

Coaggregation, Cointernalization, and Codesensitization of Adenosine A_{2A} Receptors and Dopamine D₂ Receptors*

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Antagonistic and reciprocal interactions are known to exist between adenosine and dopamine receptors in the striatum. In the present study, double immunofluorescence experiments with confocal laser microscopy showed a high degree of colocalization of adenosine A_{2A} receptors (A_{2A}R) and dopamine D₂ receptors (D₂R) in cell membranes of SH-SY5Y human neuroblastoma cells stably transfected with human D₂R and in cultured striatal cells. A_{2A}R/D₂R heteromeric complexes were demonstrated in coimmunoprecipitation experiments in membrane preparations from D₂R-transfected SH-SY5Y cells and from mouse fibroblast Ltk⁻ cells stably transfected with human D₂R (long form) and transiently cotransfected with the A_{2A}R double-tagged with hemagglutinin. Long term exposure to A_{2A}R and D₂R agonists in D₂R-cotransfected SH-SY5Y cells resulted in coaggregation, cointernalization and codesensitization of A_{2A}R and D₂R. These results give a molecular basis for adenosine-dopamine antagonism at the membrane level and have implications for treatment of Parkinson's disease and schizophrenia, in which D₂R are involved.

Adenosine and dopamine signaling exert opposite effects in the basal ganglia, a brain region involved in sensory-motor integration. Thus, adenosine agonists induce motor depression and adenosine antagonists, such as caffeine, produce motor activation (1). These opposite effects result from specific antagonistic interactions between subtypes of adenosine and dopamine receptors in the striatum, the main input structure of the basal ganglia. In fact, striatal dopamine receptors and, to some extent, adenosine receptors are segregated in the two main populations of γ -aminobutyric acid (GABA) efferent neurons. Striopallidal neurons express dopamine receptors predomi-

nantly of the D₂ subtype (D₂R)¹ and the highest density of adenosine A_{2A} receptors (A_{2A}R) in the brain (2, 3). In the rat, the strionigro-striopeduncular neurons contain dopamine receptors predominantly of the D₁ subtype (D₁R) and express exclusively adenosine receptors of the A₁ subtype (A₁R) (2–4). Experimental evidence supports the existence of antagonistic A_{2A}R/D₂R and A₁R/D₁R interactions, respectively, in the GABA striopallidal and strionigro-striopeduncular neurons (1, 5). Taken together, these data suggested proximity between receptors, which could allow them to form heteromeric complexes (directly or indirectly by means of adaptor proteins). In fact, A₁R and D₁R were found to form heteromeric complexes (6). Although antagonistic A_{2A}R/D₂R interactions have been reported to play an important role in basal ganglia function, it is still not known whether the formation of heteromeric A_{2A}R/D₂R complexes is also involved. In the present work, the existence of selective intramembrane interactions between A_{2A}R and D₂R in neuronal cells is demonstrated, including formation of heteromeric A_{2A}R/D₂R complexes and their potential involvement in cross-desensitization mechanisms via agonist-induced coaggregation and cointernalization of A_{2A}R and D₂R. These results have clinical implications for Parkinson's disease and other basal ganglia disorders where dopamine D₂R are the target for therapy (1, 5).

EXPERIMENTAL PROCEDURES

Cell Cultures—Maintenance of SH-SY5Y cells (parental and D₂R-transfected cells) as well as the pharmacological characterization and maintenance of D₂R- and D₁R-transfected mouse fibroblast Ltk⁻ cells are described in detail elsewhere (7–9). For primary cultures, striata were removed from 16-day-old Sprague-Dawley rat embryos (B&K Universal) in Ca²⁺/Mg²⁺-free PBS supplemented with 20 units/ml penicillin and 20 μ g/ml streptomycin (Invitrogen). The tissue fragments were pooled and mechanically dissociated in SFM Neurobasal serum-free medium (Invitrogen), supplemented with B27 (Invitrogen), glutamine (2 mM; Invitrogen), penicillin/streptomycin (20 units/ml/20 μ g/ml; Invitrogen), and β -mercaptoethanol (25 μ M) (Invitrogen). Cells were collected by centrifugation at 100 \times g for 5 min and resuspended in fresh medium. The resulting single-cell suspension was seeded on 24-well plates coated with gelatin (Sigma) and poly-L-lysine (Sigma), and cells were grown at 37 °C in saturation humidity with 5% CO₂.

Immunolabeling Experiments—Neuroblastoma cells were grown on glass coverslips coated with poly-L-lysine (Sigma) and exposed to vari-

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¹ The abbreviations used are: D₂R, dopamine D₂ receptor; A_{2A}R, adenosine A_{2A} receptor; HA, hemagglutinin; ANOVA, analysis of variance; PBS, phosphate-buffered saline; FITC, fluorescein isothiocyanate; IR, immunoreactive; GI, Gini's index; DOPA, 3,4-dihydroxyphenylalanine.

