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ORIGINAL ARTICLE

Computer-Assisted Image Analysis of Caveolin-1 Involvement in the Internalization Process of Adenosine A_{2A}–Dopamine D_2 Receptor Heterodimers

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Abstract

A functional aspect of horizontal molecular networks has been investigated experimentally, namely the heteromerization between adenosine A_{2A} and dopamine D_2 receptors and the possible role of caveolin-1 in the cotrafficking of these molecular complexes. This study has been carried out by means of computer-assisted image analysis procedure of laser images of membrane immunoreactivity of caveolin-1, A_{2A}, D_1, and D_2 receptors obtained in two clones of Chinese hamster ovary cells—one transfected with A_{2A} and dopamine D_1 receptors and the other one with A_{2A} and D_2 receptors. Cells were treated for 3 h with 10 μ*M* D_1 receptor agonist SKF 38393, 50 μ*M* D_2–D_3 receptor agonist quinpirole, and 200 n*M* A_{2A} receptor agonist CGS 21680. In A_{2A}–D_1-cotransfected cells, caveolin-1 was found to colocalize with both A_{2A} and D_1 receptors and treatment with SKF 38393 induced internalization of caveolin-1 and D_1 receptors, with a preferential internalization of D_1 receptors colocalized with caveolin-1. In A_{2A}–D_2-cotransfected cells, caveolin-1 was found to colocalize with both A_{2A} and D_2 receptors and either CGS 21680 or quinpirole treatment induced internalization of caveolin-1 and A_{2A} and D_2 receptors, with a preferential internalization of A_{2A} and D_2 receptors colocalized with caveolin-1. The results suggest that A_{2A} and D_2 receptors and caveolin-1 likely interact forming a macrocomplex that internalizes upon agonist treatment. These observations are discussed in the frame of receptor oligomerization and of the possible functional role of caveolin-1 in the process of co-internalization and, hence, in controlling the permanence of receptors at the plasma membrane level (prerequisite for receptor mosaic organization and plastic adjustments) and in the control of receptor desensitization.

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Index Entries: Adenosine A_{2A} receptor; dopamine D_2 receptor; caveolin-1; heteromers; internalization.

Introduction

Although it was thought for a long time that G protein-coupled receptors (GPCRs) existed mainly as monomeric entities, it is now recognized that GPCRs form homo- and heterodimers (and possibly, homo-and heteromultimers) (for recent review, *see*, e.g., Bouvier, 2001; Agnati et al., 2003). One of the most studied GPCR heteromers is the adenosine

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This paper is dedicated to Luciano Martini, Professor of Endocrinology in Milano and to Faustino Savoldi, Professor of Neurolgy in Pavia.

A_{2A}–dopamine D_2 receptor complex (Hillion et al., 2002; Agnati et al., 2003; Canals et al., 2003; Ferré et al., 2003, 2004; Ciruela et al., 2004). Both receptors are highly expressed in the striatum, where they are colocalized in the GABAergic enkephalinergic neurons (Ferré et al., 1997). These neurons play a key role in the pathophysiology of basal ganglia disorders, like Parkinson's disease, and they constitute a common pathway for the rewarding effects of addictive drugs and the antipsychotic effects of neuroleptics (Agnati et al., 2003; Ferré et al., 2003, 2004). Strong reciprocal antagonistic interactions have been found between A_{2A} and D_2 receptors. At the intramembrane level, stimulation of A_{2A} receptors decreases the affinity of D_2 receptors for agonists (Ferré et al., 1991). At the second-messenger level, stimulation of D_2 receptors antagonizes the effects secondary to A_{2A} receptor-mediated activation of adenylyl cyclase (Kull et al., 1999; Hillion et al., 2002; Lee et al., 2002). However, some studies suggest that under some conditions, synergistic A_{2A}–D_2 interactions can also be observed, with stimulation of D_2 receptors potentiating the effects of A_{2A} receptor stimulation. These conditions seem to depend on the isoform of adenylyl cyclase involved or on the interruption of previous long-term exposure to D_2 receptor agonists (Yao et al., 2002; Kudlacek et al., 2003; Vortherms and Watts, 2004).

