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Adenosine A_{2A} Receptor and Dopamine D_3 Receptor Interactions: Evidence of Functional A_{2A}/D_3 Heteromeric Complexes

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ABSTRACT

Adenosine A_{2A} and dopamine D_2 receptors have been shown previously to form heteromeric complexes and interact at the level of agonist binding, G protein coupling, and trafficking. Because dopamine D_2 and D_3 receptors show a high degree of sequence homology, A_{2A} and D_3 receptors may also interact in a similar manner. The present studies with confocal microscopy showed that A_{2A}-yellow fluorescent protein (YFP) and D_3-green fluorescent protein 2 (GFP2) receptors colocalize in the plasma membrane. Furthermore, fluorescence resonance energy transfer (FRET) analysis demonstrated that A_{2A}-YFP and D_3-GFP2 receptors give a positive FRET efficiency and are thereby likely to exist as heteromeric A_{2A}/D_3 receptor complexes. Saturation experiments with [^{3}H]dopamine demonstrated that the A_{2A} receptor agonist 4-[2-[[6-amino-9(*N*-ethyl-β-D-ribofuranuronaminoamidosyl)-9*H*-purin-2-yl]amino]ethyl]benzenepropanoic acid (CGS-21680) reduced the affinity of the high-affinity agonist binding state of the D_3 receptor for [^{3}H]dopamine. The A_{2A} and D_3 receptors seem to interact also at the level of G protein coupling, because the adenosine A_{2A} receptor agonist CGS-21680 fully counteracted the D_3 receptor-mediated inhibition of a forskolin-mediated increase in cAMP levels. Taken together, when coexpressed in the same neuron, A_{2A} and D_3 receptors seem to form A_{2A}/D_3 heteromeric receptor complexes in which A_{2A} receptors antagonistically modulate both the affinity and the signaling of the D_3 receptors. D_3 receptor is one of the therapeutic targets for treatment of schizophrenia, and therefore, the A_{2A}/D_3 receptor interactions could provide an alternative antischizophrenic treatment.

Specific subtypes of adenosine and dopamine receptors have been shown to interact not only at the intracellular signaling level, but also at the membrane level by forming heteromeric complexes (Franco et al., 2003; Fuxe et al., 2003). Increasing evidence suggests that the receptor oligomerization and particularly dimerization may play an important role in the molecular events modulating the activity of the G protein-coupled receptors (Agnati et al., 2003; Franco et al., 2003). Both confocal and coimmunoprecipitation studies have shown that adenosine A_1/dopamine D_1 receptors and adenosine A_{2A}/dopamine D_2 receptors exist as functional heteromeric complexes on the cell membrane. These interactions are subtype-specific, because A_1/D_2 receptors and A_{2A}/D_1 receptors have been shown to neither colocalize on the cell membrane nor coimmunoprecipitate (Gines et al., 2000; Hillion et al., 2002). Recent experiments with both fluorescence resonance energy transfer (FRET) and bioluminescence resonance energy transfer techniques have also demonstrated the existence of the A_{2A} and D_2 receptor heterodimers in living cells (Canals et al., 2003; Kamiya et al., 2003).

A_{2A} receptors have been shown previously to antagonistically modulate the binding characteristics of the D_2 receptors

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ABBREVIATIONS: FRET, fluorescence resonance energy transfer; B_H, density of high-affinity binding sites; CHO, Chinese hamster ovary; CGS-15943, 9-chloro-2-(2-furyl)[1,2,4]triazole[1,5-c]quinazolin-5-amine; CGS-21680, 4-[2-[[6-amino-9(*N*-ethyl-β-D-ribofuranuronaminoamidosyl)-9*H*-purin-2-yl]amino]ethyl]benzenepropanoic acid; Ro 20-1724, 4-[(3-butoxy-4-methoxyphenyl)-methyl]-2-imidazolidinone; PBS, phosphate-buffered saline; ERK1/2, extracellular signal-regulated kinases 1 and 2; GFP, green fluorescent protein; Gpp(NH)p, guanosine 5′-[*b,g*-imido]triphosphate trisodium salt; FA, field area; MGV, mean gray value; YFP, yellow fluorescent protein.

(Ferre et al., 1991; Dasgupta et al., 1996; Kull et al., 1999; Torvinen et al., 2004). Because the high-affinity state of the D_2 receptors was preferentially modulated by the A_{2A} receptor agonists, a binding assay for the physiological agonist ([^{3}H]dopamine) was used to specifically study the modulation by the A_{2A} agonist CGS-21680 of the high-affinity state of dopamine D_2 receptors. The results showed that the A_{2A} receptor agonist CGS-21680 decreased the high-affinity binding of [^{3}H]dopamine to D_2 but not to D_1 receptors (Torvinen et al., 2004).

The dopamine D_2-like receptor family consists of D_2, D_3, and D_4 receptors. D_2 and D_3 receptors have been shown to have a high degree of sequence homology, the two sharing a 46% overall amino acid homology and a 78% homology in the transmembrane domains (Giros et al., 1990). Furthermore, D_3 receptors form homodimers (Nimchinsky et al., 1997) and heterodimers with D_2 receptors (Scarselli et al., 2001), and they are codistributed in regions of the ventral striatum (Booze and Wallace 1995). Taken from previous results and from the overall sequence homology between D_2 and D_3 receptors, A_{2A} and D_3 receptors may interact directly, in a manner similar to that of A_{2A} and D_2 receptors. Therefore, it was of interest to study the potential interaction between A_{2A} and D_3 receptors, especially because indications have been obtained for A_{2A} receptor-mediated modulation of D_3 receptors in the ventral striatum (Hillefors et al., 1999a; Diaz-Cabiale et al., 2001). The present experiments focus on the interaction between A_{2A} and D_3 receptors in a stably cotransfected A_{2A}/D_3 Chinese hamster ovary (CHO) cell line, thereby eliminating the influence of other adenosine and dopamine receptor subtypes as well as the endogenous dopamine, which even in low levels affects the binding of [^{3}H]dopamine (Heikkila et al., 1983). These studies were performed using [^{3}H]dopamine binding assays and cAMP accumulation experiments. Confocal microscopy and FRET techniques were implemented to demonstrate a potential colocalization and formation of heteromeric A_{2A}/D_3 receptor complexes.

