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Adenosine A_{2A} receptors modulate the binding characteristics of dopamine D_2 receptors in stably cotransfected fibroblast cells

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Abstract

In membrane preparations from rat striatum, where adenosine A_{2A} and dopamine D_2 receptors are coexpressed, stimulation of adenosine A_{2A} receptors was found to decrease the affinity of dopamine D_2 receptors for dopamine agonists. We now demonstrate the existence of this antagonistic interaction in a fibroblast cell line (Ltk^-) stably transfected with the human dopamine D_2 (long-form) receptor and the dog adenosine A_{2A} receptor cDNAs (A_{2A}-D_2 cells). In A_{2A}-D_2 cells, but not in control cells only containing dopamine D_2 receptors (D_2 cells), the selective adenosine A_{2A} agonist 2-[*p*-(2-carboxyethyl)-phenethylamino]-5′-*N*-ethyl-carboxamido adenosine (CGS 21680) induced a 2–3-fold decrease in the affinity of dopamine D_2 receptors for dopamine, as shown in competition experiments with dopamine versus the selective dopamine D_2 antagonist [^{3}H]raclopride. By contrast, activation of the constitutively expressed adenosine A_{2B} receptors with 5′-*N*-ethyl-carboxamidoadenosine (NECA) did not modify dopamine D_2 receptor binding. In A_{2A}-D_2 cells CGS 21680 failed to induce or induced only a small increase in adenosine-3′,5′-cyclic-monophosphate (cAMP) accumulation. In D_2 cells NECA- or forskolin-induced adenylyl cyclase activation was not associated with any change in dopamine D_2 receptor binding. These results indicate that adenylyl cyclase activation is not involved in the adenosine A_{2A} receptor-mediated modulation of the binding characteristics of the dopamine D_2 (long-form) receptor.

Keywords: Adenosine; Dopamine; Adenylyl cyclase; Fibroblast; Receptor-receptor interaction

1. Introduction

Adenosine is an important neuromodulator that exerts its effects via known and cloned G-protein-coupled receptors: adenosine A_1, A_{2A}, A_{2B} and A_3 receptors (Fredholm et al., 1994). Adenosine A_{2A} receptors are highly enriched in the striatum (Jarvis et al., 1989), where they are colocalized with dopamine D_2 receptors in the striopallidal γ-aminobutyric acid (GABA)-containing neurons (Schiffmann et al., 1991; Fink et al., 1992). There is behavioural, functional and biochemical evidence for a specific antagonistic interaction between adenosine A_{2A} and dopamine D_2 receptors in the brain (for review, see Ferré et al., 1992). It was proposed that this antagonistic A_{2A}/D_2 receptor interaction could be a major mechanism of action responsible for the neuroleptic-like activity of adenosine agonists and the motor stimulant action of adenosine antagonists, like the methylxanthines caffeine and theophylline (Ferré et al., 1992). It was also proposed that the A_{2A}/D_2 receptor interaction could provide a new therapeutic approach for basal ganglia disorders, like Parkinson's disease, and for schizophrenia (Ferré et al., 1992, 1993, 1994).

Activation of adenosine A_{2A} receptors can decrease the affinity of dopamine D_2 receptors for agonists in membranes from the rat neostriatum (Ferré et al., 1991). The A_{2A} agonist 2-[*p*-(2-carboxyethyl)-phenethylamino]-5′-*N*-ethyl-carboxamido adenosine (CGS 21680) increased the equilibrium dissociation constant for low- (K_L) and high-affinity (K_H) dopamine D_2 receptor binding sites and increased the proportion of dopamine D_2 receptors in the

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high-affinity state (R_H). Such an A_{2A}/D_2 antagonistic interaction was not observed in COS-7 cells (a derivative of the CV-1 monkey cell line carrying a stably integrated portion of the SV40 virus genome) transiently transfected with dopamine D_2 receptor and adenosine A_{2A} receptor cDNAs (Snaprud et al., 1994), despite high densities of adenosine A_{2A} and dopamine D_2 receptors with normal binding properties. The failure in demonstrating the A_{2A}/D_2 interaction in COS-7 cells could have been due to the fact that a transient transfection was used. Therefore, in the present experiments we have used human dopamine D_2 receptor (long-form)-containing fibroblast cells (Ltk^- cells) (Grandy et al., 1989) after stable transfection with the dog adenosine A_{2A} receptor cDNA (Maenhaut et al., 1990). An antagonistic and selective A_{2A}/D_2 receptor interaction independent of adenylyl cyclase activation was now found in the transfected cell line.