A_{2A}–D_2 receptor interactions have been proposed to provide a new therapeutic approach for basal ganglia disorders, schizophrenia, and drug addiction (Ferré et al., 1992, 1997, 2003; Ferré, 1997). Recent clinical studies with patients with Parkinson's disease have shown that A_{2A} receptor antagonists can enhance the therapeutic effect of L-DOPA (Bara-Jimenez et al., 2003; Hauser et al., 2003). However, when considering long-term treatment with GPCR ligands, some pharmakodynamic effects have to be taken into account. Results from previous studies show that A_{2A}–D_2 interactions not only control the signaling but also the trafficking of A_{2A} and D_2 receptors. In a human neuroblastoma cell line (SH-SY5Y) with constitutive expression of A_{2A} receptors and cotranfected with D_2 receptors, long-term stimulation of either A_{2A} or D_2 receptors was associated with desensitization of A_{2A} receptors. Costimulation of both A_{2A} and D_2 receptors was necessary to induce desensitization of D_2 receptors (Hillion et al., 2002). Coadministration of A_{2A} and D_2 receptor agonists was associated with a substantial modification of the distribution of A_{2A} and D_2 receptors in the plasma membrane (coaggregation), followed by internalization of A_{2A}–D_2 receptor aggregates (Hillion et al., 2002).

Although the best-characterized mechanism for internalization of GPCRs is by clathrin-coated pits, the number of examples of clathrin-independent endocytosis is increasing. Those are mediated by caveolae and glycolipid rafts (Nabi and Le, 2003; Nichols, 2003; van Deurs et al., 2003). Caveolae are a subdomain of lipid rafts that, until recently, have been morphologically and biochemically identified as smooth invaginations of the plasma membrane that express caveolin-1 (Anderson, 1998; Kurzchalia and Parton, 1999). A most recent view proposes that the term caveolae be used as a morphological descriptor for endocytic raft-derived invaginations, with or without the presence of caveolin-1. Neverthelss, this new definition still recognizes that stable cell-surface-associated caveolae would be associated with caveolin-1 expression (Nabi and Le, 2003) and that caveolin-1 is a good marker for clathrin-independent endocytosis. Recently, it has been shown that agonist treatment induces internalization of adenosine A_1 receptors though caveolae in a smooth muscle cell line (Escriche et al., 2003). The study by Escriche et al. (2003) also suggested a direct interaction between A_1 receptor and caveolin-1.

In the present study, we use double immunofluorescence and confocal laser microscopy techniques to study the agonist-induced caveolae-mediated internalization of A_{2A} and dopamine D_1 and D_2 receptors in Chinese hamster ovary (CHO) cell lines stably cotransfected with A_{2A} and D_1 receptors or with A_{2A} and D_2 receptors.

Materials and Methods

Transfection of A_{2A}, D_{2L}, and D_1 Receptor cDNAs into CHO Cells

Transfection and characterization of the presently used A_{2A}–D_2 and A_{2A}–D_1 CHO cell line are described in detail elsewhere (Torvinen et al., 2004). In brief, CHO-K1 cells (CCL61, American Type Culture Collection, Rockville, MD) were stably transfected with a double hemagglutinin (HA)-tagged (N- and C-terminal) dog adenosine A_{2A} receptor cDNA ([a kind gift from Dr. M. Olah] 1230-kb cDNA fragment cloned into the pcDNA3.1/Hygro+, conferring resistance to hygromycin), with lipofectamine plus reagent (Life Technologies). Cell lines coexpressing A_{2A} and D_2 receptors (A_{2A}–D_2 cells) and A_{2A} and D_1 receptors (A_{2A}–D_1 cells) were created by lipofectamine-mediated stable transfection of the following

plasmids into the CHO cell line stably expressing A_{2A} receptors: the human dopamine D_{2L} (long-form) receptor cDNA cloned into the Plxsn-vector, conferring resistance to geneticin, or the rhesus macaque Myc/His-tagged dopamine D_1 receptor cloned into the pcDNA3.1, also conferring resistance to geneticin. The clones resistant to geneticin and hygromycin were selected. The CHO cell line clones with high expression of A_{2A} receptors were screened and selected by single-point binding of the A_{2A} antagonist radioligand ^{3}H-ZM-214385 at a concentration near the K_D value of the A_{2A} receptor for the radioligand. The D_2 antagonist radioligand ^{3}H-raclopride (2 n*M*) was used to select cells expressing D_2 receptors, whereas the D_1 antagonist radioligand ^{3}H-SCH-23390 (2 n*M*) was used to identify cells expressing D_1 receptors.

