Intracellular retention of the two isoforms of the D2 dopamine receptor promotes endoplasmic reticulum disruption

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SUMMARY

The dopamine D2 receptor exists as a long (D2a) and a short (D2b) isoform generated by alternative splicing of the corresponding transcript, which modifies the length of the third cytoplasmic loop implicated in heterotrimeric G-protein-coupling. Anatomical data suggested that this segment regulates the intracellular traffic and localization of the receptor. To directly address this question we used a combination of tagging procedures and immunocytochemical techniques to detect each of the two D2 receptor isoforms. Surprisingly, most of the newly synthesized receptors accumulate in large intracellular compartments, the plasma membrane being only weakly labeled, without significant difference between the two receptor isoforms. Double labeling experiments showed that this localization corresponded neither to endosomal compartments nor to the Golgi apparatus. The D2 receptor is mostly retained in the endoplasmic reticulum (ER), the long isofrom more efficiently than the short one. It is accompanied by a striking vacuolization of the ER, roughly proportional to the expression levels of the two receptor isoforms. This phenomenon is partly overcome by treatment with pertussis toxin. In addition, an intrinsic activity of the D2 receptor isoforms is revealed by [35S]-GTPγS binding and cAMP assay, which suggested that expression of weakly but constitutively active D2 receptors promotes activation of heterotrimeric G protein inside the secretory pathway. This mechanism may participate in the regulation of the cellular traffic of the D2 receptors isoforms.

Key words: Heterotrimeric G proteins, Cell compartments, Intrinsic activity

INTRODUCTION

The D2 receptor subtype mediates many important effects of dopamine in the central and peripheral nervous system of mammals (Civelli et al., 1993; Missale et al., 1998). This receptor is mainly distributed in the nigral and striatal region of the basal ganglia (where it contributes to sensory and motor control), in the olfactory bulb and retina (where it modulates sensory perception) and in the prolactin cells of the anterior pituitary gland (where it inhibits prolactin secretion). Like all the known vertebrate dopamine receptors, the D2 receptor subtype belongs to the superfamily of G-protein-coupled receptors, structurally characterized by the presence of seven-transmembrane segments (Gether and Kobilka, 1998; Missale et al., 1998; Valdenaire and Vernier, 1997). The basic molecular function of the agonist-bound form of the receptor is to act as an exchange factor for the α-subunit of heterotrimeric G proteins, the presence of the βγ subunits being strictly required (Cherfils and Chardin, 1999; Neer and Smith, 1996; Valdenaire and Vernier, 1997). The mammalian D2 receptor subtype belongs to the D2 class of dopamine receptors, this latter being functionally, structurally and evolutionarily very distant from the other dopamine receptor class, D1. As far as we know, the D2 receptor is coupled only to the class of Gαo/Gai1 proteins sensitive to pertussis toxin. It elicits a secondary interaction with different effector proteins, especially the Ca2+ and K+ voltage-sensitive channels and adenyl cyclase (Lledo et al., 1994; Missale et al., 1998).

The mammalian D2 receptor exists in two isoforms generated by the differential splicing of the pre-mRNA, which modifies the size of the third cytoplasmic loop of the receptor (Dal Toso et al., 1989; Giros et al., 1989). This cytoplasmic loop has been shown to be involved in the coupling of the receptor with heterotrimeric G proteins for several kinds of G-protein-coupled receptors. Thus the two D2 receptor isoforms (D2a, long isofrom, and D2b, short isofrom) were thought to differentially interact with heterotrimeric G protein inside the secretory pathway. This mechanism may participate in the regulation of the cellular traffic of the D2 receptors isoforms.
between the two isoforms of the D₂ receptor is that the amount of each of the corresponding mRNA varies among brain areas (Girov et al., 1989; Guivarc’h et al., 1995; Montmayeur et al., 1991). In addition, the relative abundance of the D₂ receptor isoforms is regulated by sex steroid hormones in anterior pituitary cells as well as in some brain areas (Guivarc’h et al., 1995; Guivarc’h et al., 1998), possibly modifying dopamine responses according to the physiological states of the organism. These observations suggested that the distribution of the two D₂ receptor isoforms may be modulated in a tissue-specific fashion.