Materials and Methods

Expression Vectors for FRET Analysis. The human cDNA for D_3 receptor without its stop codon was amplified using sense and antisense primers harboring unique EcoRI and KpnI sites. The fragment was then subcloned to be in-frame with a variant of GFP [pGFP2-N3 (h); PerkinElmer Life and Analytical Sciences, Boston, MA)] on the C-terminal end of the receptor. The human FLAG-A_{2A}R cDNA was cloned into the pEYFP-N1 vector in a similar fashion using the EcoRI and BamHI sites to be in-frame with the YFP fluorescent protein variant (Canals et al., 2003, 2004). The negative control cDNA coding for the chemokine receptor CXCR4-YFP was a kind gift from Dr. A. Serrano (Centro Nacional de Biotecnología–Consejo Superior de Investigaciones Cientificas, Madrid, Spain). The positive control vector used for the FRET experiments, pGFP2-EYFP cDNA, was a kind gift from Dr. R. Pepperkok's laboratory (European Molecular Biology Laboratory, Heidelberg, Germany) (Zimmermann et al., 2002).

Transient Transfections for FRET and Confocal Microscopy Analysis. HeLa cells growing in six-well dishes were transiently transfected with the cDNA encoding the indicated proteins by calcium phosphate precipitation (Jordan et al., 1996) To maintain the ratio of cDNA in cotransfections, the empty vector, pcDNA3.1, was used to equilibrate the amount of total cDNA transfected. Twenty-four hours after transfection, the medium was replaced and cells were then cultured in the same medium until harvested 48 h after transfection. For the FRET analysis, HeLa cells were transiently transfected with the plasmid DNA corresponding to D_3-GFP2 (donor) and A_{2A}-YFP or with CXCR4-YFP (acceptor) proteins using a donor-to-acceptor DNA ratio of 1:2 or with the positive control plasmid GFP2-YFP. The analysis of FRET with fluorimetry and the quantification of FRET were performed as described previously (Canals et al., 2003).

Confocal Microscopy. Transiently transfected A_{2A}-YFP/D_3-GFP2 HeLa cells were plated onto 15-mm glass coverslips and fixed in PBS containing 3.5% paraformaldehyde for 15 min at room temperature before washing with PBS and mounting onto slides. Confocal laser-scanning microscopy was performed using a Leica SP2 microscope (Leica Microsystems, Inc., Mannheim, Germany).

Confocal Microscopy and Image Analysis. The "multiply-function method" allows for the discrimination of the high-intensity/density clusters of colocalizing fluorophors (Torvinen et al., 2005). The multiply-function method performs the pixel-by-pixel multiplication of the two images and divides (always pixel-by-pixel) the result by a factor (1,. . . , 255) to avoid overflow (Fig. 2A.). Thus, by means of this procedure, the areas in which the two fluorophors simultaneously show a very high emission are detected (red, yellow, and yellowish colors in the color-coded image shown in Fig. 2A). As indicated by Manders et al. (1993), it is possible to demonstrate that the normalized product is insensitive to differences in signal intensities between the two images caused by multiplicative bias, such as different labeling, photobleaching, or different setting of the detectors. The histogram of the gray values observed in Fig. 2A was recorded, and the mean gray value (MGV) and the respective S.D. were calculated. The field area (FA) of the high emission for both fluorophors was considered to be the area in which high-intensity (and -density) A_{2A}/D_3 clusters are located. A discrimination procedure using as a threshold the equation MGV + 3 × S.D. to selectively visualize the highest emission pixels was carried out (Fig. 2B.), and the corresponding FA was considered to be the total FA of high-intensity/-density clusters of A_{2A}/D_3 receptors. By means of an interactive procedure, the high-intensity/-density clusters localized on the plasma membrane and on the cytoplasm were detected and the respective FA values measured. These FA values are expressed as percentage values of the total FA of high-intensity/-density clusters.

Functionality and Signaling of the D_3 and D_3-GFP2 Receptors. The functionality of the D_3-GFP2 receptor was tested with both [^{3}H]dopamine binding (see *Radioligand Binding Experiments*) in the transiently transfected CHO cell line and with signaling by measuring phosphorylation of extracellular signal-regulated kinases (ERK) 1 and -2 (ERK1/2) in a transiently transfected HeLa cell line.