Characterization of A_{2A}–D_2 and A_{2A}–D_1-Cotransfected CHO Cell Lines

The A_{2A}–D_1 CHO cell line was characterized by saturation analysis of the binding of the A_{2A} antagonist ^{3}H-ZM-241385 and the D_1 antagonist ^{3}H-SCH-23390. B_{max} values were 146 ± 6.1 and 282.3 ± 19.7 fmol/mg for ^{3}H-SCH-23390 and ^{3}H-ZM-241385 binding, respectively. K_D values were 1.5 ± 0.2 and 0.65 ± 0.09 n*M* for ^{3}H-SCH-23390 and ^{3}H-ZM-241385 binding, respectively. The B_{max} and K_D values determined with the A_{2A} receptor antagonist ^{3}H-ZM-241385 in A_{2A}–D_2 CHO cells were 290 ± 18 fmol/mg protein and 0.4 ± 0.07 n*M*, respectively. B_{max} and K_D values for the D_2 receptor antagonist ^{3}H-raclopride were 1900 ± 200 fmol/mg protein and 1.5 ± 0.2 n*M*, respectively (Torvinen et al., 2004).

Maintenance of Cell Lines

The stable CHO cell lines were cultured routinely at 37°C with 5% CO_2 in minimum essential medium (α-MEM) without nucleosides, supplemented with 10% fetal calf serum, 100 U/mL penicillin, 100 μg/mL streptomycin, and 2 m*M* L-glutamine. Hygromycin (300 μg/mL) was used as the selector for A_{2A} receptor cDNA. Geneticin (G-418) (400 μg/mL) was used as a specific selector for dopamine D_2 and D_1 receptor cDNAs. All cell culture products were purchased from Invitrogen.

Drugs and Protocols

For immunofluorescence staining A_{2A}–D_2 cells were grown on glass slides (Chamber Slide Culture, Labtek/Nunc) coated with poly-L-lysine (Sigma) and exposed to 200 n*M* CGS 21680 (Sigma) or 50 μ*M* quinpirole (Sigma) in serum-free medium for 3 h at 37°C; A_{2A}–D_1 cells were similarly cultured and exposed to 10 μ*M* SKF 38393 (Sigma) in serum-free medium for 3 h at 37°C. Control cells of both clones were exposed to serum-free medium for the same period.

Double-Immunolabeling Caveolin-1–A_{2A} Receptor, Caveolin-1–D_1 Receptor, and Caveolin-1-D_2 Receptor Experiments

At the end of the treatments, the cells were rinsed with PBS, fixed in 4% paraformaldehyde and 0.06 *M* sucrose for 20 min, washed with PBS containing 20 m*M* glycine, and subsequently treated with PBS containing 20 m*M* glycine and 1% BSA for 30 min at room temperature. For A_{2A}–D_1 cells, double immunostaining was performed with rabbit anti-caveolin-1 antibody (CAV 1A; 1:1000; Santa Cruz Biotechnology) and mouse anti-HA antibody (1:100; Roche) or mouse anti-His (1:100; Roche) antibody in PBS (pH 7.4), supplemented with 1% goat serum at 4°C overnight. The cells were then rinsed several times and incubated with an anti-rabbit biotinylated antibody (1:200; Amersham Pharmacia Biotech) for 1 h at room temperature. After rinsing twice in PBS, double immunofluorescence staining was performed with red-colored fluorolink Cy3™-labeled streptavidin (1:100; Amersham Pharmacia Biotech) for caveolin-1 and with green-colored Alexa fluor 488-labeled goat anti-mouse (1:100; Molecular Probes) for HA–A_{2A} or Myc–His–D_1 receptors for 1 h at room temperature. For A_{2A}–D_2 cells, double immunostaining was performed with CAV 1A antibody (1:1000) and mouse anti-HA antibody (1:100) or mouse anti-caveolin-1 antibody (CAV 1B; 1:100; BD Bioscience) and rabbit D_2 receptor antibody (1:800; a kind gift from Dr. Watson) in PBS (pH 7.4), supplemented with 1% goat serum at 4°C overnight. For caveolin-1–A_{2A} receptor and caveolin-1–D_2 receptor double immunostaining, cells were rinsed several times and incubated with an anti-rabbit biotinylated antibody (1:200; Amersham Pharmacia Biotech) and anti-mouse biotinylated antibody (1:200; Amersham Pharmacia Biotech), respectively, for 1 h at room temperature. After two rinses in PBS, the caveolin-1–A_{2A} receptor double immunofluorescence staining was performed with red-colored fluorolink Cy3™-labeled streptavidin (1:100) for caveolin-1 and with green-colored Alexa fluor 488-labeled goat anti-mouse (1:100) for HA–A_{2A} receptors. The caveolin-1–D_2 receptor double immunofluorescence

