Adenosine A\textsubscript{2A} Receptor and Dopamine D\textsubscript{3} Receptor Interactions: Evidence of Functional A\textsubscript{2A}/D\textsubscript{3} Heteromeric Complexes

Maria Torvinen, Daniel Marcellino, Meritxell Canals, Luigi F. Agnati, Carmen Lluis, Rafael Franco, and Kjell Fuxe

ABSTRACT

Adenosine A\textsubscript{2A} and dopamine D\textsubscript{3} 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\textsubscript{2} and D\textsubscript{3} receptors show a high degree of sequence homology, A\textsubscript{2A} and D\textsubscript{3} receptors may also interact in a similar manner. The present studies with confocal microscopy showed that A\textsubscript{2A}-YFP and D\textsubscript{3}-GFP2 receptors colocalize in the plasma membrane. Furthermore, fluorescence resonance energy transfer (FRET) analysis demonstrated that A\textsubscript{2A}-YFP and D\textsubscript{3}-GFP2 receptors give a positive FRET efficiency and are thereby likely to exist as heteromeric A\textsubscript{2A}/D\textsubscript{3} receptor complexes. Saturation experiments with \[^{3}H\]dopamine demonstrated that the A\textsubscript{2A} receptor agonist 4-[2-[6-amino-9(N-ethyl-β-d-ribofuranuronaminoamido)-9H-purin-2-yl]amino]ethyl]benzenepropanoic acid (CGS-21680) reduced the affinity of the high-affinity agonist binding state of the D\textsubscript{3} receptor for \[^{3}H\]dopamine. The A\textsubscript{2A} and D\textsubscript{3} receptors seem to interact also at the level of G protein coupling, because the adenosine A\textsubscript{2A} receptor agonist CGS-21680 fully counteracted the D\textsubscript{3} receptor-mediated inhibition of a forskolin-mediated increase in cAMP levels. Taken together, when coexpressed in the same neuron, A\textsubscript{2A} and D\textsubscript{3} receptors seem to form A\textsubscript{2A}/D\textsubscript{3} heteromeric receptor complexes in which A\textsubscript{2A} receptors antagonistically modulate both the affinity and the signaling of the D\textsubscript{3} receptors. D\textsubscript{3} receptor is one of the therapeutic targets for treatment of schizophrenia, and therefore, the A\textsubscript{2A}/D\textsubscript{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 communoprecipitation studies have shown that adenosine A\textsubscript{1}/dopamine D\textsubscript{1} receptors and adenosine A\textsubscript{2A}/dopamine D\textsubscript{2} receptors exist as functional heteromeric complexes on the cell membrane. These interactions are subtype-specific, because A\textsubscript{1}/D\textsubscript{2} receptors and A\textsubscript{2A}/D\textsubscript{1} receptors have been shown to neither colocalize on the cell membrane nor communoprecipitate (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\textsubscript{2A} and D\textsubscript{3} receptor heterodimers in living cells (Canals et al., 2003; Kamiya et al., 2003).

A\textsubscript{2A} receptors have been shown previously to antagonistically modulate the binding characteristics of the D\textsubscript{3} receptors.
(Ferre et al., 1991; Dasgupta et al., 1996; Kull et al., 1999; Torvinen et al., 2004). Because the high-affinity state of the D2 receptors was preferentially modulated by the A2A receptor agonists, a binding assay for the physiological agonist ([3H]dopamine) was used to specifically study the modulation by the A2A agonist CGS-21680 of the high-affinity state of dopamine D2 receptors. The results showed that the A2A receptor agonist CGS-21680 decreased the high-affinity binding of [3H]dopamine to D2 but not to D1 receptors (Torvinen et al., 2004).