HeLa cells transiently transfected with wild-type D_3R or D_3R-GFP2 were plated into 25-cm^2 flasks 24 h after transfection and were rendered quiescent by serum starvation overnight. Before mitogen-activated protein kinase phosphorylation assays, an additional 2-h incubation in fresh serum-free medium was performed immediately before the experiment to minimize basal activity. Cells were subsequently stimulated by the addition of medium with or without the D_2-like agonist quinpirole in the presence or absence of raclopride. Stimulation was terminated by a rapid rinse with ice-cold PBS, and the cell lysis was performed by the addition of 500 μl of ice-cold lysis buffer (50 mM Tris-HCl, pH 7.4, 50 mM NaF, 150 mM NaCl, 40 μM glycerophosphate, 1% Triton X-100, 20 μM phenyl-arsin oxide, 1 mM $NaVO_4$, and protease inhibitor cocktail; from Sigma-Aldrich, St. Louis, MO). The cellular debris was removed by centrifugation at 13,000*g* for 5 min, and the total protein content was measured using BCA Protein Assay reagent (Pierce Chemical, Rockford, IL). Aliquots corresponding to 15 μg of protein were mixed with SDS loading buffer, applied to 10% SDS-polyacrylamide gel electrophoresis, and analyzed with Western blot. ERK1/2 activation was assayed by incubating polyvinylidene difluoride membranes with a mouse anti-phospho-ERK1/2 antibody (Sigma-Aldrich, 1:10,000) or with

a rabbit anti-ERK1/2 antibody that recognizes both unphosphorylated and phosphorylated forms of ERK1/2 (Sigma-Aldrich, 1:40,000). The immunoreactive bands were visualized with horseradish peroxidase-linked secondary anti-mouse and anti-rabbit antibodies (DakoCytomation Ltd., Ely, Cambridgeshire, UK) and SuperSignal West Pico Chemiluminescent Substrate (Pierce).

Transfection and Screening of the Stably Expressed Cell Line. Chinese hamster ovary cells (CHO-K1 cells) stably transfected with double hemaglutinin-tagged dog adenosine A_{2A} receptor cDNA (A_{2A} CHO cells) (Torvinen et al., 2004) were used for the following experiments. For coexpression of A_{2A} and D_3 receptors, the human dopamine D_3 receptor cDNA (a kind gift from Dr J.-C. Schwartz, Centre Paul Broca de l'Institut National de la Santé et de la Recherche Médicale, Paris, France) cloned into the pRc/CMV (conferring resistance to geneticin) was transfected into the A_{2A} CHO cell line (Torvinen et al., 2004) with LipofectAMINE Plus reagent (Invitrogen, Carlsbad, CA), and the clones resistant to geneticin (G418) were selected for the screening. The clones were screened with the D_2-like receptor antagonist [^{3}H]raclopride (see *Radioligand Binding Experiments*) using single-point analysis at a radioligand concentration of 2 nM. A clone with a moderate expression of dopamine D_3 receptors was chosen for the present experiments. The stable $A_{2A}D_3$ CHO cell lines were cultured routinely at 37°C with 5% CO_2 in minimum essential medium α medium without nucleosides supplemented with 10% fetal calf serum, 100 U/ml penicillin, 100 U/ml streptomycin, 300 μg/ml hygromycin for selection of adenosine A_{2A} receptor cDNA, and 400 μg/ml G418 (all from Invitrogen) for selection of dopamine D_3 receptor cDNAs.

Radioligand Binding Experiments. The preparation for membranes is described in detail elsewhere (Ferre et al., 1998). The final protein concentration measured with Lowry's standardized protein assay was 0.2 mg/ml. Saturation experiments with the D_2-like receptor antagonist [^{3}H]raclopride are also described in detail elsewhere (Dasgupta et al., 1996; Torvinen et al., 2004). In brief, the [^{3}H]raclopride saturation experiments were carried out with 10 concentrations ranging from 0.1 to 10 nM [^{3}H]raclopride (76.8 Ci/mmol; PerkinElmer) by incubation for 30 min at room temperature. Nonspecific binding is defined as the binding in the presence of 100 μM dopamine. Expression of the A_{2A} receptors was confirmed with the A_{2A} receptor antagonist [^{3}H]ZM-241385 (17.0 Ci/mmol; Tocris Cookson Inc., Bristol, UK) saturation experiments as described previously (Torvinen et al., 2004). Competition experiments with dopamine versus [^{3}H]raclopride were performed by incubation with 20 concentrations (5 pM to 1 μM) of dopamine and 2 nM [^{3}H]raclopride for 30 min at room temperature (Dasgupta et al., 1996).

For [^{3}H]dopamine binding experiments, the following incubation buffer was used: 50 mM Tris-HCl, pH 7.4, containing 5 mM $MgCl_2$, and 1 mM dithiothreitol. Saturation experiments of [^{3}H]dopamine (47 Ci/mmol; Amersham Biosciences UK, Ltd., Little Chalfont, Buckinghamshire, UK) (range, 0.1–10 nM) were performed by incubation for 30 min at room temperature in the presence or absence of the GTP analog Gpp(NH)p (100 μM) (Sigma-Aldrich) or adenosine A_{2A} agonist CGS-21680 (100 nM) (Tocris Cookson). The concentration of 100 nM CGS-21680 used in the present study was taken from previous studies with the effects of different concentrations of CGS-21680 on D_2 receptor binding characteristics (Ferre et al., 1991; Dasgupta et al., 1996). In addition, in the present study, a [^{3}H]dopamine point analysis (2.0 nM) was carried out to study the effects of different concentrations of CGS-21680 (10 nM to 10 μM) on D_3 receptor high-affinity binding.