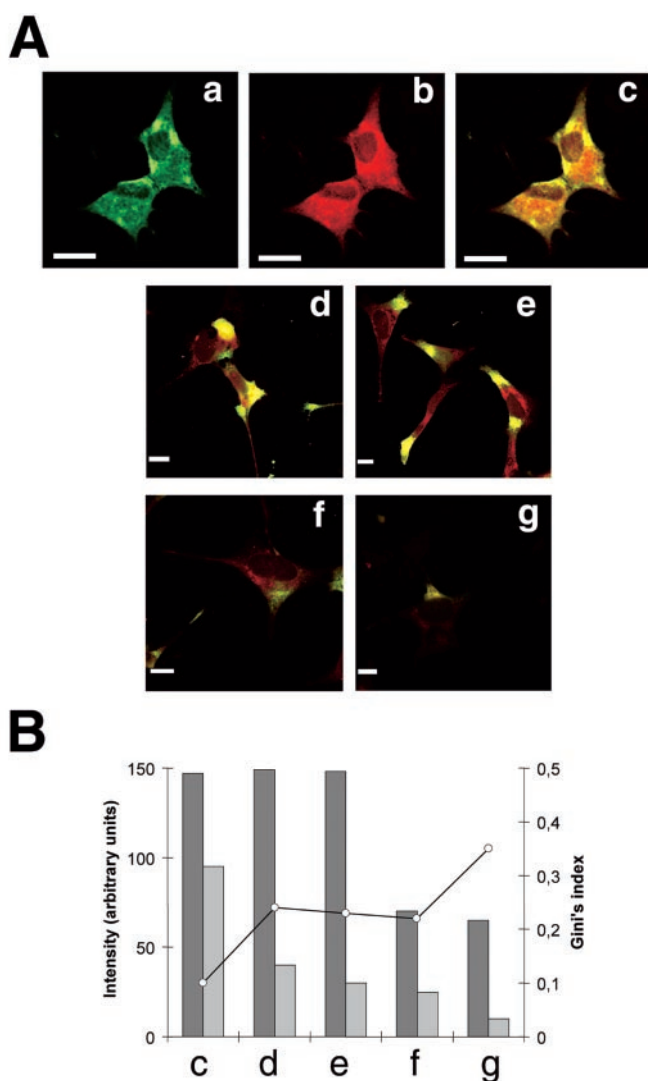


FIG. 1. *A*, double immunofluorescence staining and confocal images of SH-SY5Y neuroblastoma cells stably transfected with the cDNA encoding for human D_2R (long form). Cells were processed for immunostaining using fluorescein (green)-conjugated rabbit anti- $A_{2A}R$ antibodies and rhodamine (red)-conjugated rabbit anti- D_2R antibodies and analyzed by confocal microscopy. Superimposition of images (panels *c–h*) reveals the colocalization of $A_{2A}R$ and D_2R in yellow. Panels *a–c* show $A_{2A}R$ immunoreactivity (*a*), D_2R immunoreactivity (*b*), and $A_{2A}R$ / D_2R colocalization (*c*) in nontreated cells (no agonist preincubation). Panels *d–g* show the effects of 3 h of treatment at 37 °C with CGS-21680 (100 nM) alone (*d*), quinpirole (10 μM) alone (*e*), CGS-21680 (100 nM) and quinpirole (10 μM) (*f*), or CGS-21680 (200 nM) and quinpirole (50 μM) (*g*). Cells were processed for immunostaining after agonist preincubation. Representative images from four independent experiments/treatment are shown; scale bars, 10 μm. *B*, semiquantitation of images from panels *c–g* in *A*. The area (light gray) where diffuse IR (no or few coaggregates) and the area (dark gray) where several coaggregates could be detected were interactively selected. The median intensity values (left y axis) for these two types of areas, obtained in the four experimental conditions mentioned above (*c–g*), are given. The Gini's indexes (right y axis) for the four experimental conditions (*c–g*) are given. It should be noticed that the Gini's index is low (rather even distribution of the IR intensity among pixels) for nontreated cells (*c*), whereas the Gini's index is high (rather uneven distribution of the IR intensity among pixels) for co-treatment with CGS-21680 (200 nM) plus quinpirole (50 μM) (*g*).

ous amounts of CGS21680 (RBI) and/or quinpirole (RBI) in serum-free medium for 3 h at 37 °C. They were then rinsed with PBS, fixed in 4% paraformaldehyde and 0.06 M sucrose for 15 min, and washed with PBS containing 20 mM glycine. Cells were subsequently treated with PBS, containing 20 mM glycine, and 1% bovine serum albumin for 30 min at room temperature. Double immunostaining was performed with fluo-

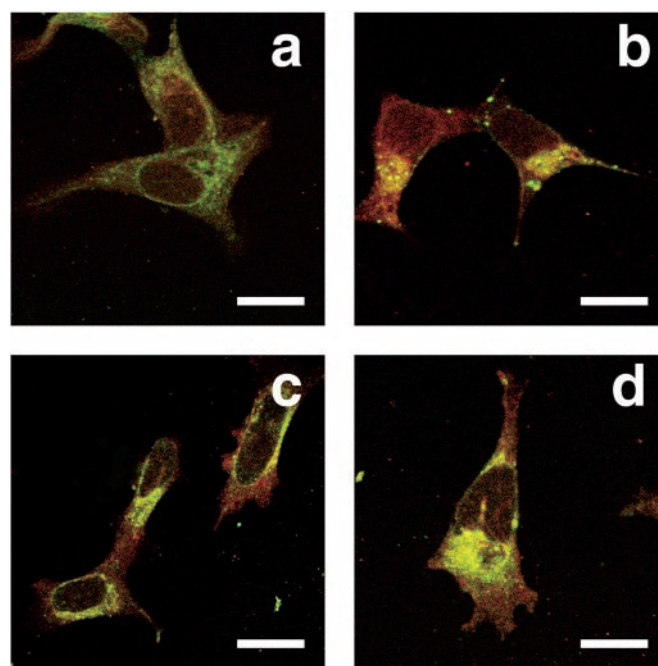


FIG. 2. Agonist-induced internalization of $A_{2A}R$ and D_2R in SH-SY5Y neuroblastoma cells stably transfected with the cDNA encoding for human D_2R (long form). Cells were processed for immunostaining using fluorescein (green)-conjugated rabbit anti- $A_{2A}R$ antibodies and rhodamine (red)-conjugated rabbit anti- D_2R antibodies and analyzed by confocal microscopy. Superimposition of images reveals the colocalization of $A_{2A}R$ and D_2R (yellow). Cells were first incubated with anti- $A_{2A}R$ and anti- D_2R antibodies for 2 h at 4 °C in the presence (*b–d*) or absence (*a*) of the agonists. The cells were then placed at 37 °C for 3 h allowing ligand-induced internalization of previously labeled receptors. Panel *a* shows the weak diffuse immunoreactivity of nonpretreated control cells (compare with Fig. 1, panels *c*). Panels *b–d* show the effects of 3 h of treatment at 37 °C with CGS-21680 (200 nM) alone (*b*), quinpirole (50 μM) alone (*c*), or CGS-21680 (200 nM) and quinpirole (50 μM) (*d*). Representative images from four independent experiments/treatment are shown; scale bars, 10 μm.