One of the most important aspects of D₂ receptor function that may result from the splicing mechanism is a different subcellular localization of the protein. Indeed, the differential distribution of D₂a and D₂b mRNAs in the rat central nervous system suggested that the sequence of the third cytoplasmic loop could be involved in the targeting of the receptor to the nerve terminals, soma or dendrites. This parameter has not yet been taken into account when the question of the differential activity of the D₂ receptor isoforms has been examined. More generally, little attention has been given to the subcellular localization of the receptor proteins inside cells, especially after the transient transfections commonly used to study pharmacological and functional characteristics of receptors. As a first step to directly address this question we modified by epitope-tagging the sequences of the two isoforms of the rat D₂ dopamine receptor and used them for transient transfection in several cell lines.

MATERIALS AND METHODS

Materials

Restriction endonucleases, Taq polymerase and plasmid preparation kits were purchased from Promega. The TA cloning kit and pcDNA3 vector were obtained from Invitrogen and the DNA sequencing kit from USB Amersham. All other chemical reagents were purchased from Prolabo (France) and cell culture reagents were obtained from Life Technologies unless indicated.

Construction of epitope-tagged D₂a and D₂b receptor expression vectors

Sequences corresponding to the c-myc epitope (amino acids EKQLISEEDL, recognized by the 9E10 monoclonal antibody; Evan et al., 1985) and the VSV-G epitope (amino acids YTDIEMNRLGK, recognized by the PS4 monoclonal antibody; Kreis, 1986) were added downstream from the translation initiation codon of the D₂a and D₂b rat sequences, respectively, and inserted in the pcDNA3 vector. The chimerical receptor sequences were obtained by PCR amplification with an upstream oligonucleotide encompassing the epitope sequences and the ten first nucleotides of the rat D₂ receptor sequence.

To fuse the C-terminal end of each isoform of the D₂ receptor to the GFP protein in the pEGFP-N1 expression vector (Clontech), the translation initiation codon of the GFP was mutated to a valine, and the stop codon of each of the D₂ receptor isoforms was mutated to a glycine. Since the cysteine residue that anchored the C-terminus of the D₂ receptor is the last of the sequence, a spacer sequence made of seven amino acids (GVCICCI for the short isoform and GVCCCGG for the long isoform) has been added to avoid, as much as possible, a steric hindrance between the receptor and the GFP. All the modified constructs were checked by full-length sequencing.

Cell culture, transfection and treatments

The different cell lines used in this study (COS-7, HeLa, HEK-293 and NG108.15) were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% (v/v) fetal calf serum and 2 mM L-glutamine and incubated at 37°C in a 5% CO₂, 95% air atmosphere. Cells were generally seeded at 4x10⁵ cells/100 mm² dishes and 6x10⁶ cells/150 mm² dishes. After overnight incubation, the cells were transfected with 10 μg DNA, most often by electroporation (Herr et al., 1994) or by the DEAE-dextran/chloroquine transfection protocol (Pari and Keown, 1997).

In the case of HeLa cells and COS-7 cells, co-expression of D₂ receptors and the II, invariant chain of MHC class II (used as an ER marker; Salamero et al., 1996) was obtained by transfecting cells with one of the pcDNA3-D₂ recombinant vectors and the pGEM-II vector, and overexpressed with the T7 polymerase recombinant virus technique. In some experiments, the D₂ receptor isoforms were co-expressed with the D₁A dopamine receptor fused to GFP at the C-terminus. The D₁A-GFP construct was produced similarly to the D₂-GFP constructs.

All cell treatments were performed in the usual culture medium. Brefeldin A (Sigma), which blocks the activity of the ARF protein and disorganizes the Golgi apparatus (Donaldson et al., 1992) was used at a concentration of 10 μg/ml for 60 minutes. At different times after cell transfection, pertussis toxin (Sigma) was added for 12 hours at a concentration of 0.1 μg/ml. Tunicamycin (Sigma), a glycosylation inhibitor, was added at 10 ng/ml or 20 ng/ml for 12 hours. Actinomycin D (Sigma), a transcription inhibitor, was used at 10 mg/ml.