Because of the potential breakdown of dopamine, this radioligand was always added last. Furthermore, because[^{3}H]dopamine is likely to degrade faster compared with the other radioligands used in this study, [^{3}H]dopamine was always used within 4 weeks from the delivery date. Nonspecific binding was defined as the binding in the presence of 1 mM apomorphine. The incubation was stopped by fast filtration through glass-fiber filters (GF/B; Whatman, Maidstone, UK) by washing three times with 2.5 ml of 50 mM ice-cold Tris-HCl, pH 7.4, with an automatic cell harvester (Brandel Inc., Gaithersburg, MD). The radioactivity content of the filters was detected by liquid scintillation spectrometry. Data from saturation experiments were analyzed by nonlinear regression analysis using GraphPad Prism (GraphPad Software Inc., San Diego, CA), and the density of the binding sites (B_{max}) and the dissociation constant (K_d) values from several independent replications were averaged to permit statistical comparisons between experiments carried out in the presence or absence of the A_{2A} agonist CGS-21680.

cAMP Accumulation Experiments. The accumulation of cAMP was measured with a [^{3}H]cAMP assay system (Amersham Biosciences) as described in the manufacturer's manual. $A_{2A}D_3$ CHO cells were preincubated for 30 min in fresh minimum essential medium α medium without serum. A sample (30 μM) of the phosphodiesterase inhibitor Ro 20-1724 (Calbiochem, San Diego, CA) was added to the media 10 min before the agonist treatment. In addition, 100 nM A_{2A} receptor agonist CGS-21680, 10 μM A_{2A} receptor antagonist CGS-15943 (NeuroSearch, Ballerup, Denmark), and/or 10 μM dopamine (Sigma-Aldrich) were incubated for 20 min at room temperature. For the experiments with forskolin (Sigma-Aldrich), 3 μM forskolin was added 10 min before the determination of cAMP levels.

Results

Confocal Microscopy and FRET Analysis. Densities of A_{2A}-YFP and D_3-GFP2 receptors were characterized in transiently cotransfected HeLa cells by [^{3}H]ZM-241385 and [^{3}H]raclopride binding, respectively. The B_{max} and K_d values for [^{3}H]ZM-241385 binding from two experiments were in the range of 148.1 to 874.2 fmol/mg protein and 0.39 to 1.68 nM, respectively. The B_{max} and K_d values for [^{3}H]raclopride binding were in the range of 122.9 to 295.7 fmol/mg protein and 4.07 to 8.57 nM, respectively. Figure 1 demonstrates the partial colocalization of A_{2A} and D_3 receptors in transiently transfected HeLa cells observed at the membrane and cytoplasmic levels. The image analysis performed on the confocal images revealed that 44 ± 11% (mean ± S.E.M.) of the high-intensity/-density A_{2A}/D_3 receptor clusters are localized at the membrane level (Fig. 2, A and B).

A potential formation of heteromeric A_{2A} and D_3 receptor complexes in the cotransfected HeLa cells was studied with FRET analysis. As shown in Fig. 3, the FRET efficiency was significantly higher ($P < 0.0001$) in HeLa cells transiently cotransfected with cDNAs corresponding to the D_3-GFP2 (donor) and the A_{2A}-YFP (acceptor) receptors compared with the negative control [the D_3-GFP2 (donor) and the chemokine receptor CXCR4-YFP (acceptor) recep-

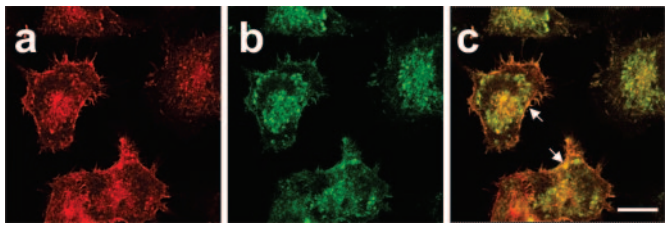

Fig. 1. Adenosine A_{2A} and dopamine D_3 receptor colocalization on the membrane of transiently transfected HeLa cells. HeLa cells transiently transfected with A_{2A}R-YFP (in red) (a) and D_3-GFP2 (in green) (b) were fixed and analyzed by confocal laser microscopy. c, colocalization of both proteins is shown in yellow at the plasma membrane (arrows). In the intracytoplasmic space, the colocalization is shown in greenish-yellow (compare with Fig. 2), because the GFP2(D_3) counteracts the YFP(A_{2A}). Scale bar, 10 μm.

tors] (paired Student's t test, mean ± S.D. of three independent experiments performed in triplicate). The strongest FRET efficiency was obtained with the chimeric positive control cDNA corresponding to GFP2-YFP protein (Zimmermann et al., 2002). The A_{2A}-GFP2/A_{2A}-YFP pair was used as a positive control for receptors forming homodimers (Canals et al., 2004). This pair gave a relatively high FRET efficiency, which was stronger than that obtained after transfection with D_3-GFP2 and A_{2A}-YFP cDNAs. The spectral signatures from cells transiently transfected with cDNAs from only one of the receptors fused to either GFP2 or YFP were used for the unmixing of the GFP2 and YFP emission spectra.

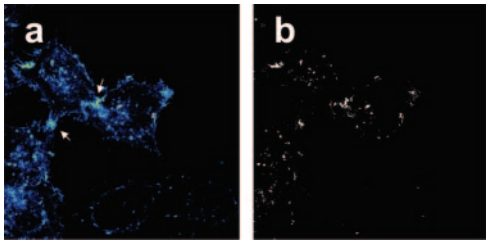

Fig. 2. Visualization of the A_{2A} and D_3 receptor clusters in a cotransfected HeLa cell line. The results were obtained by means of the multiply-function method (See *Materials and Methods*) as shown in a. The multiply-function method allows the visualization of the high-intensity/-density clusters of colocalizing fluorophors (red, yellow, and yellowish pixels). The histogram of the gray values observed in the image shown in a was recorded, and the MGV and the respective S.D. were calculated. A discrimination procedure using as a threshold the equation MGV + 3 × S.D. to selectively visualize the highest emission pixels was carried out. The result of this discrimination procedure (highest intensity/density clusters of colocalizing A_{2A}/D_3 receptor fluorophors) is shown in b (arrows).