2. Materials and methods

2.1. Transfection of fibroblast Ltk^- cells

Mouse fibroblast Ltk^- cells with the long form of the human dopamine D_2 receptor cDNA (Grandy et al., 1989) were used. Cultures were grown routinely at 37°C with 5% CO_2 in Dulbecco's minimal essential medium with 4.5 mg/ml glucose, 0.11 mg/ml sodium pyruvate, supplemented with 10% fetal calf serum, glutamine (2 mM), penicillin (50 U/ml), streptomycin (50 U/ml), G418 (geneticin, 200 μg/ml) and HEPES (10 mM) in plastic Petri dishes (Falcon). G418 was used as the selectable marker for dopamine D_2 receptors. All cell culture reagents were tissue culture grade (Gibco). The dog adenosine A_{2A} receptor cDNA RDC8 (a 2419-bp DNA fragment containing the full coding sequence of the dog adenosine A_{2A} receptor) (Maenhaut et al., 1990) cloned in BlueScript SK + (gift from G. Vassart) was subcloned into the *Eco*RI site of pZEM 228R (gift from M.G. Caron). The pZEM 228R containing the RDC8 was linearized with *Eco*RV and transfected with the calcium phosphate transfection method. Since the Ltk^- cells already were neomycin resistant, a hygromycin containing plasmid (pHyg; gift from G. Vassart) confering resistance to hygromycin B (Sigma) was used as a selectable marker. Linearized RDC8-containing pZEM 228R along with a 10-fold lower concentration of the pHyg were cotransfected. Both plasmids were incubated for 20 min at room temperature with 2 M $CaCl_2$ pH 7.01 and 2 × DNA precipitating buffer, consisting of 50 mM HEPES pH 7.05, 1.5 mM Na_2HPO_4, 10 mM KCl, 280 mM NaCl and 12 mM glucose. This mixture was added to the Ltk^- cells grown up to 70% confluence, and incubated for 7 h at 37°C with 5% CO_2. The cells were then subjected to a glycerol shock for 3 min, and then washed twice with complete medium. Selection with hygromycin B (0.3 mg/ml) was started after 48 h. A number of clones were selected on the basis of their ability to grow in the presence of hygromycin.

2.2. Analysis of RNA

Isolation of RNA from the Ltk^- cells was carried out according to the method of Chomczynski and Sacchi (1987). For preparation of the Northern blots, 15 μg of total RNA per lane was denatured in a 2.1 M formaldehyde and 50% formamide solution by heating for 2 min at 95°C, separated by electrophoresis on a 1.0% agarose/2.2 M formaldehyde gel and transferred to a nitrocellulose membrane. Blots were hybridized with ^{32}P-labelled adenosine A_{2A} receptor cDNA by the nick translation method. Following hybridization the membrane was washed and exposed to Kodak XAR-5 film with intensifying screen at −70°C. A reverse transcriptase-polymerase chain reaction analysis (RT-PCR) of adenosine A_{2A} and A_{2B} receptor mRNA was also performed. First strand cDNA was synthesized by reverse transcription of 1 μg of cytosolic RNA with 10 U/μl Moloney murine leukemia virus reverse transcriptase (Gibco) in the presence of 25 μg/ml $pd(N)_6$ random hexamers (Pharmacia-LKB), 16 mM dNTP (Pharmacia-LKB) and 1 U/μl RNasin ribonuclease inhibitor (Promega) in a buffer containing 5 mM $MgCl_2$, 50 mM KCl, 10 mM Tris-HCl pH 9.0 and Triton X-100. The samples were preincubated at 23°C for 10 min and the reaction was continued for 40 min at 42°C. The reaction was stopped by heating for 5 min at 99°C. Oligonucleotide primers for human (Furlong et al., 1992) and rat (Fink et al., 1992) adenosine A_{2A} receptors containing an *Eco*RI linker, corresponding to amino acids NLQNVT (5′-CGAATTCAACCTGCAGAACGTCACC-3′, sense) and to amino acids IAIDR (5′-TCGAATTCGCGGTC(G/A)ATGGCGAT(A/G)-3′, antisense), and degenerate oligonucleotide primers for human (Pierce et al., 1992) and rat (Stehle et al., 1992) adenosine A_{2B} receptors, corresponding to amino acids QTPTNYF (5′-CAGAC(G/C)CCCACCAACTACTT-3′, sense) and to amino acids AVLFIKI (5′-GCCACCA-(G/T)GAAGAT(C/T)TT(A/G)ATG-3′, antisense) were synthesized (Scandinavian Gene Synthesis). The sequence between the adenosine A_{2A} receptor primers corresponds to a sequence between the first and the beginning of the second cytoplasmatic loop of the receptor. The sequence between the adenosine A_{2B} receptor primers corresponds to a sequence between the first and the beginning of the third cytoplasmatic loop. Second strand synthesis of cDNA and subsequent thermocycling with 0.025 U/μl of *Taq* polymerase (Promega) were performed at a primer concentration of 200 nM, in a buffer containing 10 mM Tris-HCl pH 9.0, 50 mM KCl, 0.1% Triton X-100, 4 mM $MgCl_2$ for adenosine A_{2A} primers and 5 mM $MgCl_2$ for adenosine A_{2B} primers in a volume of 50 μl in a MiniCycler (MJ Research). Products were analyzed by electrophoresis in 1.5% (w/v) ultrapure agarose (BRL).