rescein-conjugated anti- $A_{2A}R$ antibodies (VC21-FITC, 40 μg/ml) (10) and rhodamine-conjugated anti- D_2R antibodies (D2-246-316, 30 μg/ml) (11) for 1 h at 37 °C. Antipeptide antisera against $A_{2A}R$ were raised in New Zealand White rabbits and characterized as described elsewhere (12). The specific peptide corresponds to the second extracellular loop of the receptor. Anti- $A_{2A}R$ antibodies (VC21) were purified by affinity chromatography using the specific peptide coupled to activated thiol-Sepharose 4B. The coverslips were rinsed for 40 min in the same buffer and mounted with medium suitable for immunofluorescence (ICN). Internalization assays were made as reported elsewhere (12) with some modifications. Cells were incubated in serum-free medium (4 °C, 2 h) with VC21-FITC and rhodamine-conjugated D2-246-316 in the absence (control) or presence of 200 nM CGS 21680 and/or 50 μM quinpirole. Cells were washed with the same medium (without ligands in the control experiments), and internalization of labeled receptors was induced by incubation at 37 °C for 3 h. Cells were fixed and processed as described above. Striatal cultures were exposed to various amounts of CGS21680 and/or quinpirole in the same medium for 6 h at 37 °C. They were then rinsed with PBS, fixed with 4% paraformaldehyde containing 2% picric acid for 1 h, and washed with PBS supplemented with 20 mM glycine. Double or single immunostaining was performed with fluorescein-conjugated anti- $A_{2A}R$ antibodies and rhodamine-conjugated anti- D_2R (D2-246-316) antibodies or fluorescein-conjugated anti- A_1R antibodies (PC21-FITC) (13) for 1 h at 37 °C. Microscopic observations were made in Leica TCS 4D (Leica Lasertechnik) confocal scanning laser equipment adapted to an inverted Leitz DMIRBE microscope. Image analysis was performed with a KS 400 image analyzer (Zeiss). After acquisition of the superimposed images (yellow color) of the $A_{2A}R$ / D_2R IR, the area of the cell nucleus was interactively discarded. Densitometric evaluation was performed on this new image. The median values of the histograms representing the intensity of A_2R / D_2R IR in areas with coaggregates (interactively encircled) and in areas with no or few aggregates (interactively encircled) were taken as representative val-

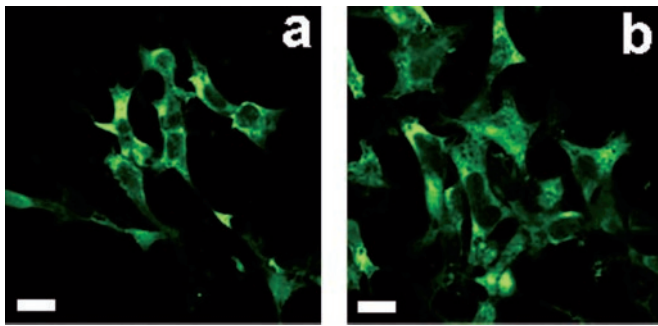


FIG. 3. **Immunostaining of parental SH-SY5Y neuroblastoma cells.** Untransfected SH-SY5Y cells were processed for immunostaining using fluorescein (green)-conjugated rabbit anti-A_{2A}R antibodies after exposure to medium in the absence (a) or presence (b) of 100 μ M quinpirole (3 h, 37 °C). Representative images from three independent experiments are shown; scale bar, 10 μ m.

ues in their respective areas. A quantitative evaluation of the degree of unevenness of the A_{2A}R/D₂R receptor codistribution was obtained by calculating the Gini's index (14, 15), which here measures the distribution of the IR intensity, pixel by pixel, in the sampled area. The Gini's index ranges from 0 (even distribution, i.e. the IR intensity is equally distributed among pixels) to 1 (maximal concentration, i.e. the IR intensity is concentrated in one single pixel) (14).

Coimmunoprecipitation Experiments—SH-SY5Y neuroblastoma cell membranes were obtained by disrupting the cells with a Polytron homogenizer (Kinematica, PTA 20TS rotor, setting 4; Brinkmann) for three 5-s periods in 50 mM Tris-HCl, pH 7.4. Membranes were separated by centrifugation at $105,000 \times g$ for 45 min at 4 °C. They were then solubilized in ice-cold lysis buffer (PBS, pH 7.4, Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS) for 1 h on ice and then centrifuged at $80,000 \times g$ for 90 min. The supernatant was immunoprecipitated as previously described (6) using anti-A_{2A}R antibodies (VC21) or an irrelevant IgG covalently coupled to protein A-Sepharose. Immunoprecipitates were resolved by SDS-PAGE, and Western blots were performed with anti-A_{2A}R, anti-A₁R (PC11), or anti-D₂R antibodies (for details, see Ref. 6).

D₂R- and D₁R transfected mouse fibroblast Ltk⁻ cells were transiently transfected with A_{2A}R cDNA double-tagged with hemagglutinin (HA-A_{2A}R-HA) (16) by calcium phosphate precipitation (8, 9). Immunoprecipitation of HA-A_{2A}R-HA was performed with anti-HA antibodies (Babco) covalently coupled to protein G-Sepharose. Immunoprecipitation and immunoblotting were done following standard protocols (6). Immunoblots were developed with ECL Western blot detection system.

cAMP Accumulation Experiments—Neuroblastoma or striatal cells were incubated with [³H]adenine (24 Ci/mmol; Amersham Biosciences; 1 μ Ci/well) for 2 h at 37 °C in serum-free medium. They were subsequently washed with PBS and incubated for 10 min at 37 °C in PBS containing 100 μ M phosphodiesterase inhibitor RO-20-1724 (Calbiochem). Various concentrations of forskolin, CGS 21680, and/or quinpirole were then added and the cells placed at 37 °C for an additional 10 min. The incubation solution was then discarded and replaced by ice-cold 0.3 M perchloric acid containing [¹⁴C]cAMP (51.3 mCi/mmol; PerkinElmer Life Sciences) as an internal standard. After sonication and centrifugation ($8,000 \times g$ for 5 min), [³H]cAMP present in the supernatant was identified by sequential chromatography (17). Formation of cAMP was expressed as the percentage of conversion of total [³H]ATP into [³H]cAMP. For statistical analysis, one-way ANOVA (followed by *post hoc* Scheffé's multiple comparison test) and Mann-Whitney's *U* test were used.