The dopamine D2-like receptor family consists of D2, D3, and D4 receptors. D3 and D4 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, D3 receptors form homodimers (Nimchinsky et al., 1997) and heterodimers with D2 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 D2 and D3 receptors, A2A and D3 receptors may interact directly, in a manner similar to that of A2A and D2 receptors. Therefore, it was of interest to study the potential interaction between A2A and D3 receptors, especially because indications have been obtained for A2A receptor-mediated modulation of D3 receptors in the ventral striatum (Hillefors et al., 1999a; Diaz-Cabiale et al., 2001). The present experiments focus on the interaction between A2A and D3 receptors in a stably cotransfected A2A/D3 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 [3H]dopamine (Heikkila et al., 1983). These studies were performed using [3H]dopamine binding assays and cAMP accumulation experiments. Confocal microscopy and FRET techniques were implemented to demonstrate a potential colocalization and formation of heteromeric A2A/D3 receptor complexes.

Materials and Methods

Expression Vectors for FRET Analysis. The human cDNA for D3 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-A2AR 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 Científicas, 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 D3-GFP2 (donor) and A2A-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 A2A-YFP/D3-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 fluorophores 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) A2A/D3 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 A2A/D3 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 D3 and D3-GFP2 Receptors. The functionality of the D3-GFP2 receptor was tested with both [3H]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 D3 or D3-GFP2 were plated into 25-cm² 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 D2-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 glycercophosphate, 1% Triton X-100, 20 µM phenyl-arsonic oxide, 1 mM NaN3, and protease inhibitor cocktail; from Sigma-Aldrich, St. Louis, MO). The cellular debris was removed by centrifugation at 13,000g 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 horse-radish 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 A2A receptor cDNA (A2A CHO cells) (Torvinen et al., 2004) were used for the PD experiments. For coexpression of A2A and D3 receptors, the human dopamine D3 receptor cDNA (a kind gift from Dr J.-C. Schwartz, Centre Paul Broca de l’Institut National de la Sante et de la Recherche Medicale, Paris, France) cloned into the pRc/CMV (conferring resistance to geneticin) was transfected into the A2A 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 D3-like receptor antagonist [3H]raclopride (see Radioligand Binding Experiments) using single-point analysis at a radioligand concentration of 2 nM. A clone with a moderate expression of dopamine D3 receptors was chosen for the present experiments. The stable A2A/D3 CHO cell lines were cultured routinely at 37°C with 5% CO2 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 A2A receptor cDNA, and 400 μg/ml G418 (all from Invitrogen) for selection of dopamine D3 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 D3-like receptor antagonist [3H]raclopride are also described in detail elsewhere (Dasgupta et al., 1996; Torvinen et al., 2004). In brief, the [3H]raclopride saturation experiments were carried out with 10 concentrations ranging from 0.1 to 10 nM [3H]raclopride (76.8 Ci/mmol; PerkinElmer) by incubation for 30 min at room temperature. Non-specific binding is defined as the binding in the presence of 100 μM dopamine. Expression of the A2A receptors was confirmed with the A3 receptor antagonist [3H]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 [3H]raclopride were performed by incubation with 20 concentrations (5 pM to 1 μM) of dopamine and 2 nM [3H]raclopride for 30 min at room temperature (Dasgupta et al., 1996).

For [3H]dopamine binding experiments, the following incubation buffer was used: 50 mM Tris-HCl, pH 7.4, containing 5 mM MgCl2, and 1 mM dithiothreitol. Saturation experiments of [3H]dopamine (47 Ci/mmol; Amersham Biosciences UK Ltd., Little Chalfont, Buckinghamshire, UK) ranging from 0.1–10 nM was 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 A2A agonist CGS-21680 (100 nM) (Tocris Cookson). The concentration of 100 nM CGS-21680 was used in the present study was taken from previous studies with the effects of different concentrations of CGS-21680 on D3 receptor binding characteristics (Ferre et al., 1991; Dasgupta et al., 1996). In addition, in the present study, a [3H]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 D3 receptor high-affinity binding.