Functional characterization of the epitope tagged-D₂ receptors expressed in COS-7 cells

Forty-eight hours after transfection, the cells were washed twice with PBS, harvested by scraping the plates, homogenized by Polytron apparatus in binding buffer (Tris 50 mM pH 7.7 at 22°C, 120 mM NaCl, 5 mM KCl, 2 mM CaCl₂, 1 mM MgCl₂ and 5 mM EDTA), and centrifuged once at 17,000 g for 20 minutes. The pellets were suspended in binding buffer, proteins quantified by Bradford protocol (Biorad) and stored at -80°C until use. The specific binding characteristics of tagged and GFP-fused D₂ receptors were defined using [³H]-spiperone as ligand (Amersham). The saturation curves were carried out with increasing concentrations of [³H]-spiperone and (+)-butaclamol 1 μM to measure the total binding and (+)-butaclamol (RBI) to estimate the nonspecific binding. The binding assays were initiated by addition of 50 μg membrane proteins to [³H]-spiperone (0.01-0.90 nM) in a total volume of 1.5 ml binding buffer. After incubation for 1 hour 30 minutes at 37°C, the reactions were stopped by filtration with GF-B glass-fibre filters (Millipore). The filters were washed twice with ice-cold wash buffer (50 mM Tris pH 7.7) and dried under vacuum. The filters were counted in 10 ml scintillation liquid. B_max and K_d values were measured after computing the crude binding values in the Kaleidagraph software (Synergy Software).

For measurements of cAMP levels, transfected cells were seeded at 1.25x10⁵ cells in 24 wells cell culture plate. Forty-eight hours after transfection, cells were incubated with 0.5 mM of the phosphodiesterase inhibitor. IBMX (3-isobutyl-1-methylxanthine; Sigma), 0.5 mM IBMX and 1x10⁻⁵ M forskolin (direct activator of the adenylyl cyclase; Sigma) or 0.5 mM IBMX, 1x10⁻⁵ M forskolin (Sigma) and 1x10⁻⁵ M bromocriptine (D₂ receptor agonist; Sigma) for 15 minutes at 37°C in 500 μl medium without fetal calf serum. The stimulation was stopped by adding 500 μl of HCl 0.2 N and kept at 4°C until use. The inhibition of forskolin-induced cAMP accumulation by D₂-specific agonist bromocriptine was determined by competition (Nordstedt and Fredholm, 1990).

Immunocytochemistry and immunofluorescence microscopy of the epitope-tagged D₂ receptor isoforms and cellular markers

The transfected cells were grown at low density on coverslips for 48 hours before being processed for immunocytochemistry. After three
washes in ice-cold PBS, cells were fixed in 3% paraformaldehyde (Sigma) freshly made in phosphate-buffered saline (PBS pH7.4, 0.1 mM CaCl2 and 0.1 mM MgCl2) for 15 minutes at room temperature. Cells were incubated in PBS containing 50 mM NH4Cl (Sigma) as a blocking agent for 30 minutes and washed again in PBS. Cell permeabilization was performed in PBS containing 0.1% saponin (Sigma) and 0.2% BSA (Sigma) for 30 minutes. The permeabilized cells were then incubated with primary antibodies (1/200) for 60 minutes at room temperature or overnight at 4°C in PBS containing 0.1% saponin and 3% BSA, and washed three times in PBS. Then, the cells were incubated for 2 hours at room temperature with secondary antibodies (1/300) and washed three times in PBS. The anti-D2 receptor antibody (SM) is a rabbit polyclonal antibody raised against the sequence of the third cytoplasmic loop of the receptor fused to GST (Maltais, 2000). The secondary antibodies were either an IgG anti-mouse or an IgG anti rabbit immunoglobulin, labeled with FITC/GFP and Rhodamine/Texas-Red. The fluorescence was selected with an appropriate double fluorescence dichroic mirror and band pass filters (AOTF). Simultaneous double fluorescence acquisitions were performed with the same buffer and dried under vacuum. The radioactivity of the filters was determined by liquid scintillation counting.

RESULTS

Functional characterization of modified dopamine D2 receptors transiently expressed in cells

Since the two isoforms of the rat D2 receptor were modified by heterologous sequences added either to the N-terminus or to the C-terminus, the modified receptors had to be functionally characterized. For the N-terminal epitope-tagged sequences, binding assays performed with [3H]-spiperone revealed that high levels of D2 receptors were expressed 48 hours after transfection in COS-7 cells (300-400 fmol/mg proteins) and that the ligand Kd of the two receptor isoforms were similar and virtually identical to those of wild-type receptors. Essentially similar results were obtained for the GFP-fused D2 receptor isoforms (Table 1).

Accordingly, when stimulated by the agonist bromocriptine (10-5M), these epitope-tagged receptors were as potent as the two unmodified receptor isoforms to inhibit fosfokin-induced cAMP accumulation in transfected cells (Table 1). Incidentally, we did not find a significant difference between the two isoforms for their efficiency to inhibit adenylyl cyclase in COS-7 cells. Therefore, as far as the functional properties of the proteins are concerned, they did not appear to be significantly affected by the N- and C-terminal modifications, as reported for several others G-protein-coupled receptors and their epitope-tagged receptors (Barak et al., 1997; Drmota et al., 1998; Liu et al., 1999; Schulein et al., 1998; Tarasova et al., 1997).

