isoforms in cultured cells. We therefore developed a rapid assay that allowed us to transiently express these receptors, in different cell types, and to study their functional coupling to G proteins. We were able to monitor in a transfection assay the activity of the reporter gene whose transcription is sensitive to stimulation or inhibition of the cAMP pathway. Hence, this assay is likely to provide evidence for the receptor–G protein–effector interaction. In particular, we were able to show that both D_2 dopamine receptor isoforms expressed in a human choriocarcinoma cell line, JEG-3, inhibit transcription induced by a receptor that activates the cAMP pathway, but at different levels. Furthermore, pertussis toxin (PTX) studies suggest coupling of these receptors to G_i proteins, even though a differential dose–response to the PTX concentration is obtained, depending on the receptor isoform tested. Taken together these data suggest a differential coupling of these two receptors to G proteins.

MATERIALS AND METHODS

Reagents. Forskolin, 3-hydroxytyramine hydrochloride, (−)-epinephrine (+)-bitartrate salt, (−)-isoproterenol (+)-bitartrate salt, and PTX were purchased from Sigma; (+)-butaclamol was purchased from Research Biochemical (Natick, MA); LY171555 (quinpirole) and ICI118551 were a generous gift from N. Amlaiky (URA DO 589 Centre National de la Recherche Scientifique).

Expression Vectors. For eukaryotic expression, the following expression vectors were used: $pSVD_2A^+$ (sense orientation), $pSVD_2A^-$ (antisense orientation), and $pSVD_2B^+$ or $pSVD_2B^-$ containing the D_2A and D_2B cDNAs in pSG5 (3). The reporter plasmid pSomCAT (7) was used; it contains a 20-base-pair (bp) synthetic oligodeoxynucleotide complementary to the rat somatostatin cAMP-responsive promoter element (CRE) sequence cloned upstream of the herpes simplex virus thymidine kinase promoter, from position −109 to +57, linked to the bacterial chloramphenicol acetyltransferase (CAT) gene. pKSVTF is a vector containing the human β_2-adrenergic receptor and it was a generous gift from R. J. Lefkowitz (8). As a transfection efficiency control we used the plasmid pCH110 carrying the β-galactosidase gene (Pharmacia).

Cell Culture and Transient Expression Assay. JEG-3, a human cell line derived from a choriocarcinoma, was used.

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Abbreviations: G protein, guanine nucleotide-binding protein; G_i, inhibitory G protein; CRE, cAMP-responsive promoter element; CREB, cAMP-responsive element-binding protein; CAT, chloramphenicol acetyltransferase; PTX, pertussis toxin.

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Cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum.

Cells (10^6 per plate) were transfected by the calcium phosphate technique (9) with 2 μg of plasmids pSomCAT and pKSVTF per plate and 2 μg of one of the following plasmids per plate: pSVD$_2$A$^+$, pSVD$_2$A$^-$, pSVD$_2$B$^+$, and pSVD$_2$B$^-$. In each transfection 1 μg of pCH110 was added to monitor the transfection efficiency. Twenty-four hours after transfection the appropriate ligands in H_2O [(+)-butaclamol in 50% methanol] were added at the concentrations indicated in Figs. 1–3. PTX was added to the cells 12 hr after transfection at the concentration indicated in Fig. 3. Eight to 10 hr after the addition of drugs, cells were harvested. Before CAT assays (10) extracts were normalized for proteins (11) and β-galactosidase activity (12).

RNA Analysis. Northern blot analysis was performed using 10 μg of total RNA (3) from different cell lines. Synthetic 60-mer oligonucleotides were synthesized corresponding to the human $G_{\alpha i\text{-}1}$, $G_{\alpha i\text{-}2}$, or $G_{\alpha i\text{-}3}$ sequences between amino acids 107 and 128, labeled by T4 polynucleotide kinase. Hybridizations were performed under standard conditions in 40% formamide at 42°C, with washing in 0.3 M NaCl/30 mM sodium citrate/0.1% SDS at 45°C.