staining was performed with red-colored fluorolink Cy3™-labeled streptavidin (1:100) for caveolin-1 and with green-colored Alexa fluor 488-labeled goat anti-rabbit (1:100) for D_2 receptor for 1 h at room temperature. Finally, the slides were rinsed three times in PBS and mounted with a medium suitable for immunofluorescence (30% Mowiol, Calbiochem).

Image Analysis

Microscopic observations were made by means of Leica TCS 4D (Leica Laser Technik) confocal scanning laser equipment adapted to an inverted Leica DMIRBE microscope interfaced with an argon-krypton laser set at a power of 8 mW in each line (488, 568, 647 nm) and operating in the simultaneous acquisition mode of red (568 nm) and green (519 nm) emissions at a primary magnification of ×100 (Leica planapochromatic ×100/NA 1.4 objective). To avoid any brightness overflow and cross talk between the two channels, single-stained samples were used to properly fix the settings of the detectors. To minimize the noise and to keep a low photobleaching rate, we selected an acquisition time of 1s per scan and averaged 16 scans to produce each 512 × 512-pixel image. A sampling step of 80 nm in the plane of section and 0.25 μm in the axial direction were applied, thus meeting the requirements of the Nyquist theorem (Webb and Dorey, 1995). To get further noise reduction, images were convolved with a 3 × 3 median filter (Landmann, 2002). The contribution of unspecific staining was estimated in each image as the mean brightness value exhibited by the cell nucleus plus 1 S.D. Background-corrected brightness values were then obtained by subtracting pixel by pixel such a value from the original image. A region of interest (ROI) was also interactively defined to restrict the analysis to a spatially confined area of the image corresponding to a single-cell profile.

Analysis of Colocalization

A global evaluation of the degree of colocalization exhibited by the two patterns of fluorescence was obtained by estimating the overlap coefficient, according to Manders et al. (1993). It is simply the sum over all pixels in the ROI of the following normalized product of channel intensities:

$$\pi(x, y) = \frac{R(x, y) \cdot G(x, y)}{\sqrt{\left(\sum_{x,y} R(x, y)^2 \cdot \sum_{x,y} G(x, y)^2\right)}}$$

where $R(x,y)$ and $G(x,y)$ indicate red and green fluorescence intensities at the image location (x,y).