RESULTS

Colocalization of A_{2A}R and D₂R in Nontreated SH-SY5Y Neuroblastoma Cells: Immunolabeling Experiments—A_{2A}R/D₂R interactions were first studied in a previously characterized human neuroblastoma cell line (SH-SY5Y) stably transfected with human D₂R (long form) (about 1100 fmol/mg of protein) (7). This cell line constitutively expresses functional A_{2A}R (about 200 fmol/mg of protein), the activation of which decreases the affinity of the transfected D₂R for dopamine and counteracts D₂R-mediated intracellular [Ca²⁺]_i response (7). Double immunofluorescence experiments including image

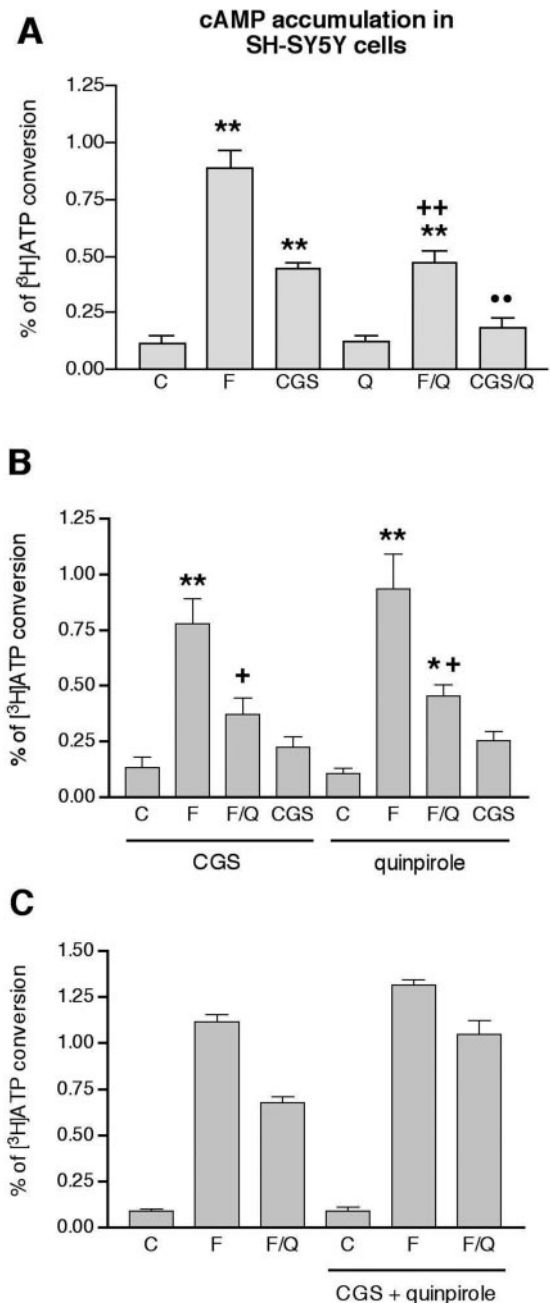


FIG. 4. **cAMP accumulation experiments in SH-SY5Y neuroblastoma cells stably transfected with D₂R (long form).** Results represent means \pm S.E. and are expressed as percentage of conversion of total [³H]ATP to [³H]cAMP ($n = 6-8$). **A**, effects of 10 μ M forskolin (F), 1 μ M CGS 21680 (CGS), and 1 μ M quinpirole (Q), alone or in combination, without agonist preincubation. Quinpirole significantly decreased forskolin- and CGS-induced cAMP accumulation. **, $p < 0.01$ compared with control (C); +, $p < 0.05$ compared with forskolin; ++, $p < 0.01$ compared with CGS; one-way ANOVA and *post hoc* Scheffé's multiple comparison test. **B**, effects of 10 μ M forskolin (F), 1 μ M CGS 21680 (CGS), and 1 μ M quinpirole (Q), alone or in combination, after preincubation for 3 h with either 200 nM CGS 21680 or 50 μ M quinpirole. Preincubating the cells with either agonist counteracted CGS 21680-induced cAMP accumulation. ** and *, $p < 0.01$ and $p < 0.05$, respectively, compared with control (C); +, $p < 0.05$ compared with forskolin; one-way ANOVA and *post hoc* Scheffé's multiple comparison test. **C**, effects of 10 μ M forskolin in the absence (F) and presence (F/Q) of 1 μ M quinpirole with and without preincubation for 3 h with 200 nM CGS 21680 and 50 μ M quinpirole. The counteractive effect of quinpirole on forskolin-induced cAMP accumulation was significantly lower after simultaneous preincubation with A_{2A}R and D₂R agonists (17.1%/11.4%, in median/interquartile range; $n = 7$) than without preincubation (40.7%/5.8%, in median/interquartile range; $n = 7$) ($p < 0.01$; Mann-Whitney *U* test).