2.3. Radioligand binding experiments

The Ltk$^-$ cells were lifted from Petri dishes with a cell scraper. Harvested cells were washed twice with ice-cold PBS and once with ice-cold Tris-HCl buffer (50 mM, pH 7.6) containing 0.01% L-(+)-ascorbic acid and 1 mM EDTA, 5 mM $MgCl_2$. The same Tris buffer was used to sonicate the cells (30 s) and the homogenate was centrifuged at 3000 rpm for 10 min at 4°C. The precipitated nucleic fraction was discarded and the supernatant centrifuged at 19000 rpm for 40 min at 4°C. The membrane pellet was then resuspended in the same buffer by sonication. Saturation experiments with the dopamine D_2 receptor antagonist [^{3}H]raclopride were performed at 10 concentrations (0.5–15 nM) of [^{3}H]raclopride (3.0 TBq/mmol; NEN) by incubation for 30 min at room temperature. Non-specific binding was defined as the binding in the presence of dopamine (1 mM). Saturation experiments with the adenosine A_{2A} receptor agonist [^{3}H]CGS 21680 were performed at 10 concentrations (5.5–160 nM) of [^{3}H]CGS 21680 (1.5 TBq/mmol; NEN) by incubation for 90 min at room temperature. Membranes were previously preincubated for 30 min at 37°C with adenosine deaminase (Boehringer-Mannheim; 10 U/ml) to remove endogenous adenosine. Non-specific binding was defined as the binding in the presence of 100 μM 2-chloradenosine. Competition experiments with dopamine versus [^{3}H]raclopride were performed by incubation with 20 concentrations (10 pM–1 mM) of dopamine and 2 nM [^{3}H]raclopride for 30 min at room temperature in the presence or absence of the adenosine A_{2A} receptor agonist CGS 21680 at concentrations of 10, 30, 100 and 300 nM. The effect of CGS 21680 (100 nM), the non-selective adenosine agonist 5′-*N*-ethylcarboxamidoadenosine (NECA) (1 μM) and forskolin (30 μM) were also tested in cells only containing dopamine D_2 receptors (D_2 cells). Competition experiments with and without CGS 21680 were also performed in the presence of the adenosine receptor antagonist 8-phenyl-theophylline (8-PT). The incubation was stopped by automatically washing the membranes (Brandel) three times with 5 ml ice-cold Tris buffer over Whatman GF/B filters (Millipore). The radioactivity content of the filters was detected by liquid scintillation spectrometry. To avoid the variability of the binding parameters associated with the assay conditions, such as cell confluency (around 80%) and number of passages (up to 15), the same membrane preparation was used to study the effect of different drugs on the binding characteristics of dopamine D_2 receptors and each experiment was independently analyzed. Data from saturation experiments were analyzed by non-linear regression analysis for the determination of dissociation constants (K_d) and the number of receptors (B_{max}). Data from competition experiments were analyzed for the determination of K_H, K_L and R_H values. The amount of nonspecific binding was calculated by extrapolation of the displacement curve. Points deviating more than two residuals from the fitted curve were discarded. Curves with more than three discarded points were not included for statistical analysis. To achieve homogeneity of variance and allow parametric statistical analysis, K_H and K_L values were logarithmically transformed and analyzed by Student's paired *t*-test or by repeated measures analysis of variance (ANOVA) followed by Fisher's protected least-square difference test (PLSD).

2.4. cAMP accumulation experiments

After scraping the cells off the culture plates they were washed twice with PBS (phosphate-buffered saline, Gibco) and resuspended in serum-free media at a concentration of $0.5–1.2 \times 10^6$ cells/ml. Aliquots of 0.2 ml were transferred to test tubes along with the phosphodiesterase inhibitor rolipram (30 μM) added to a final volume of 0.3 ml. Different concentrations of CGS 21680 and NECA in the presence and absence of the adenosine receptor antagonist theophylline (30 μM) were studied. The effect of forskolin (30 μM) alone and the effect of different doses of the dopamine D_2 receptor agonist quinpirole (1, 10 and 100 nM) on forskolin-induced adenosine-3′,5′-cyclic-monophosphate (cAMP) accumulation were also analyzed. The reaction was terminated with 50 μl perchloric acid to a final concentration of 0.1 M after 10 min incubation at 37°C. Samples were neutralized with 4 M KOH/1 M Tris and the cAMP content in the supernatants determined with a protein binding assay (Nordstedt and Fredholm, 1990). One-way ANOVA was used for statistical analysis followed by PLSD test.