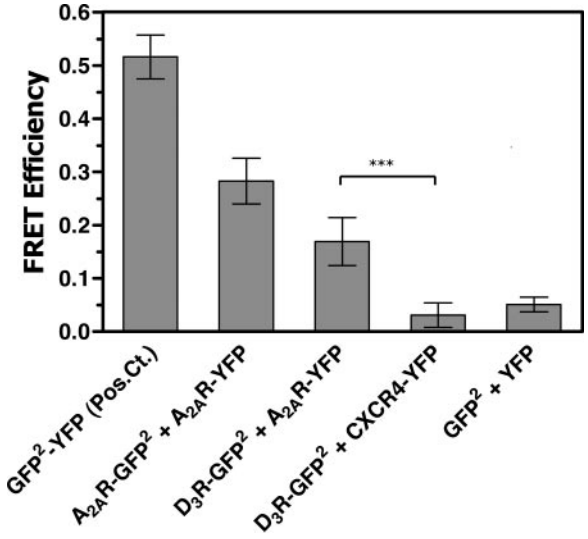

Fig. 3. FRET efficiency of the D_3-GFP2 and A_{2A}-YFP pair by sensitized emission in living cells. HeLa cells were transiently transfected with the plasmid DNA corresponding to D_3-GFP2 (donor) and A_{2A}-YFP or with CXCR4-YFP (acceptor) proteins using a donor-to-acceptor DNA ratio of 1:2 or with the positive control plasmid GFP2-YFP. Fluorescence readings were performed 48 h after transfection as described under *Materials and Methods*. The A_{2A} receptor, which has been shown to form homodimers, was used as positive control for interacting receptors, whereas CXCR4-YFP was used to determine the specificity of the D_3-GFP2/A_{2A}-YFP interaction. Linear unmixing of the emission signals was applied to the data (see *Materials and Methods*), and the results are shown as the sensitized emission of the acceptor when the cells were excited at 400 nm. Data are the mean ± S.D. of three independent experiments performed in triplicate. Significance was evaluated using unpaired Student's t test (***, $P < 0.0001$).

Functionality of the D_3-GFP2 Receptors. Saturation experiments with [^{3}H]dopamine from membrane preparations of a CHO cell line transiently transfected with the D_3-GFP2 receptor cDNA showed a specific [^{3}H]dopamine binding (B_H and K_d values were 80.0 ± 0.7 fmol/mg protein and 1.8 ± 0.04 nM, respectively; mean ± S.E.M., $n = 3$), with a high affinity similar to that found in the nontagged D_3 receptor (Table 1).

The functionality for both D_3 and D_3-GFP2 receptors was also tested by ERK1/2 phosphorylation assay (Cussac et al., 1999). The results show that the D_2-like agonist quinpirole increased dose-dependently (1–100 nM) the phosphorylation of ERK1/2 in HeLa cells expressing D_3 or D_3-GFP2 receptors (results not shown). This effect was antagonized by the D_2-like antagonist raclopride (results not shown).

Modulation of Dopamine Binding to D_3 Receptors by A_{2A} Receptor Agonist CGS-21680. $A_{2A}D_3$ CHO cell line stably expressing A_{2A} and D_3 receptors was established for the [^{3}H]dopamine binding studies as described under *Materials and Methods*. The density and affinity of the A_{2A} receptors in membrane preparations from the $A_{2A}D_3$ CHO cells were characterized by saturation analysis with the A_{2A} antagonist [^{3}H]ZM-241385. The B_{max} and K_d values were 253.0 ± 7.0 fmol/mg protein and 0.46 ± 0.04 nM, respectively (mean ± S.E.M., $n = 4$) (Fig. 4A). The density and affinity of D_3 receptors was characterized by saturation analysis with the D_2-like antagonist [^{3}H]raclopride. The B_{max} and K_d values for this antagonist were 1953.0 ± 50.66 fmol/mg protein and 1.4 ± 0.1 nM, respectively (mean ± S.E.M., $n = 4$) (Fig. 4B). Competition experiments using dopamine versus [^{3}H]raclopride in membrane preparations from the $A_{2A}D_3$ CHO cell line showed a significantly better fit for two binding sites than for one binding site (F test, $P < 0.0001$). The dissociation constants for the high- (K_H) and low-affinity (K_L) binding sites (shown as means with ± S.E.M. in parentheses) were estimated to be 2.6 (±0.17) and 280 (±0.01) nM, respectively ($n = 4$). The number of D_3 receptors in the high-affinity state was studied by saturation experiments using [^{3}H]dopamine as agonist with concentrations in the range of 0.1 to 10 nM. The density of high-affinity [^{3}H]dopamine binding sites (B_H) was found to be substantially lower than the total density of receptors determined with the D_2-like antagonist [^{3}H]raclopride binding, probably reflecting the fact that 18.5% of the D_3 receptors were in the high-affinity state

TABLE 1

The effects of adenosine A_{2A} receptor agonist CGS-21680 on [^{3}H]dopamine binding to dopamine D_3 receptors

Saturation analysis of [^{3}H]dopamine binding in the $A_{2A}D_3$ CHO cell line, given as mean ± S.E.M., $n = 4$. Only the high-affinity [^{3}H]dopamine binding sites are studied (see *Results*).

	K_d	B_H
	nM	*fmol/mg protein*
Control	1.1 ± 0.2	361.5 ± 16.0
CGS-21680 100 nM	3.2 ± 0.4**	437.7 ± 9.9*
Gpp(NH)p 100 μM	–	–

** $P < 0.01$, Student's unpaired t test.
* $P < 0.05$, Student's unpaired t test.
–, very low specific binding; neither K_d nor B_H values could be calculated.