Because of the potential breakdown of dopamine, this radioligand was always added last. Furthermore, because[3H]dopamine is likely to degrade faster compared with the other radioligands used in this study, [3H]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 (Bmax) and the dissociation constant (Kd) values from several independent replications were averaged to permit statistical comparisons between experiments carried out in the presence or absence of the A2A agonist CGS-21680.

cAMP Accumulation Experiments. The accumulation of cAMP was measured with a [3H]cAMP assay system (Amersham Biosciences) as described in the manufacturer’s manual. A2A/D3 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 A2A receptor agonist CGS-21680, 10 μM A2A 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 A2A-YFP and D3-GFP2 receptors were characterized in transiently cotransfected HeLa cells by [3H]ZM-241385 and [3H]raclopride binding, respectively. The Bmax and Kd values for [3H]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 Bmax and Kd values for [3H]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 A2A and D3 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 A2A/D3 receptor clusters are localized at the membrane level (Fig. 2, A and B).

A potential formation of heteromeric A2A and D3 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 D3-GFP2 (donor) and the A2A-YFP (acceptor) receptors compared with the negative control (the D3-GFP2 (donor) and the chemokine receptor CXCR4-YFP (acceptor) recep-

Fig. 1. Adenosine A2A and dopamine D3 receptor colocalization on the membrane of transiently transfected HeLa cells. HeLa cells transiently transfected with A2A-R-YFP (in red) (a) and D3-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(D3) counteracts the YFP(A2A). Scale bar, 10 μm.
Fused to either GFP2 or YFP were used for the unmixing of the GFP2 and YFP emission spectra.

**Functionality of the D₃-GFP2 Receptors.** Saturation experiments with [³H]dopamine from membrane preparations of a CHO cell line transiently transfected with the D₃-GFP2 receptor cDNA showed a specific [³H]dopamine binding (B₄ and Kᵢ 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 D₃ receptor (Table 1).

The functionality for both D₃ and D₄-GFP2 receptors was also tested by ERK1/2 phosphorylation assay (Cussac et al., 1999). The results show that the D₃-like agonist quinpirole increased dose-dependently (1–100 nM) the phosphorylation of ERK1/2 in HeLa cells expressing D₃ or D₄-GFP2 receptors (results not shown). This effect was antagonized by the D₃-like antagonist raclopride (results not shown).

**Modulation of Dopamine Binding to D₃ Receptors by A₂ₐ Receptor Agonist CGS-21680.** A₂ₐD₃ CHO cell line stably expressing A₂ₐ and D₃ receptors was established for the [³H]dopamine binding studies as described under Materials and Methods. The density and affinity of the A₂ₐ receptors in membrane preparations from the A₂ₐD₃ CHO cells were characterized by saturation analysis with the A₂ₐ antagonist [³H]ZM-241385. The B₄ and Kᵢ 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 D₃ receptors was characterized by saturation analysis with the D₃-like antagonist [³H]raclopride. The B₄ and Kᵢ 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 [³H]raclopride in membrane preparations from the A₂ₐD₃ CHO cell line showed a significantly better fit for two binding sites than for one binding site (P test, P < 0.0001). The dissociation constants for the high- (K₄) and low-affinity (Kᵢ) 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 D₃ receptors in the high-affinity state was studied by saturation experiments using [³H]dopamine as agonist with concentrations in the range of 0.1 to 10 nM. The density of high-affinity [³H]dopamine binding sites (B₄) was found to be substantially lower than the total density of receptors determined with the D₃-like antagonist [³H]raclopride binding, probably reflecting the fact that 18.5% of the D₃ receptors were in the high-affinity state

**RESULTS**

**Table 1**

<table>
<thead>
<tr>
<th></th>
<th>K₄</th>
<th>[³H]Dopamine binding to dopamine D₃ receptors</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Control</strong></td>
<td>1.1 ± 0.2</td>
<td>361.5 ± 16.0</td>
</tr>
<tr>
<td><strong>CGS-21680</strong></td>
<td>3.2 ± 0.4³</td>
<td>437.7 ± 9.9⁴</td>
</tr>
<tr>
<td><strong>Gpp(NH)p</strong></td>
<td>100 μM</td>
<td>–</td>
</tr>
</tbody>
</table>

**Notes:**

**K₄** values could be calculated.

* P < 0.05, Student’s unpaired t test.