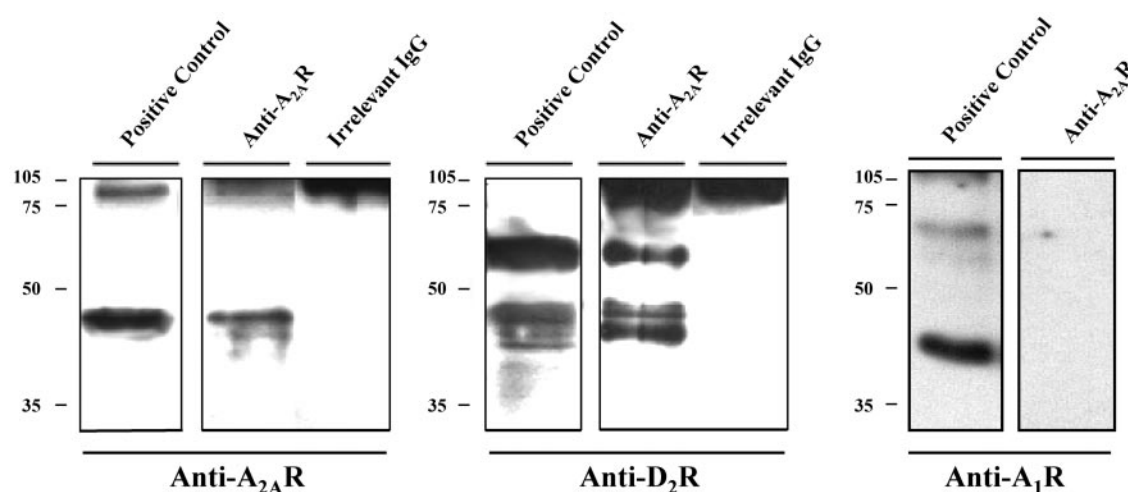


FIG. 5. Coimmunoprecipitation of $A_{2A}R$ and D_2R from membrane preparations of SH-SY5Y neuroblastoma cells stably transfected with D_2R (long form). Immunoprecipitation with anti- $A_{2A}R$ antibodies was followed by Western blotting with antibodies against $A_{2A}R$ (left), against D_2R (middle), or against A_1R (right). For positive control an aliquot of cell membrane extracts was used.

analysis were performed in nonpermeabilized cells, using fluorescein-conjugated anti- $A_{2A}R$ antibodies (green color) and rhodamine-conjugated anti- D_2R antibodies (red color). As seen in Fig. 1A (panels a and b), there is a clear spatial predominance of D_2R versus $A_{2A}R$ immunoreactivity, which is in agreement with the higher D_2R density found in previous binding experiments (7). Confocal analysis revealed substantial colocalization of both receptors at the membrane level (yellow color in Fig. 1A, panel c), which indicates that in the absence of exogenous agonists the lateral distance between the two receptor proteins is less than 0.1 μm . A semi-quantitative analysis of the distribution of the yellow was done by evaluating the intensity of the color (which directly reflects the amount of coexisting receptors) and calculating the Gini's index or GI (a measure of unevenness, which evaluates the proportion of coaggregates in the sampled area). These calculations were completed on the whole cell body membrane, as well as on the areas of the cell body membrane containing coaggregates and on the parts with few or no coaggregates (Fig. 1B). In nontreated cells (Fig. 1A, panel c), high amounts of coexisting receptors were found both in the cell body membrane with coaggregates and in the cell body membrane displaying a more diffuse distribution of $A_{2A}R/D_2R$ IR. The GI value of these cells was 0.10, indicating a rather even distribution of the colocalized $A_{2A}R/D_2R$ IR and few coaggregates of the two receptors (Fig. 1B).

Coaggregation of $A_{2A}R$ and D_2R in CGS 21680- or Quinpirole-treated SH-SY5Y Cells: Immunolabeling Experiments—Substantial modifications of $A_{2A}R$ and D_2R distribution were obtained when the cells were treated with the $A_{2A}R$ agonist CGS 21680 or the D_2R agonist quinpirole. Hence, 3 h of treatment with either CGS 21680 (100 nM, Fig. 1A, panel d) or quinpirole (10 μM , Fig. 1A, panel e) induced an aggregation of both $A_{2A}R$ and D_2R . Similar effects were also observed with shorter incubation times at higher agonist concentrations (data not shown). Thus, 3 h of incubation at these concentrations give a maximal effect. The computer-assisted analysis of the confocal images showed an increase in unevenness of the yellow distribution (GI for the entire cell body membrane was 0.23 for CGS 21680- and 0.20 for quinpirole-treated cells). Furthermore, the yellow intensity value was still high for the cell body membrane with $A_{2A}R/D_2R$ coaggregates but very low for the cell body membrane with diffusely distributed $A_{2A}R/D_2R$ IR (Fig. 1B). Altogether, this analysis indicates the presence on the cell body membrane of an increased proportion of $A_{2A}R/D_2R$ coaggregates.

Cointernalization of $A_{2A}R$ and D_2R in CGS 21680- or Quinpirole-treated SH-SY5Y Cells: Immunolabeling Experiments—Cotreatment with CGS 21680 (100 nM) and quinpirole (10 μM) markedly decreased the yellow intensity values of the cell body membrane associated with coaggregates and diffusely distributed $A_{2A}R/D_2R$ IR as compared with untreated cells (Fig. 1, A (panel f) and B). This indicates that cotreatment with CGS 21680 (100 nM) and quinpirole (10 μM) induces coaggregation followed by cointernalization of $A_{2A}R$ and D_2R . A stronger reduction in D_2R IR (red) was, however, obtained with higher concentrations of quinpirole (50 μM) and CGS 21680 (200 nM) (Fig. 1A, panel g). At these concentrations of the agonists, the Gini's index was high (0.30) reflecting an uneven distribution of $A_{2A}R/D_2R$ IR. In addition, the values for the yellow intensity in the cell body membrane with $A_{2A}R/D_2R$ coaggregates and the cell body membrane with diffusely distributed $A_{2A}R/D_2R$ IR were, respectively, low and very low (Fig. 1B). This suggests that a cotreatment of the cells with high doses of the agonists potentiates both coaggregation and cointernalization of $A_{2A}R$ and D_2R . Ligand-induced cointernalization was confirmed in experiments where cells were first labeled with anti- $A_{2A}R$ and anti- D_2R antibodies for 2 h at 4 $^{\circ}C$ in the absence (Fig. 2, panel a) or in the presence of CGS 21680 (200 nM) and/or quinpirole (50 μM) (Fig. 2, panels b–d). Subsequently, the ligand-induced internalization of labeled receptors was allowed to occur by incubating the cells for 3 h at 37 $^{\circ}C$. Confocal analysis revealed colocalized $A_{2A}R/D_2R$ aggregates inside the cell after pretreatment with either CGS 21680 (200 nM) or quinpirole (50 μM) (Fig. 2, panels b and c), which became more pronounced after pretreatment with both compounds (Fig. 2, panel d).

Absence of Quinpirole-induced Aggregation of $A_{2A}R$ in Parental SH-SY5Y Cells: Immunolabeling Experiments—The involvement of D_2R in quinpirole-mediated effects was assessed using parental neuroblastoma SH-SY5Y cells, which express very low levels of D_2R (7). Hence, quinpirole at 100 μM for 3 h did not induce any modification of $A_{2A}R$ immunolabeling pattern (Fig. 3).