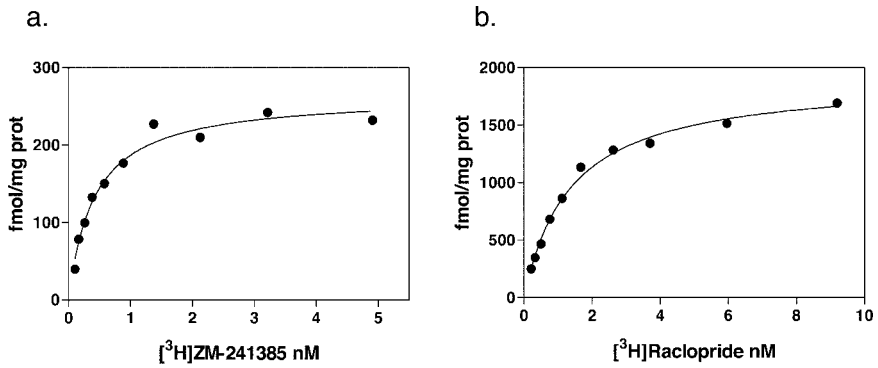

Fig. 4. Representative saturation curves of the specific A_{2A} receptor antagonist [^{3}H]ZM-241385 (a) and D_2-like antagonist [^{3}H]raclopride (b) binding in crude membrane preparations from $A_{2A}D_3$ CHO cell line. The B_{max} and K_d values for [^{3}H]ZM-241385 binding were 261.8 fmol/mg protein and 0.4 nM, respectively. The B_{max} and K_d values for [^{3}H]raclopride binding were 1908.0 fmol/mg protein and 1.3 nM, respectively.

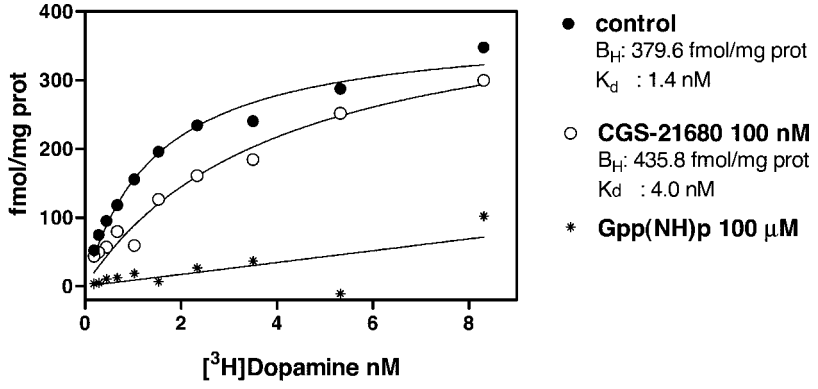

Fig. 5. Representative saturation curves of specific binding of [^{3}H]dopamine in crude membrane preparations from the $A_{2A}D_3$ CHO cell line. Modulation by adenosine A_{2A} receptor agonist CGS-21680 (100 nM). For details, see the text.

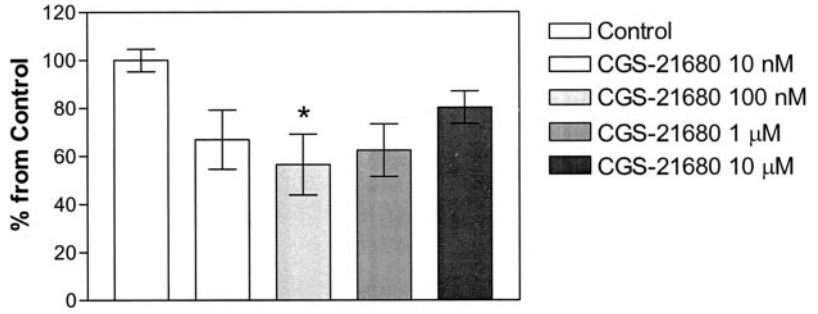

Fig. 6. Specific binding of [^{3}H]dopamine in crude membrane preparations from the $A_{2A}D_3$ CHO cell line. Point analysis with ~2.0 nM [^{3}H]dopamine. Modulation by adenosine A_{2A} receptor agonist CGS-21680. For details, see the text; $n = 3$ experiments, mean and S.E.M.; ⋆, $P < 0.05$ compared with control, one-way analysis of variance with Dunnett's multiple comparison test.

(B_H = 361.5 ± 16.0 and B_{max} = 1953.0 ± 50.66 fmol/mg protein for [^{3}H]dopamine and [^{3}H]raclopride binding, respectively) (Figs. 4B and 5). The effect of the A_{2A} agonist CGS-21680 (10 nM to 10 μM) on the affinity of D_3 receptors for [^{3}H]dopamine were studied with [^{3}H]dopamine point analysis (2.0 nM), and the results showed a peak effect with 100 nM CGS-21680 for a decrease in the affinity of the D_3 receptors for dopamine; therefore, this concentration were used for the [^{3}H]dopamine saturation experiments (Fig. 6). The A_{2A} agonist CGS-21680 (100 nM) significantly ($P < 0.01$, Student's t test) increased by 290% the K_d value of the high-affinity [^{3}H]dopamine binding sites (Fig. 5 and Table 1). The density of high-affinity dopamine D_3 receptor binding sites was weakly but significantly increased by CGS-21680 ($P < 0.05$, Student's t test) (Fig. 5 and Table 1).