**TABLE 1**

**The effects of adenosine A₂ₐ receptor agonist CGS-21680 on [³H]dopamine binding to dopamine D₃ receptors**

**Saturation analysis of [³H]dopamine binding in the A₂ₐD₃ CHO cell line, given as mean ± S.E.M., n = 4. Only the high-affinity [³H]dopamine binding sites are studied (see Results).**

---

**FIGURES**

**Fig. 2. Visualization of the A₂ₐ and D₃ 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 fluorophores (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 x S.D. to selectively visualize the highest emission pixels was carried out. The result of this discrimination procedure (highest intensity/density clusters of colocalizing A₂ₐ/D₃ receptor fluorophors) is shown in b (arrows).

**Fig. 3. FRET efficiency of the D₃-GFP2 and A₂ₐ-YFP pair by sensitized emission in living cells.** HeLa cells were transiently transfected with the plasmid DNA corresponding to D₃-GFP2 (donor) and A₂ₐ-YFP or with CXC41-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 A₂ₐ receptor, which has been shown to form homodimers (Canals et al., 2004). This pair gave a relatively high FRET efficiency, which was stronger than that obtained after transfection with D₃-GFP2 and A₂ₐ-YFP cDNAs. The spectral signatures from cells transiently transfected with cDNAs from only one of the receptors

---

**TIONS**

**triplicate. Significance was evaluated using unpaired Student’s t test (*** P < 0.0001).**
The affinity of D₃ receptors for [³H]dopamine were studied with [³H]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 D₃ receptors for dopamine; therefore, this concentration were used for the [³H]dopamine saturation experiments (Fig. 6). The A₂A agonist CGS-21680 (100 nM) significantly (P < 0.01, Student’s t test) increased the Kᵋ value of the high-affinity [³H]dopamine binding sites (Fig. 5 and Table 1). The density of high-affinity dopamine D₃ receptor binding sites was weakly but significantly increased by CGS-21680 (P < 0.05, Student’s t test) (Fig. 5 and Table 1).

Adenosine A₂A and Dopamine D₃ Receptor Interactions at the Second-Messenger Level. The effects of both A₂A and D₃ receptor agonists on cAMP levels were studied in the A₂A-D₃ 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 A₂A receptor agonist CGS-21680 significantly increased the cAMP accumula-

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 A₂A and dopamine D₂ receptors have been described previously to form functional hetero-

Fig. 4. Representative saturation curves of the specific A₂A receptor antagonist [³H]ZM-241385 (a) and D₂-like antagonist [³H]raclopride (b) binding in crude membrane preparations from A₂A-D₃ CHO cell line. The Bₘₐₓ and Kᵋ values for [³H]ZM-241385 binding were 261.8 fmol/mg protein and 0.4 nM, respectively. The Bₘₐₓ and Kᵋ values for [³H]raclopride binding were 1908.0 fmol/mg protein and 1.3 nM, respectively.

Fig. 5. Representative saturation curves of specific binding of [³H]dopamine in crude membrane preparations from the A₂A-D₃ CHO cell line. Modulation by adenosine A₂A receptor agonist CGS-21680 (100 nM). For details, see the text.

Fig. 6. Specific binding of [³H]dopamine in crude membrane preparations from the A₂A-D₃ CHO cell line. Point analysis with ~2.0 nM [³H]dopamine. Modulation by adenosine A₂A 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.

<table>
<thead>
<tr>
<th>Bₘₐₓ</th>
<th>Kᵋ</th>
<th>CGS-21680 10 nM</th>
<th>CGS-21680 100 nM</th>
</tr>
</thead>
<tbody>
<tr>
<td>379.6 fmol/mg prot</td>
<td>1.4 nM</td>
<td>435.8 fmol/mg prot</td>
<td>4.0 nM</td>
</tr>
</tbody>
</table>