To discriminate among the variety of red and green combinations characterizing dual-labeled pixels—those expressing the highest level of association between the two fluorochromes—the following steps were applied. The distribution of the normalized products in the ROI was obtained and the 99th percentile (p99) calculated. Pixels showing the highest normalized product (i.e., with $([x,y] > p99)$ were then selected. These highly correlated pixels (hcps) are the locations giving the highest contribution to the general overlap coefficient, so they can be considered as locations where high association between the two signals certainly occurs. Their mean green/red (G/R) ratio was then evaluated together with the corresponding G/R S.D. The whole set of pixels with G/R ratio in the range G/R ± 2 S.D. was finally discriminated, and the colocalization factors M_R and M_G (Manders et al., 1993) of this pattern estimated. The parameters used for statistical analysis were overlap coefficient and M-factors (MD_1, MD_2, MA_{2A}, and M_{Cav}). M-factor is the amount of immunoreactivity of each fluorochrome within the hcp (number of the hcp multiplied by the mean intensity of that fluorochrome within the hcp) as a fraction of its total immunoreactivity (total field area multiplied by the mean intensity of that fluorochrome within the entire image). Overlap coefficient is the overall evaluation of the correspondence of the red and green intensities within the entire image. The coefficient is equal to 1 and to 0 for the perfect match and mismatch between the two fluorochromes, respectively. Each experiment was performed five times, and in each experimental session about 20 cells per treatment were evaluated. The mean values of the measured parameters obtained in the treated cell cultures were then compared with the value observed in the corresponding control group by Student's *t*-test for paired data (GraphPad Prism 3.03, GraphPad Software).

Results

Experiments on Colocalization of A_{2A} and D_1 receptors With Caveolin-1 and Internalization Upon Agonist Treatment

Under basal conditions there was evidence for colocalization of A_{2A} and D_1 receptors with caveolin-1. An example of the results obtained is shown in Fig. 1, where treatment with SKF 38393 causes a relative excess of caveolin-1 immunoreactivity with respect to

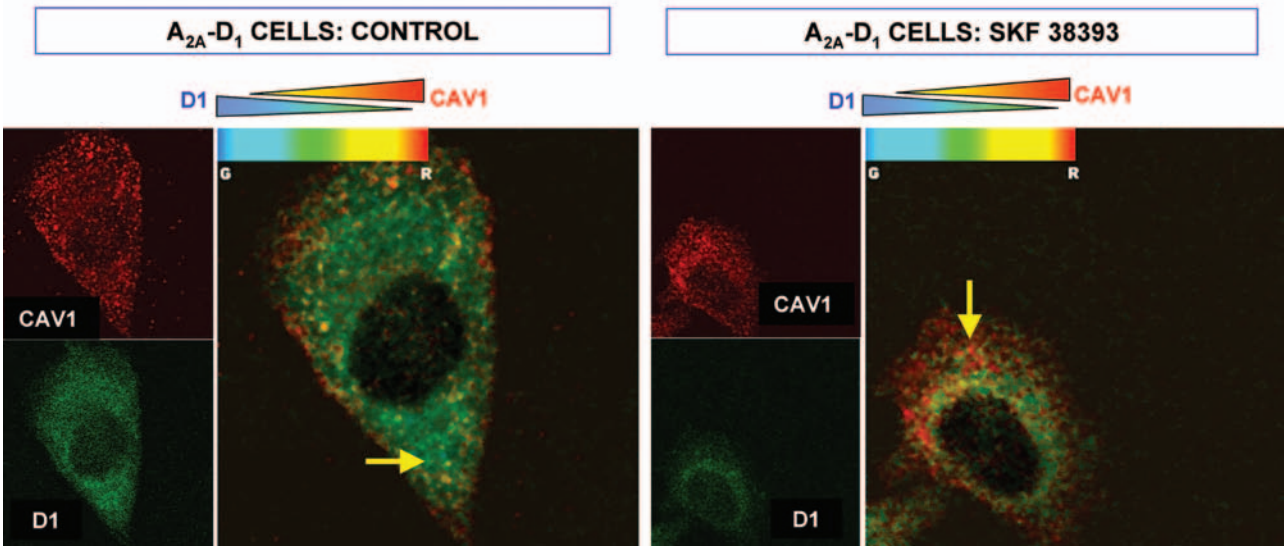

Fig. 1. Example of the results observed in A_{2A}–D1 cells upon treatment with 10 μ*M* D_1 receptor agonist SKF 38393 (3 h). Pixels are coded according to their G/R ratio with a color table going from blue (pixels positive only to D_1 receptor) to red (pixels positive only to caveolin-1). The arrow in the left panel points to a small cluster of blue pixels and, hence, a relative excess of D_1 receptor immunoreactivity with respect to caveolin-1 immunoreactivity. The right panel shows (*see* arrow) a marked excess of pixels positive for caveolin-1, indicating the preferential internalization of D_1 receptors.