Codesensitization of $A_{2A}R$ and D_2R in CGS 21680- and Quinpirole-treated SH-SY5Y Cells: cAMP Accumulation Experiments—Under basal conditions 1 μM CGS 21680 significantly increased cAMP accumulation in SH-SY5Y neuroblastoma cells (Fig. 4A). Quinpirole (1 μM) did not modify cAMP levels but significantly counteracted both forskolin (10 μM)-induced and CGS 21680-induced increases in cAMP accumulation (Fig. 4A). These results confirm the existence of functional and in-

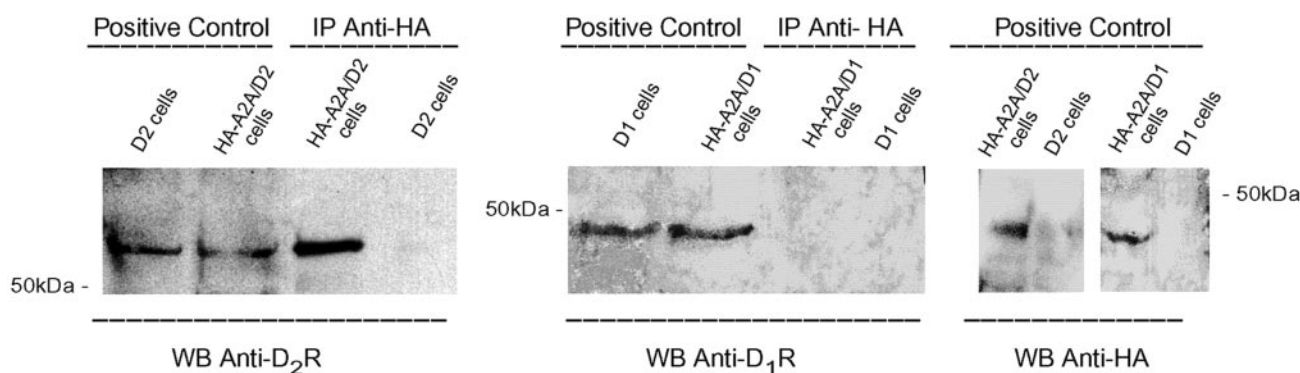


FIG. 6. **Coimmunoprecipitation of HA-A_{2A}R-HA and D₂R from membrane preparations of mouse Ltk⁻ fibroblasts.** Ltk⁻ fibroblasts stably transfected with either human dopamine D₂R (long form) or human dopamine D₁R were transiently transfected with either double-tagged HA-A_{2A}R-HA (HA-A_{2A}R-HA/D₂R and HA-A_{2A}R-HA/D₁R cells) or an irrelevant plasmid (D₂R and D₁R cells). An aliquot of cell membrane extracts was used as a positive control. WB, Western blot; IP, immunoprecipitation.

teracting A_{2A}R and D₂R in D₂R-transfected neuroblastoma cells (7). After 3 h of incubation with either 200 nM CGS 21680 or 50 μ M quinpirole (and subsequent withdrawal of the agonists, see “Experimental Procedures”), CGS 21680 was no longer able to significantly increase cAMP levels (Fig. 4B). This demonstrates the existence of both homologous and D₂R-mediated heterologous desensitization of A_{2A}R. Instead, the inhibitory effect of quinpirole on forskolin-induced elevation of cAMP levels was about the same (40–50% inhibition) under basal conditions and after a 3-h incubation with the A_{2A}R or the D₂R agonist (Fig. 4, A and B). Thus, as already described, A_{2A}R clearly desensitized after A_{2A}R agonist incubation (18, 19) and D₂R (long form) showed resistance to D₂R agonist-induced desensitization (20). However, after 3 h of incubation with both quinpirole (50 μ M) and CGS 21680 (200 nM), quinpirole (1 μ M) inhibited forskolin-induced cAMP accumulation by only 17.1%/11.4% (median/interquartile range) (Fig. 4C). This effect, when compared with the effect of quinpirole on untreated cells, was significantly different (Mann-Whitney *U* test; *p* < 0.01). Hence, quinpirole was found to inhibit forskolin-induced cAMP accumulation by 40.7%/5.8% (median/interquartile range) (Fig. 4C). Therefore, costimulation of A_{2A}R and D₂R accelerates D₂R desensitization, likely by causing increased D₂R internalization (see above).

Coimmunoprecipitation of A_{2A}R and D₂R in Membrane Preparations from SH-SY5Y Neuroblastoma Cells and Ltk⁻ Fibroblast Cells—The possible existence of A_{2A}R/D₂R heteromeric complexes was then analyzed by coimmunoprecipitation performed on membrane preparation of D₂R-transfected SH-SY5Y neuroblastoma cells. Immunoprecipitation with anti-A_{2A}R antibodies followed by Western blotting with anti-D₂R antibodies revealed three bands of 43, 47, and 63 kDa (Fig. 5), corresponding to different glycosylated states of the D₂R (21). The same three bands were also obtained in control lysate preparations (Fig. 5). On the other hand, immunoprecipitation with anti-A_{2A}R antibodies followed by Western blotting with anti-A₁R did not reveal any band corresponding to A₁R, indicating the absence of A_{2A}R-A₁R co-immunoprecipitation (Fig. 5). A Western blotting performed with control lysate preparations showed a band of ~40 kDa, which corresponds to the A₁R (Fig. 5). A_{2A}R/D₂R interactions were also examined in mouse fibroblast Ltk⁻ cells stably transfected with the human D₂R (long form) or human D₁R (8, 9). These two cell lines express a similar density of transfected dopamine receptors (2.8 pmol/mg protein of D₂R in the D₂R-Ltk⁻ cell line and 4.2 pmol/mg protein of the D₁R in the D₁R-Ltk⁻ cell line (Refs. 8 and 9)). Both cell lines were transiently transfected with either dog A_{2A}R cDNA double-tagged with hemagglutinin (HA-A_{2A}R-HA) or an irrelevant plasmid. Antibodies against the hemagglutinin tag (anti-HA)

were able to precipitate a band of ~65 kDa detected in Western blot with the anti-D₂R antibodies but only from cells that express both D₂R and HA-A_{2A}R-HA (Fig. 6). This band corresponds to the highly glycosylated state of D₂R (21). As a positive control, we showed that this band could also be obtained from lysates of D₂R-transfected Ltk⁻ cells, thus being independent of the presence of HA-A_{2A}R-HA. Antibodies against anti-HA failed to coimmunoprecipitate D₁R in cells expressing D₁R/HA-A_{2A}R antibodies, assessing for the specificity of the A_{2A}R/D₂R coimmunoprecipitation (Fig. 6). These results demonstrate for the first time that A_{2A}R and D₂R assemble into heteromeric complexes in two different cell lines that coexpress both receptors and that these complexes exist in the absence of exogenous agonists.