Characterization of the detection of the two D2 receptor isoforms after transient expression in several cell lines

The subcellular distribution and intracellular transport of the long (D2a) and short (D2b) isoforms was first analyzed by the
immunocytochemical detection of the VSV-G epitope and c-myc epitope with specific monoclonal antibodies (see Materials and Methods). Forty-eight hours after transfection in COS-7 cells, the labeling corresponding to the epitope-tagged receptors was surprisingly found to be localized almost exclusively in intracellular compartments. The plasma membrane appeared only very weakly decorated and even undetectable in many cells (Fig. 1A,B). In general, the long isoform seemed to be more strongly accumulated in intracellular compartments than the short isoform. To eliminate the possibility of an artefact due to receptors overexpression in peculiar cells, different cell lines were transfected by each of the two D2 receptor isoforms. In HeLa fibroblast cells (Fig. 1C,D) and HEK-293 cells, the pictures obtained were comparable with those obtained in COS-7 cells. Identical data have also been obtained in the NG108.15 neuroblastoma-glioma hybrid (data not shown). It suggested that the predominant intracellular localization depended essentially on the intrinsic properties of the receptor, and not on the cell types used for transient expression. The appearance of receptor staining did not correspond to a delayed transport to the plasma membrane. Indeed, the overall receptor distribution remained the same up to 96 hours after cell transfection, the number of labeled cells decreasing progressively with time. Treatment with the transcription inhibitor, actinomycin D, given 48 hours after transfection, did not modify this labeling, indicating that the receptors were retained into intracellular compartments.

At this point, several control experiments were undertaken to search for some of the problems that may have accounted for the predominant intracellular detection, somewhat unexpected for a cell surface receptor. Several conditions of cell fixation, permeabilization and antibody incubation were used without significantly modifying the labeling appearance. Since the interaction between the N-terminal tags and the glycosyl moieties may have impaired antibody recognition, we treated the transfected cells by tunicamycin, a glycosylation inhibitor. However, in these conditions, the detection of the D2 receptor isoforms at the cell surface was further decreased in a dose dependent manner (Fig. 2) since inhibition of protein glycosylation prevents the transport of the receptors to the plasma membrane.

Thus we chose to use different anti-receptor antibodies, although these reagents cannot discriminate between the two D2 receptor isoforms. A first D2-specific receptor polyclonal antibody (a kind gift from B. Ciliax, Emory University) (Yung et al., 1995) gave approximately the same kind of labeling as that obtained with the anti-tag antibodies (data not shown). By contrast, the pictures obtained with a second polyclonal antibody (see Materials and Methods) directed against the third
cytoplasmic loop of the receptor showed, for the two isoforms, a clear staining at the plasma membrane, although most of the immunoreactivity was observed scattered inside the cells (Fig. 3C).

To verify that the polyclonal anti-D2 antibody (SM) recognized the D2 receptors without any bias (Grayson et al., 1998), we used D2b receptor-GFP fusion proteins (Fig. 3). The labeling obtained with the polyclonal anti-receptor antibody (see Materials and Methods; Fig. 3C) completely overlapped with that of D2b receptor-GFP fusion proteins (Fig. 3A) in transfected cells, whereas monoclonal anti-N terminal tag antibodies revealed preferentially D2 receptors localized in intracellular compartments (Fig. 3B,D). However, the situation was different for the two D2 receptor isoforms. If the localization of the GFP-fused short isoform strikingly resembled that obtained without C-terminal fusion, the GFP-fused long isoform displayed a weird intracellular fluorescence concentrated inside the nucleus. The D2a-GFP and D2b-GFP receptor mRNA levels measured by northern blots showed that the amounts of each of the transcripts were about the same throughout the time course of transient expression in COS-7 cells. Thus it is probable that some degree of degradation of the GFP-tagged D2a receptor occurred (data not shown).

To test the possibility of an obligatory interaction between the two D2 isoforms we co-expressed the D2b-GFP construct with the unmodified D2a receptor in COS-7 cells. Nevertheless, no apparent changes in D2 receptor localization could be seen in these experiments (data not shown).