RESULTS

Activated β_2-Adrenergic Receptor Stimulates CAT Activity Dependent on a CRE-Containing Promoter. A well-characterized effect of stimulation of the adenylyl cyclase pathway is activation of the cAMP-dependent protein kinase (PKA) (13). One of the substrates of the PKA is a transcription factor, the cAMP-responsive element-binding protein (CREB; ref. 14), which binds to CRE (14), and, once phosphorylated, induces transcriptional activation.

Using a CAT (10) reporter plasmid containing a CRE in its promoter we were able to show that it is possible to monitor variations of the intracellular cAMP levels induced by transient transfections of membrane receptors. These could be either activators of the adenylyl cyclase pathway, such as the β_2-adrenergic receptor (1), or inhibitors, such as the D$_2$ dopamine receptors.

In a first attempt to test our system we transfected the reporter plasmid pSomCAT in cultured cells in the presence or absence of forskolin, a constitutive activator of adenylyl cyclase. Most cell lines responded to the addition of forskolin with a strong induction of the CAT activity (data not shown). Among all cell lines tested, we chose JEG-3, a human choriocarcinoma cell line, because of the total absence of basal expression of pSomCAT (Fig. 1, lane 1) and also because of the very strong induction that follows the addition of forskolin into the medium (data not shown) (15). To further challenge our system we asked whether a membrane receptor activator of the adenylyl cyclase pathway could elicit the same activation over CAT activity as forskolin in the presence of physiological concentrations of its specific agonist. No endogenous β_2-adrenergic receptors were present in this cell line, as observed by the absence of CAT activity when pSomCAT was transfected and (−)-isoproterenol (up to 100 μM) was added into the medium (data not shown). A β_2-adrenergic receptor expression vector, pKSVTF, was therefore cotransfected with pSomCAT (Fig. 1). The expression of these two plasmids did not evoke any spontaneous CAT activity in JEG-3 cells (Fig. 1, lanes 2 and 3). When increasing concentrations of (−)-isoproterenol were added to the medium, a clear, dose-dependent and saturable induction of CAT was observed (EC_{50} = 70 nM) (Fig. 1, lanes 4–8). (−)-Epinephrine induced a similar response (EC_{50} = 700 nM; see Table 1). Addition to the medium of ICI118551, a β_2-specific antagonist, at a final concentration of 10 μM completely blocked the observed stimulation (Fig. 1, lanes 9–12).

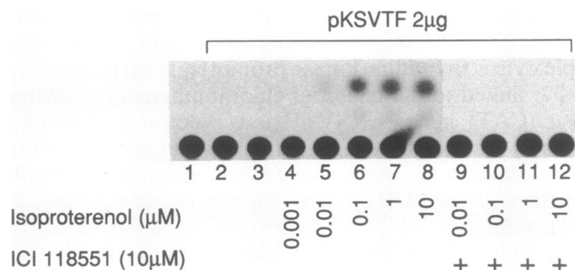

Fig. 1. Stimulation of CAT activity by the β_2-adrenergic receptor. Two micrograms of pKSVTF and pSomCAT was cotransfected in JEG-3 cells. Lane 1, pSomCAT alone; lanes 2 and 3, pSomCAT and pKSVTF; lanes 4–8, pSomCAT and pKSVTF in the presence of increasing concentrations of (−)-isoproterenol as indicated; lanes 9–12, same as lanes 5–8 plus 10 μM ICI118551.