(Bₜₐ = 361.5 ± 16.0 and Bₘₐₓ = 1953.0 ± 50.66 fmol/mg protein for [³H]dopamine and [³H]raclopride binding, respectively) (Figs. 4B and 5). The effect of the A₂A agonist CGS-21680 (10 nM to 10 μM) on the affinity of D₃ receptors for [³H]dopamine were studied with [³H]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 D₃ receptors for dopamine; therefore, this concentration were used for the [³H]dopamine saturation experiments (Fig. 6). The A₂A agonist CGS-21680 (100 nM) significantly (P < 0.01, Student’s t test) increased by 290% the Kᵋ value of the high-affinity [³H]dopamine binding sites (Fig. 5 and Table 1). The density of high-affinity dopamine D₃ receptor binding sites was weakly but significantly increased by CGS-21680 (P < 0.05, Student’s t test) (Fig. 5 and Table 1).
of both receptors. Furthermore, the interactions between adenosine and dopamine receptors have been shown previously to be subtype-specific (between A1/D1 and A2A/D2 receptors) (Fuxe et al., 1998; Gines et al., 2000; Hillion et al., 2002). Because the homology between D2-like receptors is high, the question arises whether D3 and D4 receptors interact with the A2A receptor in a manner similar to that of the D3 receptor.

The multiply-function method image analysis of the confocal images allows a discrimination of the high-intensity/density clusters of the colocalized A2A and D3 receptors. Results from the confocal microscopy accompanied by a subsequent image analysis showed that 44% of the high-intensity/density clusters of the A2A and D3 receptors colocalize on the cell membrane. It has been shown previously that both A2A (Canals et al., 2004) and D3 (Nimchinsky et al., 1997) receptors form homodimeric complexes. It is therefore tempting to hypothesize that a certain amount of the A2A and D3 receptors on the membrane form either A2A or D3 homodimers or A2A/D3 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 A2A and D3 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 A2A and D3 receptors can form heterodimers even in the absence of agonist treatment in a manner similar to that of A2A and D2 receptors (Canals et al., 2003; Kamiya et al., 2003).

The competition experiments using [3H]raclopride versus dopamine showed that D3 receptors have two binding sites for dopamine, with a dissociation constant (Kd) of 2.6 nM for the high-affinity state. Because the high-affinity component of [3H]dopamine binding to D3 receptors was selectively studied, the concentration range used in the

![Fig. 7. cAMP accumulation experiments in the A2A/D3 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; 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.](https://www.aspetjournals.org/asp.pdf?doi=10.1124/jpharm.117.024716)
sion levels (low A2A to high D3 receptor density). However, high-order heteromeric A2A/D3 receptor complexes with monomeric A2A receptors and D3 dimers and/or tetramers may exist, in which activated A2A receptors may enhance negative cooperativity in D3 dimers and D3 tetramers. Such a hypothesis can explain the present antagonistic A2A/D3 receptor interactions in the presence of high and low densities of D3 and A2A receptors, respectively. Furthermore, selective A2A/D3 interactions only with the high-affinity state D3 receptors should also be considered.

Taken together, these findings open up a possibility that A2A and D3 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 A2A and D3 receptors have been shown previously. Therefore, future studies in primary neuronal cultures and in endogenous tissue will show the potential formation of heteromeric A2A/D3 receptor complexes in neurons. To engage in these studies, there is a need for specific commercial D3 receptor antibodies that work with communoprecipitation and immunocytochemistry in primary neuronal cultures and in endogenous tissues. Furthermore, there is a need for more specific D3 receptor agonists and antagonists that differentiate between the D2-like receptor subtypes.

Blockage of dopamine D3 receptors has been indicated to be an important feature of atypical neuroleptics (Schwartz et al., 2000; Schwartz, 2003). The present results describing the formed A2A/D3 receptor heterodimeric complexes suggest a new therapeutic possibility to antagonistically modulate D3 receptors, namely via an activation of the A2A receptors. Furthermore, a similar kind of interactions may exist between the A2A and dopamine D3 receptors in view of previous studies with microdialysis showing an antagonistic A2A and D3 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 D2-like antipsychokinetic agents have been shown to behave as potent agonists for the D3/D2 heterodimers (Maggio et al., 2003). Thus, in diseases involving the dopaminergic D2 receptor family (Missale et al., 1998; Schwartz et al., 2000), new drugs may preferentially be directed toward the adenosine A2A receptors, which are a part of the A2A/D3 and A2A/D2 heteromeric complexes (Agnati et al., 2003; Franco et al., 2003). Therefore, A2A 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.