**Identification of the intracellular compartments where the D2 receptor isoforms accumulate**

Confocal examinations of the transfected cells confirmed that most of the receptor labeling was concentrated in intracellular compartments; the staining spread over endoplasmic reticulum (ER) and Golgi apparatus. When the N-tagged receptors were labeled with the monoclonal antibodies, it appeared that the long D2a isoform is distributed widely over the cell cytoplasm, with a fine-grained aspect, whereas, the D2b isoform is generally more densely packed close to cell nucleus (Fig 1). No significant differences between the two isoforms could be seen for the localization at the plasma membrane. In addition, given the variability of labeling obtained from one transfected cell to another, it was difficult to firmly substantiate a differential subcellular localization for the two receptor isoforms. We used plasma membrane biotinylation of the cells transfected by each of the isoforms to better quantify the proportion of receptors localized at the plasma membrane and inside the cells. By this technique, 20 to 30% of the receptors that bound [3H]-spiperone were retained on the biotinylated membranes, with no significant difference between the two isoforms (Fig. 4). These results confirmed that a large majority of the two D2 receptor isoforms were held into intracellular compartments.

To ascertain the precise localization of the two D2 receptor isoforms, the simultaneous visualization of different organelle markers was examined using double-immunofluorescence analyzed by confocal microscopy. These observations were carried out in COS-7 and HeLa cells. We examined the possibility that the intracellular localization of the D2 receptors could correspond to an accumulation in the endocytotic pathway promoted by a large retrieval of the receptors from the plasma membrane. Colocalization of the receptor with rhodamine-labeled transferrin (well known to be endocytosed in clathrin-coated vesicles) was checked at different periods of time (5 minutes, 10 minutes, 15 minutes, 1 hour and 4 hours) after a 10 minute exposure to this ligand. Either after a short delay (5 minutes), when transferrin is essentially localized in peripheral early endosomes, or at longer chase time (60 minutes), when transferrin is found concentrated in the pericentriolar early endosomes (Fig. 5C,F), the labeling of the D2 receptors (Fig. 5A,D) and the endosomal marker were always mutually exclusive (Fig. 5B,E). This observation strongly suggests that, at least when cells are not exposed to D2 receptor agonist, the receptor is not significantly present in the clathrin-dependent endocytosis-recycling pathway.

Another possibility was that D2 receptors accumulate in the secretary pathway, especially in the Golgi apparatus. The Rab6 immunoreactivity, used as a marker of the Golgi complex, colocalized poorly with the two D2 receptor isoforms (Fig. 6A-D). It indicated that the receptors were not accumulating...
significantly in the Golgi sub-compartments characterized by this low molecular weight G protein. Further evidence for the localization of the receptors outside Golgi membranes was provided by cell treatment with brefeldin A (BFA). This antibiotic disrupts the organization of Golgi saccules and promotes a relocalization of Golgi proteins in upstream membrane compartments (Donaldson et al., 1992). In BFA-treated cells, the expected redistribution of the Rab6 immunofluorescence all over the cytoplasm was observed (Fig. 6H). By contrast, the intracellular distribution of the two isoforms of the dopamine receptor was not significantly modified by the BFA treatment (Fig. 6G).

The localization of D2 receptors in the ER compartments was examined with reference to the invariant chain (Ii) of MHC class II molecules, transfected together with the D2 receptor isoforms (see Materials and Methods). Here, a significant proportion of the receptor labeling appeared to be colocalized with the Ii marker (Fig. 7A-D). However, a general observation was that Ii labeling looked excluded from the sites where the receptor was the most abundant. The long D2a isoform was, in general, more widely distributed together with the ER marker than the D2b isoform (Fig. 7A,C). This evidence supported our previous observations of the long isoform being retained further upstream and more strongly than the short isoform in early compartments of the secretory pathway. In addition, as clearly visualized by the Ii labeling, it became obvious that this retention of the D2 receptors in the ER was accompanied by a dramatic change in the ER morphology. This latter appeared generally enlarged and transformed in large vacuoles (Fig. 7B,D). This morphological change is only seen in D2 receptor-transfected cells and it looked broadly proportional to the expression levels of the receptors in the cell.
The D_2_ receptor-induced vacuolization of the endoplasmic reticulum is partly due to heterotrimeric G protein activation

The observation of the large accumulation of the D_2_ receptor isoforms in intracellular compartments and of the ER vacuolization prompted us to further study the mechanisms of this striking effect. The first evidence of a disturbance of membrane protein transport was provided by the observation that co-expression of each of the D_2_ receptor isoforms together with the D_1A receptor almost completely blocked the transport of this latter protein (Fig. 8B), which otherwise is able to reach the plasma membrane (Fig. 8A). This phenomenon accounted for a rather general blockage of the intracellular transport of transmembrane proteins, at least of the pathway used by G-protein-coupled receptors.