Inhibition of CAT-Stimulated Activity by the D$_2$ Receptors. The CAT-stimulated level, either by forskolin or by the β_2-adrenergic receptor, can be brought back to very low levels by the cotransfection of pSVD$_2$A$^+$ or pSVD$_2$B$^+$, but only in the presence of dopamine. D$_2$A and D$_2$B have been shown to possess similar binding affinities (16, 17). By saturation binding experiments we observed that our two D$_2$ dopamine receptors' expression vectors behaved similarly. In particular, [^{3}H]spiperone-binding assays indicated that a similar number of receptor's molecules displaying equivalent affinity (K_d = 400 nM) were produced (N. Amlaiky and E.B., unpublished data). We chose to stimulate the CAT activity through the β_2-adrenergic receptor, instead of forskolin, because it reflects better the *in vivo* conditions. To activate the transfected β_2-adrenergic receptor, 10 μM (−)-isoproterenol was added to the medium. Fig. 2*A* is illustrative of such an experiment in which either pSVD$_2$A$^+$ (lanes 2–9) or pSVD$_2$B$^+$ (lanes 10–16) was used to attenuate the CAT activity stimulated by the β_2-adrenergic receptor in the presence of increasing concentration of dopamine (Fig. 2*A*: D$_2$A, lanes 4–9; D$_2$B, lanes 11–16). The observed inhibition correlates with a reduction of the intracellular cAMP levels, as measured by radioimmunoassay (data not shown). This inhibition was blocked by addition to the medium of (+)-butaclamol, a specific D$_2$ antagonist, at 2 μM final concentration (Fig. 2*C*: D$_2$A, lanes 2–4; D$_2$B, lanes 6–8). Transfections of the antisense plasmids pSVD$_2$A$^-$ (Fig. 2*B*, lanes 2 and 3) and pSVD$_2$B$^-$ (Fig. 2*B*, lanes 4 and 5), either in the absence or in the presence of dopamine, did not elicit any effect on the stimulated CAT activity, demonstrating also the absence of endogenous D$_2$ dopamine receptors.

The reduction elicited by expression of the D$_2$B isoform was stronger than D$_2$A at any of the dopamine concentrations used (Fig. 2*A*, compare lanes 4–9 to lanes 11–16; Fig. 3*A*). Quantification of the radioactive acetylated forms of chloramphenicol shows that at 0.1 μM dopamine, 64% reduction of CAT activity was observed when the system was challenged by the expression of the D$_2$B isoform (Fig. 3*A*, ●). The

Table 1. EC_{50} values (nM) of β_2-adrenergic, D$_2$A, and D$_2$B receptors

Stimulatory agent	Receptor		
	β_2-adrenergic	D$_2$A	D$_2$B
(−)-Epinephrine	700		
(−)-Isoproterenol	70		
Dopamine		72	58
LY171555		630	320

EC_{50} values were determined after transient expression of the appropriate receptors in JEG-3 cells. EC_{50} values represent the average of values required to observe the half-maximal CAT activity from at least five experiments.