Adenosine A_{2A} and Dopamine D_3 Receptor Interactions at the Second-Messenger Level. The effects of both A_{2A} and D_3 receptor agonists on cAMP levels were studied in the $A_{2A}D_3$ CHO cell line (Fig. 7). Dopamine did not change the cAMP levels compared with the control but markedly and significantly counteracted the forskolin-mediated increase in cAMP accumulation. Treatment with the A_{2A} receptor agonist CGS-21680 significantly increased the cAMP accumulation compared with the control, an effect counteracted by dopamine. The A_{2A} receptor antagonist CGS-15943 abolished the effects of the A_{2A} agonist CGS-21680 and significantly enhanced the inhibitory effects of dopamine on CGS-21680–induced cAMP accumulation (Fig. 7A).

CGS-21680 treatment fully counteracted the dopamine-mediated inhibition of forskolin-induced cAMP accumulation despite its ability to produce relatively small increases in the cAMP levels (Fig. 7B). Furthermore, CGS-15943 not only counteracted this action of CGS-21680 but also led to an enhanced inhibitory action of dopamine on forskolin-induced increases of cAMP levels (Fig. 7B).

Discussion

Growing evidence suggests that many subfamilies of the G protein-coupled receptors exist as oligomers. Agonist-induced conformational changes of the interacting receptors within the formed oligomers modify their pharmacology, signaling, and/or trafficking (Agnati et al., 2003; Franco et al., 2003; Fuxe et al., 2003). Adenosine A_{2A} and dopamine D_2 receptors have been described previously to form functional heteromeric complexes, affecting the signaling and the trafficking

of both receptors. Furthermore, the interactions between adenosine and dopamine receptors have been shown previously to be subtype-specific (between A_1/D_1 and A_{2A}/D_2 receptors) (Fuxe et al., 1998; Gines et al., 2000; Hillion et al., 2002). Because the homology between D_2-like receptors is high, the question arises whether D_3 and D_4 receptors interact with the A_{2A} receptor in a manner similar to that of the D_2 receptor.

The multiply-function method image analysis of the confocal images allows a discrimination of the high-intensity/-density clusters of the colocalized A_{2A} and D_3 receptors. Results from the confocal microscopy accompanied by a subsequent image analysis showed that 44% of the high-intensity/-density clusters of the A_{2A} and D_3 receptors colocalize on the cell membrane. It has been shown previously that both A_{2A} (Canals et al., 2004) and D_3 (Nimchinsky et al., 1997) receptors form homodimeric complexes. It is therefore tempting to hypothesize that a certain amount of the A_{2A} and D_3 receptors on the membrane form either A_{2A} or D_3 homodimers or A_{2A}/D_3 receptor heteroligomers, in which additional receptors and/or proteins cannot be excluded. This may lead to a formation of high-order heteromeric receptor complexes at the plasma-membrane level, forming "receptor mosaics" (Agnati et al., 2004). The FRET analysis demonstrates that A_{2A} and D_3 receptors can form heterodimers in the transiently cotransfected HeLa cells, with the receptor expression levels in the range of the endogenous expression of both receptors (see *Results*). This analysis verifies that A_{2A} and D_3 receptors can form heterodimers even in the absence of agonist treatment in a manner similar to that of A_{2A} and D_2 receptors (Canals et al., 2003; Kamiya et al., 2003).

The competition experiments using [^{3}H]raclopride versus dopamine showed that D_3 receptors have two binding sites for dopamine, with a dissociation constant (K_H) of 2.6 nM for the high-affinity state. Because the high-affinity component of [^{3}H]dopamine binding to D_3 receptors was selectively studied, the concentration range used in the [^{3}H]dopamine binding experiments was limited to concentrations between 0.1 and 10 nM. In this range, it was possible to estimate the density of the high-affinity state D_3 receptors. It is well known that agonists bind with high affinities to hepta-spanning membrane receptors when they are bound to G proteins. Because the G protein uncoupling of D_3 receptors with the GTP-analog Gpp(NH)p abolished the specific [^{3}H]dopamine binding between 0.1 to 10 nM, only the D_3 receptors in a high-affinity state were studied. The small increase found in the density of the D_3 receptor may reflect the reduced conversion of D_3 receptors from their high-affinity to low-affinity states, possibly related to the A_{2A} receptor-mediated antagonistic regulation of D_3 receptor signaling (see below). The modulation summarized in Table 1 indicates that the A_{2A} receptor agonist CGS-21680 is able to significantly and strongly reduce the affinity of the high-affinity agonist binding state of the D_3 receptors for dopamine. As in the previously described A_{2A} receptor-mediated modulation of D_2 receptors (Ferre et al., 1991), the maximal antagonistic effect of CGS-21680 was obtained at a concentration of 100 nM. [^{3}H]CGS-21680 has a K_d value of ~30 nM in A_{2A} receptor-expressing CHO cell lines (Klotz, 2000). According to previous studies (Ferre et al., 1991), the modulatory effect of CGS-21680 on D_2 receptor affinity disappears with higher concentrations, probably related to a process of desensitization.