Colocalization and Coaggregation of A_{2A}R and D₂R in Cultured Striatal Neurons: Immunolabeling Experiments—A_{2A}R/D₂R interactions were also studied in neuronal primary cultures of rat striatum. Cells were grown for 2 weeks and immunostained for A_{2A}R and D₂R (see above). Immunolabeling of A₁R on nonpermeabilized cells was also performed. Most neurons exhibited A_{2A}R and D₂R IR in the soma and in the dendrites, however, with predominance of D₂R to A_{2A}R IR in the dendrites (Fig. 7A). As for neuroblastoma cells, confocal analysis revealed a high degree of A_{2A}R/D₂R colocalization in the absence of exposure to exogenous agonists (Fig. 7A). Treatment (6 h) with either the A_{2A}R agonist CGS 21680 (100 nM) or the D₂R agonist quinpirole (10 μ M) induced aggregation of both A_{2A}R and D₂R (Fig. 7A) and reduction in IR intensity. However, no synergism was observed when cells were cotreated with CGS 21680 (100 nM) and quinpirole (10 μ M) for the same time (Fig. 7A). To assess the specificity of A_{2A}R/D₂R interactions, cells were incubated with 10 μ M quinpirole for 6 h and immunostained with anti-A₁R antibodies. No change in the pattern of distribution of A₁R present in the soma and dendrites was observed (Fig. 7B). In addition and instead of A_{2A}R/D₂R, no aggregates were seen, which confirms the specificity of A_{2A}R/D₂R interactions. Cointernalization experiments analogous to those described in Fig. 2 could not be performed in cultured striatal cells, which did not tolerate the temperature conditions of the experimental internalization protocol (cells changed morphology and fell off the coverslip).

Interaction between A_{2A}R and D₂R in Cultured Striatal Neurons: cAMP Accumulation Experiments—Both basal and forskolin-induced cAMP accumulation were about 10 times higher than in neuroblastoma cells (Fig. 8A). This could explain why, unlike what was observed in neuroblastoma cells, CGS 21680 (1 μ M) did not induce any significant increase in cAMP accumulation compared with basal values (Fig. 8A). As for neuroblastoma cells, quinpirole (1 μ M) did not modify cAMP levels

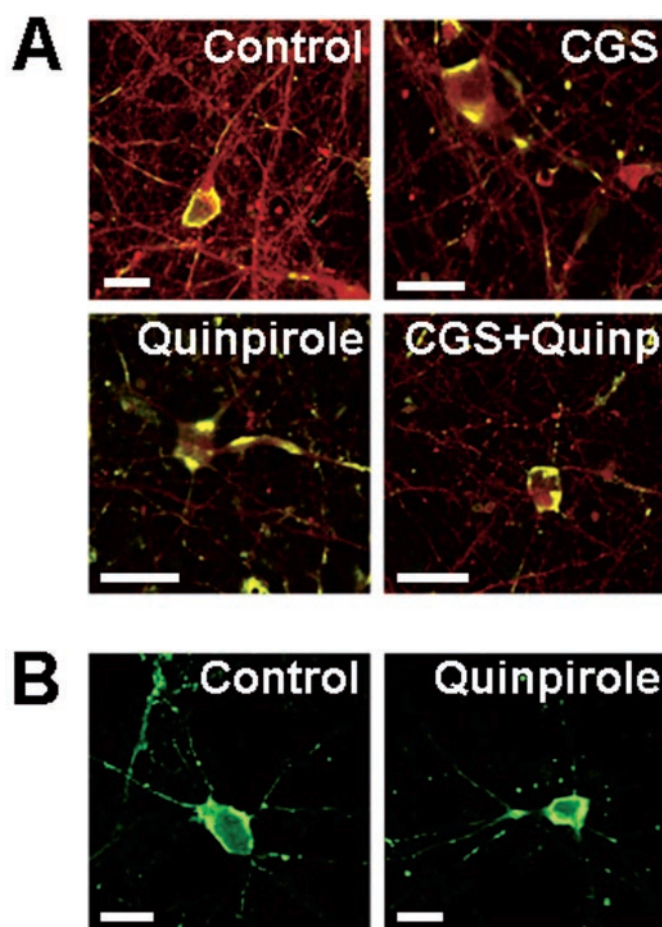


FIG. 7. **Double immunofluorescence staining and confocal images of striatal neurons in culture.** Panel A, cells were exposed for 6 h to either 100 nM CGS-21680, 10 μ M quinpirole, or both and were processed for immunostaining using fluorescein (green)-conjugated rabbit anti- A_{2A} R antibodies and rhodamine (red)-conjugated rabbit anti- D_2 R antibodies. The cells were analyzed by confocal microscopy. Superimposition of images reveals the colocalization of A_{2A} R and D_2 R in yellow. Panel B, staining of adenosine A_1 R with fluorescein (green)-conjugated anti- A_1 R antibodies. Note the lack of effect of 10 μ M quinpirole (6 h). Representative images from four to five independent experiments/treatment are shown; scale bar, 10 μ m.

but significantly reduced forskolin (10 μ M)-induced cAMP accumulation (Fig. 8B). Finally, CGS 21680 (1 μ M) counteracted the effect of quinpirole on forskolin-induced cAMP accumulation (Fig. 8B). These results provide a functional demonstration of the antagonistic A_{2A} R/ D_2 R interactions in striatal neurons in culture.