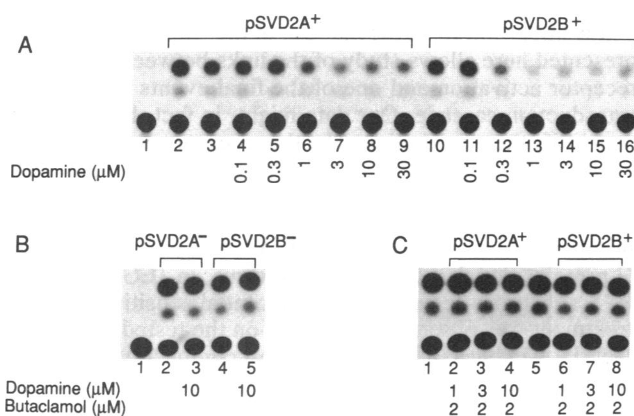

FIG. 2. Specific inhibition of CAT-stimulated activity by D_2 receptor isoforms. (*A*) Two micrograms of pSomCAT, pKSVTF, and either $pSVD_2A^+$ or $pSVD_2B^+$ was cotransfected in JEG-3 cells. Lane 1, pSomCAT alone; lane 2, pSomCAT and pKSVTF in the presence of 10 μM (−)-isoproterenol; lanes 3–9 and 10–16, as in lane 2 plus $pSVD_2A^+$ (lanes 3–9) or $pSVD_2B^+$ (lanes 10–16), with increasing concentration of dopamine as indicated. (*B*) Antisense plasmid $pSVD_2A^-$ or $pSVD_2B^-$ cotransfected with 2 μg of pSomCAT and pKSVTF in the presence of 10 μM (−)-isoproterenol. Lane 1, pSomCAT alone; lanes 2 and 4, pSomCAT and pKSVTF plus 2 μg of either one of the antisense expression vectors as indicated; lanes 3 and 5, as in lanes 2 and 4 plus 10 μM dopamine. (*C*) (+)-Butaclamol (2 μM) completely blocks D_2-induced inhibition of CAT activity. Same points as in *A*, lanes 2, 6, 7, 8, 10, 13, 14, and 15, but with the addition of (+)-butaclamol.

inhibition reached its maximal level at 1 μM dopamine, with 74% reduction, and remained constant at 10 μM. At 100 μM dopamine, only 59% reduction was observed.

In comparison, D_2A elicited a repression of 43% at 0.1 μM dopamine and a maximum of 61% at 10 μM; at 100 μM the inhibition was 51% (Fig. 3*A*, ■). The inhibition elicited from D_2A and D_2B is dose-dependent (EC_{50} of D_2A = 72 nM; EC_{50} of D_2B = 58 nM) and saturable. The obtained values are in a similar range of the ones found *in vivo* (18). Similar results were obtained by activation of the D_2 receptors by the D_2-specific agonist LY171555 (data not shown) (see Table 1).

PTX Affects the Inhibition Induced by the D_2 Receptors. We decided to investigate whether the observed inhibition of CAT activity by the two isoforms was due to coupling of these receptors to the same G_i or whether different G_is were involved in this process. To gain insights into this question, we pursued the same experiments described above in the presence of increasing concentrations of PTX. PTX ADP-ribosylates G_i protein and G_o protein (G protein of unknown function), rendering them inactive (19). PTX was used at 0.01–100 ng/ml to treat transfected JEG-3 cells for 16 hr; the results are shown in Fig. 3 *B–F*.

Upon addition of dopamine to the medium a differential response to PTX treatment was observed depending on which of the two isoforms was expressed. The inhibition effect over CAT expression evoked by the transfection of D_2A was almost completely blocked by PTX at 1 ng/ml (Fig. 3*D*). When D_2B was expressed, a PTX concentration of 100 ng/ml had to be used to abolish the inhibiting effect (Fig. 3*F*).

It has recently been reported that a D_2 receptor isolated from the pituitary gland associates with higher selectivity with $G_{\alpha i\text{-}2}$ but could also associate less efficiently with $G_{\alpha i\text{-}1}$ or $G_{\alpha i\text{-}3}$ (20). Therefore we decided to characterize which G_i proteins were expressed in JEG-3 cells. To do this we designed oligonucleotides able to detect $G_{\alpha i}$ mRNAs (21, 22) in a Northern blot analysis of JEG-3 RNAs as well as RNAs from other cell types. The results of this hybridization are shown in Fig. 4. This study reveals the presence of a band at ≈3 kilobases that correspond to $G_{\alpha i\text{-}1}$ and $G_{\alpha i\text{-}3}$ (Fig. 4; $G_{\alpha i\text{-}1}$ and $G_{\alpha i\text{-}3}$) (21, 22); at the level corresponding to the size of $G_{\alpha i\text{-}2}$ we were unable to detect any band in the RNA from JEG-3 cells (Fig. 4; $G_{\alpha i\text{-}2}$), whereas a band of the expected size was obtained in other human (HeLa), rat (AtT20), mouse (NIH 3T3), and monkey (COS) cell lines present on the same blot (Fig. 4). Similar amounts of RNA were used, as quantified by orange acridine staining of RNA in the gel and by hybridization of the blot to a human α-actin probe (not shown).