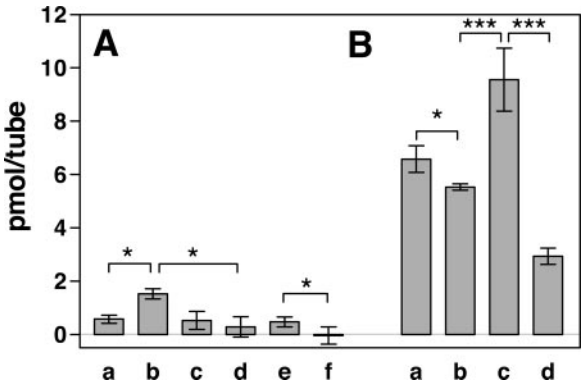

Fig. 7. cAMP accumulation experiments in the $A_{2A}D_3$ CHO cell line. Experiments were performed as described under *Materials and Methods*. The values are given as picomoles per incubation tube. Results represent the mean ± S.E.M. of three independent experiments in which each point was determined in triplicate. A, a, control; b, CGS-21680; c, CGS-15943; d, CGS-21680 + CGS-15943; e, CGS-21680 + dopamine; and f, CGS-21680 + CGS-15943 + dopamine. B, a, forskolin; b, forskolin + dopamine; c, forskolin + dopamine + CGS-21680; and d, forskolin + dopamine + CGS-21680 + CGS-15943. Concentrations used: 100 nM CGS-21680, 10 μM CGS-15943, 10 μM dopamine, and 3 μM forskolin. ⋆, $P < 0.05$; ⋆⋆⋆, $P < 0.001$ using one-way analysis of variance and post hoc Newman-Keuls multiple comparison test.

The antagonistic interactions between A_{2A} and D_3 receptors in the $A_{2A}D_3$ CHO cell line were also determined at the level of second messengers. D_3 receptor activation by dopamine did not change the basal cAMP levels; however, dopamine did partially and significantly counteract the forskolin-induced cAMP accumulation. Treatment with the A_{2A} receptor agonist CGS-21680 increased significantly although modestly the basal cAMP levels, and this effect was counteracted by simultaneous dopamine treatment. Moreover, CGS-21680 fully counteracted the dopamine-mediated strong inhibition of forskolin-induced cAMP accumulation. The A_{2A} antagonist CGS-15943 counteracted the effects of CGS-21680, showing the specificity of the actions of this agonist. The A_{2A} antagonist CGS-15943 also enhanced the dopamine-mediated inhibitory action on cAMP levels, which most probably is a result of a tonic activation of the A_{2A} receptors by endogenous adenosine.

These results demonstrate that A_{2A} and D_3 receptors expressed in the CHO cell line are functional and interact antagonistically at the level of D_3 receptor agonist recognition and signaling. These antagonistic A_{2A}/D_3 receptor interactions were obtained even though the density of the D_2-like receptor antagonist [^{3}H]raclopride binding sites were seven times higher compared with the A_{2A} receptor antagonist [^{3}H]ZM-241385 binding sites. Still, the levels of A_{2A} and D_3 receptor expression in the stably cotransfected $A_{2A}D_3$ CHO cell line are in the range of the endogenous expression of both receptors in the striatum. Density of the A_{2A} receptors is ~250 fmol/mg protein in rat striatum (Lillrank et al., 1999), whereas the density of the D_3 receptors is shown to be between 130 and 1000 fmol/mg protein in rat, depending on the age and the specific area in the striatum (highest density in the ventral striatum) (Hillefors et al., 1999a,b). Thus, a strong antagonistic modulation of D_3 receptors by A_{2A} receptors in the A_{2A}/D_3 CHO cell line was evident despite the differences in the expres-

sion levels (low A_{2A} to high D_3 receptor density). However, high-order heteromeric A_{2A}/D_3 receptor complexes with monomeric A_{2A} receptors and D_3 dimers and/or tetramers may exist, in which activated A_{2A} receptors may enhance negative cooperativity in D_3 dimers and D_3 tetramers. Such a hypothesis can explain the present antagonistic A_{2A}/D_3 receptor interactions in the presence of high and low densities of D_3 and A_{2A} receptors, respectively. Furthermore, selective A_{2A}/D_3 interactions only with the high-affinity state D_3 receptors should also be considered.

Taken together, these findings open up a possibility that A_{2A} and D_3 receptors may form heteromers also in nerve-cell membranes in various regions in the dorsal and the ventral striatum, in which an expression of both A_{2A} and D_3 receptors have been shown previously. Therefore, future studies in primary neuronal cultures and in endogenous tissue will show the potential formation of heteromeric A_{2A}/D_3 receptor complexes in neurons. To engage in these studies, there is a need for specific commercial D_3 receptor antibodies that work with coimmunoprecipitation and immunocytochemistry in primary neuronal cultures and in endogenous tissues. Furthermore, there is a need for more specific D_3 receptor agonists and antagonists that differentiate between the D_2-like receptor subtypes.

Blockage of dopamine D_3 receptors has been indicated to be an important feature of atypical neuroleptics (Schwartz et al., 2000; Schwartz, 2003). The present results describing the formed A_{2A}/D_3 receptor heteromeric complexes suggest a new therapeutic possibility to antagonistically modulate D_3 receptors, namely via an activation of the A_{2A} receptors. Furthermore, a similar kind of interactions may exist between the A_{2A} and dopamine D_4 receptors in view of previous studies with microdialysis showing an antagonistic A_{2A} and D_4 receptor interaction in the corticostriatal glutamate systems (Tanganelli et al., 2004).

A formation of heteromeric receptor complexes increases the alternative strategies for drug design. Some D_2-like antiparkinsonian agents have been shown to behave as potent agonists for the D_3/D_2 heterodimers (Maggio et al., 2003). Thus, in diseases involving the dopaminergic D_2 receptor family (Missale et al., 1998; Schwartz et al., 2000), new drugs may preferentially be directed toward the adenosine A_{2A} receptors, which are a part of the A_{2A}/D_3 and A_{2A}/D_2 heteromeric complexes (Agnati et al., 2003; Franco et al., 2003). Therefore, A_{2A} receptor agonists, which already have been shown to exhibit atypical antipsychotic properties (Rimondini et al., 1997; Andersen et al., 2002), offer a potential novel treatment for schizophrenia.

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