D_1 receptor immunoreactivity, indicating a preferential disappearance of D_1 immunoreactivity. The quantitative analysis indicates that the D1 receptor agonist SKF 38393 causes a reduction of D_1 receptor and caveolin-1 immunoreactivities, a significant reduction of caveolin-1–D_1 receptor colocalization (overlap coefficient, $p < 0.05$), a significant reduction of the fraction of D_1 receptor immunoreactivity colocalized with caveolin-1 immunoreactivity (M_{D1}, $p < 0.05$), and a significant reduction of the fraction of caveolin-1 colocalized with D_1 receptors (M_{Cav}, $p < 0.05$) (Fig. 2, left panel). On the other hand, SKF 38393 treatment did not modify A_{2A} receptor or caveolin-1 immunoreactivity (Fig. 2, right panel). These results suggest that SKF 38393 induces co-internalization of caveolin-1 and D_1 receptors and that the fraction of D_1 receptors colocalized with caveolin-1 are more susceptible to internalize upon agonist treatment.

Experiments on Colocalization of A_{2A} and D_2 Receptors With Caveolin-1 and Internalization Upon Agonist Treatment

Under basal conditions there was evidence for colocalization of A_{2A} and D_2 receptors with caveolin-1. The quantitative analysis indicates that both the D_2 receptor agonist quinpirole and the A_{2A} receptor agonist CGS 21680 cause a reduction of D_2 receptor, A_{2A} receptor, and caveolin-1 immunoreactivities,

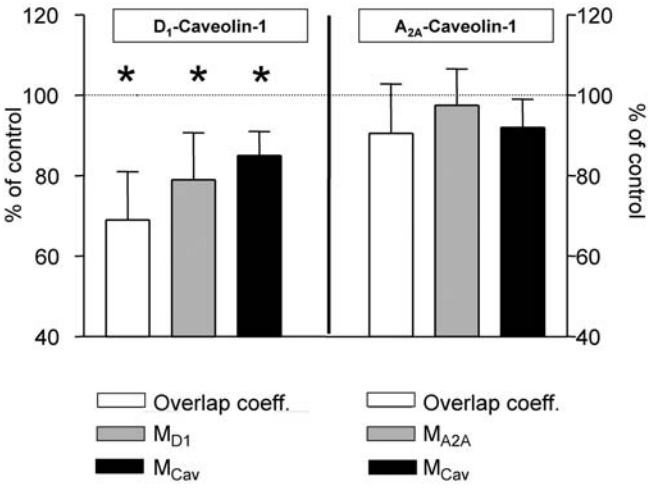

Fig. 2. Quantitative evaluation of colocalization between the D_1 receptor with caveolin-1 and the A_{2A} receptor with caveolin-1 in A_{2A}–D_1 cells after treatment with 10 μ*M* D_1 receptor agonist SKF 38393 (3 h). The overlap coefficient is a measure of colocalization, and M-factor (MD_1, MA_{2A}, and M_{Cav}) is the amount of immunoreactivity of each fluorochrome within the highly correlated pixels as a fraction of its total immunoreactivity (see Materials and Methods). The results suggest that SKF 38393 induces co-internalization of caveolin-1 and D_1 receptors and that the fraction of D_1 receptors colocalized with caveolin-1 are more susceptible to internalize upon agonist treatment. (*) Significantly different compared to control (Student *t*-test: $p < 0.05$).