Since the mammalian D_2_ receptor is known to be coupled to the G_ia_ and G_id_ class of heterotrimeric G proteins and since activation of G_ia/G_id_ proteins was shown to affect intracellular transport (Leyte et al., 1992), we decided to examine to what extent heterotrimeric G protein activation by the expressed D_2_ receptors may account for the modification of the ER morphology. To do that, we treated the cells with pertussis toxin (PTX), either just after transfection by the D_2_ receptor isoforms (Fig. 7E,F) or 2 hours after transfection (Fig. 7G,H). Interestingly, only when PTX was applied early after the transfection, the vacuolization of the ER appeared significantly overcome. In this condition, it became difficult to see ER modification, except in the cells that exhibited the highest levels of receptor expression. Thus, it seemed probable that some degree of constitutive activity of the D_2_ receptor (Hall, 1997) was able to turn on the PTX-sensitive G protein present in the ER or the cis-Golgi. This activation seemed responsible, at least in part, of the ER vacuolization.

In order to test the hypothesis of D_2_ receptor intrinsic activity, we performed two kinds of experiments. The first looked at the binding of [^35]S-GTPgammaS to indicate G protein activation and the second assayed cAMP levels, which reflect the effect of receptors on signaling pathways. We used a well-
described D2 receptor agonist (bromocriptine) and two antagonists (butaclamol(+) and sulpiride(−)), which have been shown to behave as inverse agonist (Hall, 1997). As expected, bromocriptine elicited a significant increase of [35S]-GTPγS binding (Table 2). By contrast, butaclamol had no effect on this phenomenon, behaving as a true antagonist. Interestingly, sulpiride clearly decreased [35S]-GTPγS binding at high concentrations (10−5 M) compared with low concentrations (10−10 M), thus acting as an inverse agonist, demonstrating the intrinsic activity of the two D2 receptor isoforms. Surprisingly, only about half of the value obtained in untransfected COS-7 cells (37.3±6.5 d.p.m./μg of protein) was observed in COS-7 cells expressing D2 receptor isoforms (14.4±1.1 d.p.m./μg of protein) for the D2b and 22.4±1.8 d.p.m./μg of protein for the D2a). When cAMP levels were assayed in COS-7 cells (Table 2), we consistently observed that the level of cAMP in COS-7 cells expressing the D2 receptors was about half of that in untransfected cells (32.7% for the D2b and 46.1% for the D2a). Nevertheless, activation of D2 receptors by bromocriptine decreased forskolin-stimulated cAMP accumulation (16% for the D2b and 32% for the D2a).

Table 2. Constitutive activity of the D2 receptors expressed in COS-7 cells

<table>
<thead>
<tr>
<th></th>
<th>D2b</th>
<th>D2a</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>B_max (fmol/mg of protein)</td>
<td>1302.00±89.0</td>
<td>1560.00±103.0</td>
<td>–</td>
</tr>
<tr>
<td>[35S]-GTPγS binding (%)</td>
<td>14.6±6</td>
<td>4.5±5</td>
<td>–</td>
</tr>
<tr>
<td>Bromocriptine stimulation (10−10 M to 10−5 M)</td>
<td>0.5±3.2</td>
<td>0.1±3.2</td>
<td>–</td>
</tr>
<tr>
<td>Butaclamol(+) inhibition (10−10 M to 10−5 M)</td>
<td>29.6±9</td>
<td>26.1±3.9</td>
<td>–</td>
</tr>
<tr>
<td>Sulpiride(−) inhibition (10−10 M to 10−5 M)</td>
<td>12.1±4*</td>
<td>9.7±2*</td>
<td>18±6*</td>
</tr>
<tr>
<td>cAMP accumulation (pmol/mg of protein)</td>
<td>82.5±32</td>
<td>99±29</td>
<td>78.5±21</td>
</tr>
<tr>
<td>Basal</td>
<td>69±22</td>
<td>67±18</td>
<td>70±16</td>
</tr>
<tr>
<td>FSK</td>
<td>67±18</td>
<td>70±16</td>
<td>70±16</td>
</tr>
<tr>
<td>FSK+Bromo</td>
<td>67±18</td>
<td>70±16</td>
<td>70±16</td>
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The B_max and K_d values for the binding of [3H]-spiperone to membranes of transfected cells are given (see Materials and Methods). Percentage of stimulation or inhibition of the [35S]-GTPγS binding in COS-7 cells for different treatments are indicated and the cellular levels of cAMP are expressed in pmol/mg of protein (n=3-8). FSK, forskolin-treated cells; FSK+Bromo, forskolin+bromocriptine-treated cells. *P<0.05 versus control.