DISCUSSION

Two isoforms of D_2 dopamine receptors are generated by alternative splicing mechanisms. The existence of two cDNAs

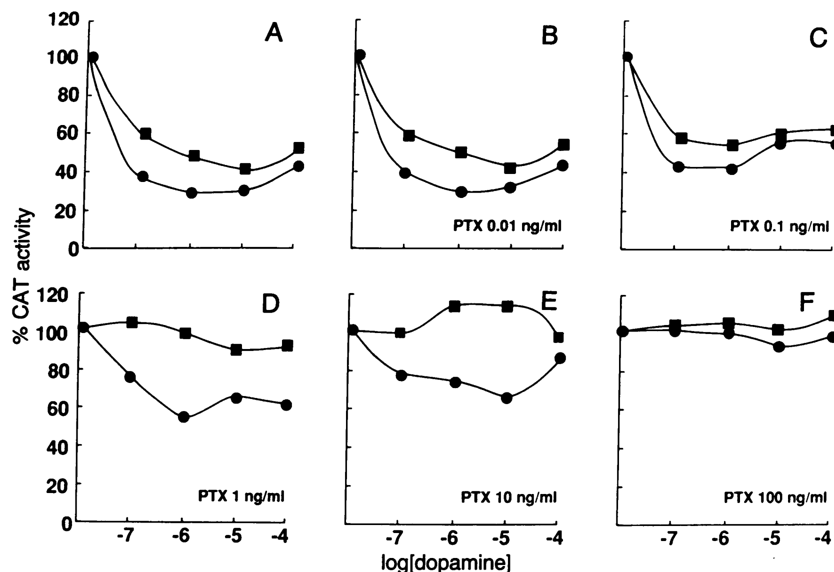

FIG. 3. PTX effects on D_2 receptor inhibition. One-hundred percent CAT activity was arbitrarily chosen as the maximal stimulation obtained by the cotransfection of pSomCAT and β_2-adrenergic receptor in the presence of 10 μM (−)-isoproterenol. Data were evaluated by assaying the acetylated forms of chloramphenicol cut from the TLC plates after organic separation in a scintillation spectrometer. Each panel shows data obtained by cotransfection of pSomCAT, pKSVTF, and $pSVD_2A^+$ (■) or $pSVD_2B^+$ (●) in the presence of 10 μM (−)-isoproterenol and at the dopamine concentrations indicated. (*A*) Inhibition profiles of the D_2 isoforms observed in absence of PTX. (*B–F*) Inhibition profiles with increasing concentration of PTX as indicated. The results shown here are representative of at least five individual experiments.

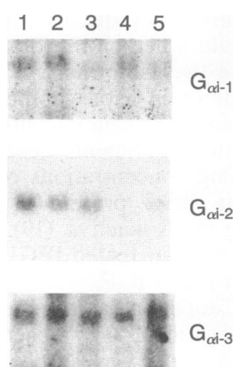

FIG. 4. Analysis of $G_{\alpha i\text{-}1}$, $G_{\alpha i\text{-}2}$, and $G_{\alpha i\text{-}3}$ mRNA present in various cell lines. Ten micrograms of total RNA was used. Lane 1, NIH 3T3; lane 2, COS; lane 3, AtT20; lane 4, JEG-3; lane 5, HeLa.

encoding D_2 dopamine receptors raised interest in understanding how the effects elicited by these isoforms could be regulated. These cDNAs differ by an 87-nucleotide insertion in the third intracytoplasmic domain. This finding is quite puzzling, since this domain is thought to play a pivotal role in the receptor–G protein interaction, as demonstrated for the β_2- and α_2-adrenergic receptors (6).

Since isolation of D_2A and D_2B, extensive studies have been pursued to reveal discriminating differences between them, on their binding affinities or on the inhibition of the adenylyl cyclase, but no clear distinction has yet been made. A recent report shows that D_2B could elicit a different physiological response depending on the cell type in which it is tested, suggesting a differential coupling in various cell types (5). It appears clear from these and other data (23) that receptor function is not only dependent on its own structure but also on features characteristic of a determined cell type, such as the presence of particular G proteins.