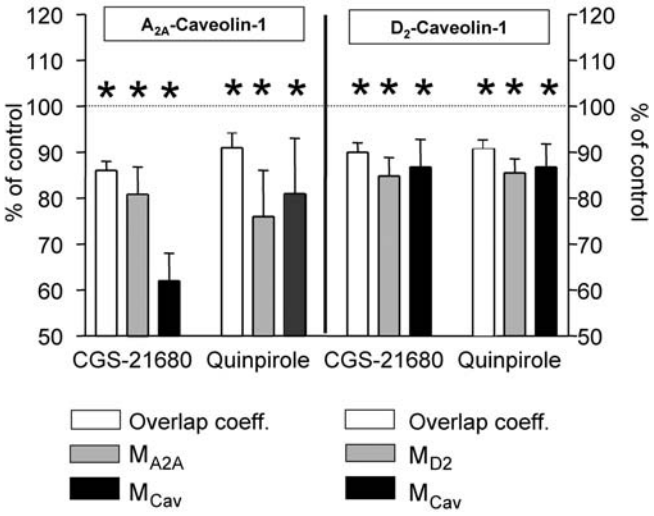

Fig. 3. Quantitative evaluation of colocalization between the D_2 receptor with caveolin-1 and the A_{2A} receptor with caveolin-1 in A_{2A}–D_2 cells after treatment with 50 μ*M* D_2 receptor agonist quinpirole (3 h) or 200 n*M* A_{2A} receptor agonist CGS 21680 (3 h). The overlap coefficient is a measure of colocalization, and M-factor (MD_2, MA_{2A}, and M_{Cav}) is the amount of immunoreactivity of each fluorochrome within the highly correlated pixels as a fraction of its total immunoreactivity (see Materials and Methods). The results suggest that either quinpirole or CGS 21680 induces co-internalization of caveolin-1 and D_2 and A_{2A} receptors, and that the fractions of D_2 and A_{2A} receptors colocalized with caveolin-1 are more susceptible to internalize upon agonist treatment. (*) Significantly different compared to control (Student *t*-test: $p < 0.05$).

a significant reduction of caveolin-1–D_2 receptor and caveolin-1–A_{2A} receptor colocalization (overlap coefficient, $p < 0.05$ in both cases), a significant reduction of the fraction of D_2 receptor and A_{2A} receptor immunoreactivities colocalized with caveolin-1 immunoreactivity (M_{D2} and MA_{2A}, $p < 0.05$), and a significant reduction of the fraction of caveolin-1 colocalized with D_2 and A_{2A} receptors (M_{Cav}, $p < 0.05$ in both cases) (Fig. 3). These results suggest that quinpirole and CGS 21680 induce co-internalization of caveolin-1 and D_2 and A_{2A} receptors, and that the fraction of D_2 and A_{2A} receptors colocalized with caveolin-1 are more susceptible to internalize upon agonist treatment.

Discussion

Although caveolae are well recognized for their involvement in clathrin-independent endocytosis, the role of caveolin-1 is less clear. It was initially thought that caveolin-1 would play a structural role in the formation of caveolae, but the new view is that it plays a regulatory rather than structural function (Liu et al., 2002; Nabi and Le, 2003; Nichols, 2003). In addition to its involvement in endocytosis, caveolin-1 seems to be involved in lipid traffic and signal transduction as well (Smart et al., 1999; Liu et al., 2002). These multiple functions of caveolin-1 are related to its ability to bind to many different lipids and proteins (Smart et al., 1999; Liu et al., 2002), including GPCRs such as adenosine A_1 and serotonin 5-HT_{2A} receptors (Escriche et al., 2003; Bhatnagar et al., 2004). As mentioned in the Introduction, caveolin-1 is a marker of caveolae and clathrin-independent endocytosis. Therefore, the colocalization of A_{2A}, D_2, and D_1 receptors with caveolin-1 in CHO cells shown in the present immunohistochemical study indicates that these receptors are, at least in part, localized in caveolae and, therefore, potentially able to internalize through clathrin-independent endocytosis upon agonist treatment.