The observation of a predominantly intracellular distribution of the transfected D2 receptor isoforms was very surprising at first glance and raised many embarrassing questions. The problem is not the strong accumulation of the D2 receptors in the secretory pathway, but mainly the default of membrane localization. Some technical issues certainly accounted for the poor detection of the N-terminal tags when the receptors are localized at the plasma membrane. It does not depend on the antibody itself since this phenomenon occurred either with the 9E10 anti-myc monoclonal antibody (labeling the short isoform of the D2 receptor) or with the PSD4 anti-VSV-G monoclonal antibody (used to detect the long D2 receptor isoform). The reasons for this are not clear, although some kind of interaction between the receptor N-terminus and components of the extracellular matrix may prevent antibodies from good access to the epitopes. The use of different approaches to visualize receptors allowed us to partly solve this issue. The polyclonal anti-D2 receptor antibody, which is directed against the third cytoplasmic loop of the receptor, provided a much better plasma membrane decoration than the monoclonal antibody. The short isoform of the D2 receptor fused to GFP gave essentially the same labeling pattern as that of the anti-D2 receptor antibody, suggesting that they both provided a faithful picture of the subcellular localization of the receptor isoforms.

Fig. 5. Localization of the D1A receptor isoform co-transfected with the D2b receptor in COS-7 cells. D1A-GFP fusion construct expressed in COS-7 cells is mainly present at the plasma membrane (A; confocal microscope image). In cells where it is co-expressed with the D2b receptor it appears retained into altered intracellular compartments (B). The co-expressed myc-tagged D2 receptor is visualized with the monoclonal 9E10 antibody coupled to Texas-Red (C). Bars, 2 μm.

**DISCUSSION**

This work was intended to analyze in detail the subcellular localization of the mammalian dopamine D2 receptor in several types of heterologous cells. Our initial aim was to detect differential distribution in cell compartments and differential regulation between the two splicing isoforms of the receptor. Indeed, besides their basic function of modulating the activity of heterotrimeric G proteins, the precise subcellular localization of membrane receptors are likely to be major determinants of their physiological role (Valdenaire and Vernier, 1997). The demonstration that the differential splicing of the D2 dopamine receptor is regulated in mammals (Guivarc’h et al., 1995; Guivarc’h et al., 1998) and the existence of a differential localization of the two transcripts in the brain suggested possible differences in the intracellular targeting of this physiologically important receptor.
does not prevent a large proportion of the receptors from reaching the plasma membrane in the case of the D1 dopamine receptors (Fig. 8) or β-adrenoreceptors (Von Zastrow et al., 1993) or αβ3-receptors (Fonseca et al., 1995; Hirasawa et al., 1997). However, in some instances, a predominant intracellular distribution has been described for a few receptors of the G-protein-coupled receptor superfamily such as the α2C-adrenoreceptor (Daunt et al., 1997), the α1A-adrenoreceptor (Hirasawa et al., 1997), the 5HT1B receptor (Langlois et al., 1996) and the thrombin receptor (Hein et al., 1994). Whether these observations have a common mechanism and whether they correspond to a natural situation is not known yet.

In the case of the D2 receptor, several hypothesis may have accounted for this puzzling observation and some of them have been tested in the present study. First, this phenomenon is not cell-specific since it has been observed in HeLa, COS-7, and HEK-293 cells, as well as in the NG108.15 neuroblastoma-glioma hybrid. Second, it does not depend on a delayed transport to the plasma membrane, as shown by the labeling not being altered with time. Incidentally, it is worth mentioning that the two isoforms showed no significant difference in their presence at the cell surface, thus indicating that the alternative splicing is not affecting the protein targeting to the plasma membrane, as shown by the labeling of the immature receptors in CHO cells by Fishburn et al., who showed that the long D2a receptor isoform exhibited a glycosylation pattern reminiscent of poorly transported membrane proteins (Fishburn et al., 1995).