Taking advantage of the knowledge regarding the promoter elements responsible for the transcriptional regulation induced by cAMP (i.e., CRE) we designed a rapid assay, which allows study of the transcriptional modification induced by membrane receptors that activate or inhibit the adenylyl cyclase pathway in different cell types. Its sensitivity enables us to work with limited amounts of DNA, thus reducing possible transfection artifacts. We demonstrated that transfection of a β_2-adrenergic receptor, in JEG-3 cells, resulted in a strong stimulation of CAT activity upon addition of isoproterenol to the medium. This activation is dose-dependent and saturable; it was obtained at isoproterenol concentrations similar to those needed for a direct measurement of intracellular cAMP levels (24). When dopamine D_2 receptors (which act as inhibitors of the adenylyl cyclase) were cotransfected with the β_2-adrenergic receptor, the CAT activity was brought back to low levels (Fig. 2). These results clearly show that cAMP-induced transcription can be modulated by β_2-adrenergic and D_2 dopamine receptors' activation in a dose-dependent manner. Therefore we concluded that the observed data reflect well the events happening upstream at the adenylyl cyclase level. D_2B seems to inhibit CAT activity more efficiently than D_2A, suggesting a more selective coupling of D_2B to the G proteins present in these cells. At high dopamine concentrations we observed that the inhibition was weaker than at lower concentrations. These same results were obtained when the activation of D_2 receptors was induced by LY171555, instead of dopamine, ruling out the possibility of a nonspecific activation of other receptors. We do not exclude the possibility that alternative pathways (25) could have been activated at these concentrations, whose consequence would be stimulation of factors activating the CRE, by cross-talk phenomena (7).

It is important to stress that use of the transfection system presented here allows study of the links between membrane receptor activation and one of the final events in the signal transduction cascade. Our data might, in fact, better reflect or amplify the effects elicited by the receptors on the adenylyl cyclase. This might result in a more physiological analysis of the whole signal transduction process. The inhibition provoked by the activation of the D_2 receptors is a consequence of the coupling to G_i proteins (4). As shown in our data, the G proteins coupled to both D_2 isoforms, in JEG-3 cells, are PTX substrates, even though differential sensitivity to PTX treatment was observed depending on the tested isoform. In fact, D_2A-induced inhibition is almost completely suppressed at a PTX concentration 100 times lower than the one used to block D_2B effects. These findings could be interpreted in different ways: (*i*) Both receptors couple to the same G_i, but D_2A needs higher ratio of G_i:receptor than D_2B. Therefore ADP ribosylation of even a small amount of the specific G protein (low PTX concentration) renders D_2A unable to elicit its effects. In similar conditions D_2B could still function. (*ii*) The two D_2 receptor isoforms could be coupled to different G_i proteins, displaying a differential sensitivity to PTX treatment. (*iii*) One or both receptors couple to multiple G proteins. One or more of these G proteins is missing in JEG-3 cells.

Analysis of RNA from JEG-3 cells showed the presence of $G_{\alpha i\text{-}1}$ and $G_{\alpha i\text{-}3}$, whereas no signal was detected for mRNA corresponding to $G_{\alpha i\text{-}2}$ in these cells. This finding could be interpreted as the absence in JEG-3 cells or the presence of a low amount of the specific $G_{\alpha i\text{-}2}$ mRNA.

Experiments performed in a cell line containing $G_{\alpha i\text{-}2}$-specific mRNA, using the same assay, show a similar behavior for the two D_2 isoforms (unpublished data). Taken together, these data suggest that the two isoforms display a differential selectivity for the G_i protein(s) present in JEG-3 cells. Is D_2A more selectively coupled to $G_{\alpha i\text{-}2}$ than to other G_i proteins? Future experiments must be designed to test this hypothesis by cotransfecting $G_{\alpha i}$-specific expression vectors with the D_2 receptor isoforms.

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