DISCUSSION

The main findings of the present work are first that A_{2A} R and D_2 R are colocalized and form heteromers in untreated neuronal cells and that they coaggregate upon long term exposure to either agonist. The formation of A_{2A} R/ D_2 R heteromers and aggregates is receptor subtype-specific because co-expression of A_{2A} R/ D_1 R (present study) or A_1 R/ D_2 R (6) does not lead to formation of heteromers. Furthermore, A_{2A} R did not form heteromeric complexes with A_1 R (present study). This phenomenon may therefore constitute the molecular basis for the selective A_{2A} R/ D_2 R interactions observed *in vitro*, like A_{2A} R modulation of D_2 binding characteristics (7, 8, 22, 23), counteraction of D_2 R-mediated intracellular $[Ca^{2+}]_i$ responses (7), and inhibition of cAMP accumulation (see Ref. 23 and present paper). The same type of antagonism was observed *in vivo* with the A_{2A} R inhibition of D_2 R-mediated regulation of GABA release in the globus pallidus and of D_2 R-mediated

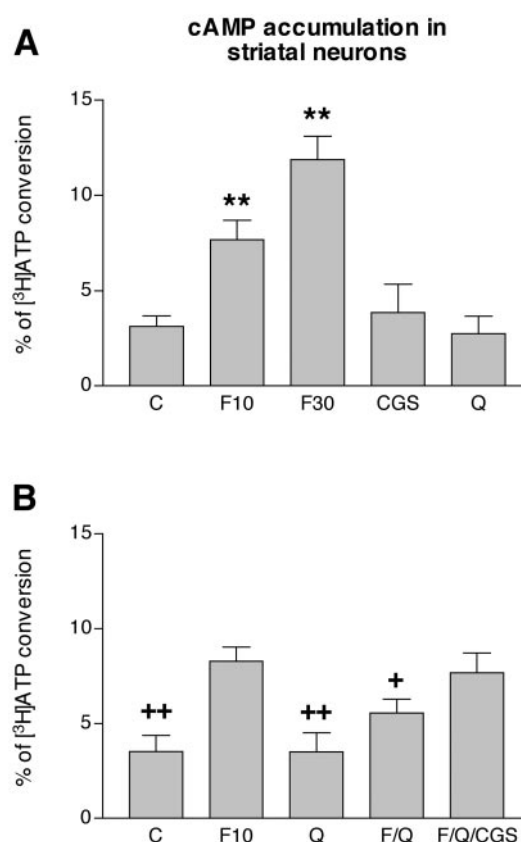


FIG. 8. **cAMP accumulation experiments in striatal neurons in culture.** Results represent means \pm S.E. and are expressed as percentage of conversion of total $[^3H]$ ATP to $[^3H]$ cAMP ($n = 10-14$). A, effects of 10 and 30 μ M forskolin (F10 and F30, respectively), 1 μ M CGS 21680 (CGS), and 1 μ M quinpirole (Q). Forskolin, but not CGS 21680, significantly increased cAMP accumulation. **, $p < 0.01$ compared with control (C); one-way ANOVA. B, effects of 10 μ M forskolin (F), 1 μ M CGS 21680 (CGS), and 1 μ M quinpirole (Q), alone or in combination. CGS 21680 counteracted the inhibitory effect of quinpirole on forskolin-induced cAMP accumulation. + and ++, $p < 0.05$ and $p < 0.01$, respectively, compared with forskolin; one-way ANOVA and *post hoc* Scheffé's multiple comparison test.

increases in motor activity (1, 5). Besides acute antagonistic actions, the A_{2A} R/ D_2 R heteromers may be involved in receptor trafficking, because long term exposure to either A_{2A} R or D_2 R agonists induces aggregation and cotreatment with these agonists induces internalization of both receptors. Moreover, prior exposure to A_{2A} R or D_2 R agonists can produce a desensitization of the A_{2A} R in terms of cAMP accumulation associated with coaggregation. In contrast, co-exposure to A_{2A} R and D_2 R agonists, but not to any of the two agonists alone, causes desensitization of D_2 R-mediated inhibition of cAMP accumulation, which is associated with cointernalization. The present observation that A_{2A} R and D_2 R functions are simultaneously altered after long exposure to agonists can aid in understanding behavioral findings involving cross-tolerance and cross-sensitization between dopamine agonists and compounds active at adenosine receptors (such as caffeine) (24, 25). Together with other recently reported findings, the present results suggest that changes in A_{2A} R function may be involved in the secondary effects observed after chronic intermittent treatment with L-DOPA such as reduced antiparkinsonian activity and dyskinesia (26). Adenosine is a feedback detector of neuronal activation, in view of the fact that it allows the neuronal network to return into a resting state (27). Adenosine is therefore expected to increase in the striatal extracellular fluid from patients with Parkinson's disease mainly after chronic intermittent L-DOPA

treatment and in agreement with the evidence of increased striatal glutamate drive (28). Hence, striatal extracellular levels of adenosine have been found to increase in the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine model of Parkinson's disease (29). Thus, the wearing off of the antiparkinsonian action of L-DOPA treatment may in part be caused by the simultaneous chronic activation of A_{2A}R and D₂R that, according to the present results, may lead to substantial cointernalization of both receptors. The coadministration of A_{2A}R antagonists together with L-DOPA or dopamine D₂R agonists may therefore provide a new therapeutic approach lacking the secondary effects of chronic L-DOPA treatment (30, 31). Overall, the present and previous (1, 5) data implicate that the membrane interactions taking place between A_{2A}R and D₂R via heteromeric complexes represent a crucial mechanism influencing D₂R-mediated transmission. This prompts development of adenosine and dopamine antagonists/agonists compounds preferentially active on A_{2A}R/D₂R heteromers as new drugs for the treatment of neuropsychiatric diseases, such as Parkinson's disease (30, 31), schizophrenia (32, 33), Huntington's disease (34), and dystonia (35), in which D₂R have been implicated.

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**MECHANISMS OF SIGNAL
TRANSDUCTION:
Coaggregation, Cointernalization, and
Codesensitization of Adenosine A_{2A}
Receptors and Dopamine D₂ Receptors**

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