Three observations provided clues to explain the intracellular retention of the D2 receptor isoforms. The first one is that a glycosylation defect, due to an imperfect folding of the protein, could theoretically promote a fast retrieval of the receptors from the intermediate compartment of the Golgi complex (Gahmberg and Tolvanen, 1996). In the case of the D2 receptors (Fig. 2), a defect in the maturation of the polysaccharide moities of the receptor appears to be the consequence of impaired transport out of the ER, but not its initial cause, in agreement with previous studies (Fishburn et al., 1995).

The second possibility is that the retention of the receptor in the ER may be dependent, at least in part, on the activation of PTX-sensitive G proteins by constitutively active D2 receptors. The existence of a significant intrinsic activity of the D2 receptors was first suggested by Hall and Strange (Hall and Strange, 1997). This hypothesis is supported by data showing that activation of PTX-sensitive-heterotrimeric G proteins was able to block the formation of secretory vesicles from the TGN (Leyte et al., 1992). A similar mechanism may have accounted for the impaired transport of the long isoform of the D2 receptor early in the secretory pathway. This contention is further supported by the fact that this receptor localization is insensitive to BFA, suggesting that the immature receptors are retained in a membrane compartment that could be excluded from the Golgi bi-directional traffic. In addition, the transport of receptors otherwise normally present at the cell surface (such as the D1 dopamine receptor), is also impaired by the simultaneous presence of the D2 receptor (Fig. 8). This indicates that the modification of the membrane protein traffic induced by the D2 receptor is more general and that it affects at least one other polytopic transmembrane protein. Whether this phenomenon may be elicited by other G-protein-coupled receptors retained intracellularly is not known. However, in the case of GABAB R1 subunit which, alone, is both unable to go to the plasma membrane and unable to activate G proteins, no perturbation of the ER or other membrane compartments are elicited (Couve et al., 1998).

A third possibility suggested by a recent study (Vickery and von Zastrow, 1999) that provides evidence for a constitutive endocytosis of the D2 receptor in a dynamin-independent, clathrin-independent pathway, as analyzed by the endocytosis of antibodies directed against N-terminal tagged receptor. Our data do not exclude this possibility and two of the observations made by these authors fit with our own data: (1) that constitutive endocytosis is very likely to correspond to a constitutive activation of the receptor; and (2) an accumulation of the D2 receptors inside the cells is also observed in these experiments. However, in the steady-state conditions we used, most of this intracellular accumulation predominantly corresponded to a blocked transport in the biosynthetic pathway (as supported by tunicamycin treatment) and not to constitutive endocytosis.

From a different perspective, the poor localization of the D2 receptors at the plasma membrane may rely on the lack of a component, a molecular partner that would be required for the maintenance of the receptor at the plasma membrane in the transfected cells. In particular, heterologous receptor dimerization should be a requirement for a proper targeting to the plasma membrane, as recently shown for the GABAB and GABAB receptor subtypes (Kaupmann et al., 1998; White et al., 1998). Although the possibility of self-dimerization of the D2 receptor has been reported by some authors (Ng et al., 1996), no evidence exists for the association of the D2 receptor with another type of G-protein-coupled receptor or even for an association between the two D2 receptor isoforms (this study). In addition, the requirement of some type of ‘scaffolding proteins’ may be envisaged, such as PDZ-domain proteins, but no consensus for PDZ binding is found for the D2 receptor.

Although the previous hypothesis may account for the unusual localization of the D2 receptors after transient expression in cells, the provocative observation of a massive vacuolization of the ER is related, at least in part, to receptor-dependent activation of heterotrimeric G proteins. The salutary effect of PTX, based on the ER morphology analysis, implies that G protein activation has been elicited by the endogenous activity of the D2 receptors. The mechanism of PTX inhibition of G protein stimulation relies on the impairment of the direct interaction of the receptor with the C-terminus of the α subunit of the G protein which is ADP ribosylated by the toxin. The phenomenon strongly resembles that promoted by the pore-forming toxin aerolysin (Abrami et al., 1998). Although the mechanisms of vacuolization promoted by aerolysin are not completely understood, the toxin affects early steps of protein secretion, as did the dopamine D2 receptors. In addition, it involves pertussis-toxin sensitive G proteins and certainly calcium release from internal stores (Krause et al., 1998). In this respect, the D2 receptor, which can also modulate calcium entry via G protein activation (Lledo et al., 1994), may be similar to this pore forming toxin.

The observation of the intracellular localization of the D2...


Subcellular localization of D₂ dopamine receptor isoforms