References
Canals M, Marcellino D, Canals R, Frigerio F, Burguero J, and Fuxe K (2003) A2A/D3 receptor interactions in the presence of high and low densities of D3 and A2A receptors, respectively. Further- more, selective A2A/D3 interactions only with the high-affinity state D3 receptors should also be considered.

Taken together, these findings open up a possibility that A2A and D3 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 A2A and D3 receptors have been shown previously. Therefore, future studies in primary neuronal cultures and in endogenous tissue will show the potential formation of heteromeric A2A/D3 receptor complexes in neurons. To engage in these studies, there is a need for specific commercial D3 receptor antibodies that work with communoprecipitation and immunocytochemistry in primary neuronal cultures and in endogenous tissues. Furthermore, there is a need for more specific D3 receptor agonists and antagonists that differentiate between the D2-like receptor subtypes.

Blockage of dopamine D3 receptors has been indicated to be an important feature of atypical neuroleptics (Schwartz et al., 2000; Schwartz, 2003). The present results describing the formed A2A/D3 receptor heterodimeric complexes suggest a new therapeutic possibility to antagonistically modulate D3 receptors, namely via an activation of the A2A receptors. Furthermore, a similar kind of interactions may exist between the A2A and dopamine D3 receptors in view of previous studies with microdialysis showing an antagonistic A2A and D3 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 D2-like antipsychokinetic agents have been shown to behave as potent agonists for the D3/D2 heterodimers (Maggio et al., 2003). Thus, in diseases involving the dopaminergic D2 receptor family (Missale et al., 1998; Schwartz et al., 2000), new drugs may preferentially be directed toward the adenosine A2A receptors, which are a part of the A2A/D3 and A2A/D2 heteromeric complexes (Agnati et al., 2003; Franco et al., 2003). Therefore, A2A 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.

References
Canals M, Marcellino D, Canals R, Frigerio F, Burguero J, and Fuxe K (2003) A2A/D3 receptor interactions in the presence of high and low densities of D3 and A2A receptors, respectively. Furthermore, selective A2A/D3 interactions only with the high-affinity state D3 receptors should also be considered.

Taken together, these findings open up a possibility that A2A and D3 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 A2A and D3 receptors have been shown previously. Therefore, future studies in primary neuronal cultures and in endogenous tissue will show the potential formation of heteromeric A2A/D3 receptor complexes in neurons. To engage in these studies, there is a need for specific commercial D3 receptor antibodies that work with communoprecipitation and immunocytochemistry in primary neuronal cultures and in endogenous tissues. Furthermore, there is a need for more specific D3 receptor agonists and antagonists that differentiate between the D2-like receptor subtypes.

Blockage of dopamine D3 receptors has been indicated to be an important feature of atypical neuroleptics (Schwartz et al., 2000; Schwartz, 2003). The present results describing the formed A2A/D3 receptor heterodimeric complexes suggest a new therapeutic possibility to antagonistically modulate D3 receptors, namely via an activation of the A2A receptors. Furthermore, a similar kind of interactions may exist between the A2A and dopamine D3 receptors in view of previous studies with microdialysis showing an antagonistic A2A and D3 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 D2-like antipsychokinetic agents have been shown to behave as potent agonists for the D3/D2 heterodimers (Maggio et al., 2003). Thus, in diseases involving the dopaminergic D2 receptor family (Missale et al., 1998; Schwartz et al., 2000), new drugs may preferentially be directed toward the adenosine A2A receptors, which are a part of the A2A/D3 and A2A/D2 heteromeric complexes (Agnati et al., 2003; Franco et al., 2003). Therefore, A2A 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.


**Address correspondence to:** Dr. Kjell Fuxe, Department of Neuroscience, Karolinska Institute, 17177 Stockholm, Sweden. E-mail: kjell.fuxe@neuro.ki.se