In a previous study in a human neuroblastoma cell line (SH-SY5Y), with constitutive expression of A_{2A} receptors and cotranfected with D_2 receptors, A_{2A} and D_2 receptors were shown to coaggregate and co-internalize upon long-term exposure with either A_{2A} or D_2 receptor agonists (Hillion et al., 2002). Similar results were obtained in the present study in CHO cells cotransfected with both A_{2A} and D_2 receptors. Long-term stimulation of either A_{2A} or D_2 receptors with selective agonists induced internalization of both A_{2A} and D_2 receptors, measured as a significant decrease in cell-surface receptor immunoreactivity in nonpermeabilized cell preparations. Furthermore, we were able to show the specificity of this A_{2A}–D_2 receptor co-internalization; in CHO cells cotransfected with both A_{2A} and D_1 receptors, the D_1 receptor agonist was able to induce internalization of D_1, but not A_{2A}, receptors. The most probable mechanism responsible for the selective A_{2A}–D_2 receptor heterointernalization is the existence of A_{2A}–D_2, but not A_{2A}–D_1, heteromeric receptor complexes, as demonstrated previously with coimmunoprecipitation experiments from mouse Ltk fibroblasts cotransfected with A_{2A} and D_1 or D_2 receptors (Hillion et al., 2002). Agonist exposure was always associated with caveolin-1 internalization and with a significant reduction in the degree of colocalization between A_{2A}, D_2, or D_1 receptors and caveolin. Furthermore, the present results suggest that the fraction of receptors colocalized with caveolin-1 is more susceptible to internalize upon agonist exposure, supporting the involvement of clathrin-independent endocytosis for the internalization of D_1, D_2, and A_{2A} receptors. For A_{2A} and D_2 receptors, there is increasing evidence for a

predominance of homo- and heterodimeric versus monomeric forms (Armstrong and Strange, 2001; Canals et al., 2003; Guo et al., 2003).

The fact that a similar degree of internalization of A_{2A} and D_2 receptors is found with exposure to either agonist suggests that A_{2A}–D_2 heterodimers might be more susceptible to be internalized than A_{2A} or D_2 homodimers. This could be associated with a reduced adenosine-mediated control of dopaminergic transmission and, therefore, be involved in some of the secondary effects of long-term treatment with dopaminergic drugs, such as L-DOPA-induced dyskinesia in Parkinson's disease. Recent studies have shown up-regulation of striatal A_{2A} receptors in an animal model of Parkinson's disease upon intermittent treatment with L-DOPA (Tomiyama et al., 2004) and in Parkinson's disease patients with dyskinesias (Calon et al., 2004), which could be related to a compensatory increase of A_{2A} receptor homodimers. Experiments are in progress to determine the possible preferential internalization of A_{2A}–D_2 heteromeric complexes upon agonist exposure.

Recently, it has been demonstrated that caveolin-1 functions as a negative regulator of caveolae or raft-like membrane microdomains (Nabi and Le, 2003; Nichols, 2003; van Deurs et al., 2003), making caveolae highly immobile and slowing down internalization. It might be surmised, therefore, that caveolin-1 can contribute to stabilize raft-like domains; this is obviously a necessary condition to have signaling molecules organized into horizontal molecular networks (HMNs) functionally connected with vertical molecular networks (VMNs). Horizontal molecular networks (HMNs) are formed by different classes of molecules, and some of them are targets for other cytoplasmic molecules. Of obvious importance are those acting on the GPCRs (hence, on receptor mosaics [RMs]) as they regulate the level of inputs to the cell biochemical machinery, that is, to the VMNs. Among these molecules, one could mention (1) molecules controlling GCPR function (e.g., regulators of G-protein signaling proteins [Ishii and Kurachi, 2003]); (2) molecules controlling GPCR internalization (e.g., β-arrestins and caveolins [Gainetdinov et al., 2004]); and (3) molecules controlling GPCR desensitisation (e.g., G protein-coupled receptor kinases, which are key modulators of GPCR signaling and can also interact with caveolins [Penela et al., 2003]).

These are some of the molecules that likely keep under control the inputs to the VMNs, address the signal impinging on the cell only to some of the VMNs among all those that are potentially interconnected with the HMN receiving the extracellular signals, and can give off their own signals to the intracellular biochemical machinery (Miller and Lefkowitz, 2001).

Our data show that caveolin-1 interacts with D_1, D_2, and A_{2A} receptors and that the A_{2A}–D_2 receptor heterodimers are at least in part located within caveolae. Furthermore, there are indications that caveolin-1 controls the internalization of these receptors, likely allowing in the basal condition a sufficient time of permanence at plasma membrane level to give rise to RMs and HMN organization, but also being involved in the internalization (hence, stopping the A_{2A}, D_1, D_2 receptor function) with strong and permanent activation of these receptors.

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