3. Results

3.1. Analysis of adenosine A_{2A} receptor mRNA expression

Expression of the adenosine A_{2A} receptor mRNA was verified by Northern blot analysis. Three of the Ltk$^-$ cell lines expressing moderate levels of adenosine A_{2A} receptor message (A_{2A}-D_2 cells) were isolated and named clones 7, 8 and 9. Ltk$^-$ cells expressing only the dopamine D_2 receptor (D_2 cells) showed no adenosine A_{2A} receptor message. In addition RT-PCR analysis was used to determine which adenosine A_2 receptor mRNA was expressed. As seen in Fig. 1, both A_{2A}-D_2 and D_2 cells showed a band corresponding to adenosine A_{2B} receptor mRNA. In addition, A_{2A}-D_2 cells showed a band corresponding to adenosine A_{2A} receptor mRNA, which was not seen in D_2 cells.

3.2. Saturation experiments with [^{3}H]raclopride and [^{3}H]CGS 21680

A high specific [^{3}H]raclopride binding (more than 90% around the K_d values) with a better fit for one binding site

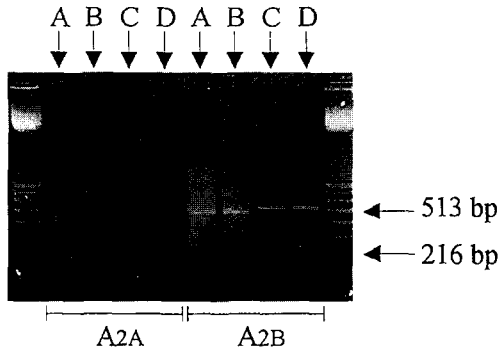

Fig. 1. RT-PCR analysis showing the presence of adenosine A_{2B} receptor mRNA in D_2 cells (A), and of adenosine A_{2A} and A_{2B} receptor mRNAs in A_{2A}-D_2 cells (B: clone 7; C: clone 8; D: clone 9).

(data not shown) was obtained. B_{max} values for the dopamine D_2 receptor binding sites labeled with [^{3}H]raclopride in membrane preparations from clone 7, clone 8, clone 9 and D_2 cells were (in mean ± S.E.M.) 3426 ± 72, 1597 ± 78, 1807 ± 34 and 2837 ± 83 fmol/mg protein, respectively. K_d values for those dopamine D_2 receptor binding sites were (in mean ± S.E.M.) 6.4 ± 0.7, 11.9 ± 0.8, 14.0 ± 0.7 and 7.2 ± 0.9 nM, respectively (n = 3–4). A low specific [^{3}H]CGS 21680 binding (about 40% around the K_d values) with a better fit for one binding site (data not shown) was obtained (Fig. 2). B_{max} values for the adenosine A_{2A} receptor binding sites labeled with [^{3}H]CGS 21680 in membrane preparations from clone 7, clone 8 and clone 9 were (in mean ± S.E.M.) 732 ± 23, 557 ± 33 and 625 ± 52 fmol/mg protein, respectively. K_d

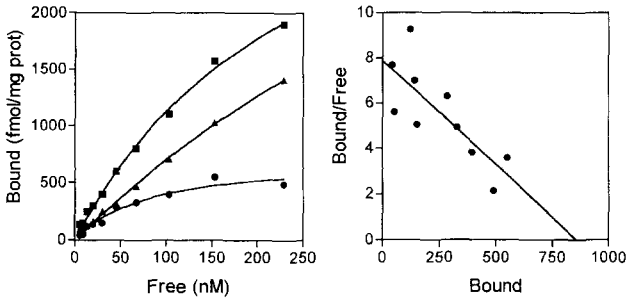

Fig. 2. Representative binding isotherms (left graph) and the corresponding Scatchard plot (right graph) of [^{3}H]CGS 21680 binding in membrane preparations from Ltk$^-$ cells containing both dopamine D_2 and adenosine A_{2A} receptors. B_{max} and K_d values obtained by non-linear regression analysis were 729 fmol/mg protein and 80.2 nM, respectively. Squares: total binding; triangles: nonspecific binding; circles: specific binding.

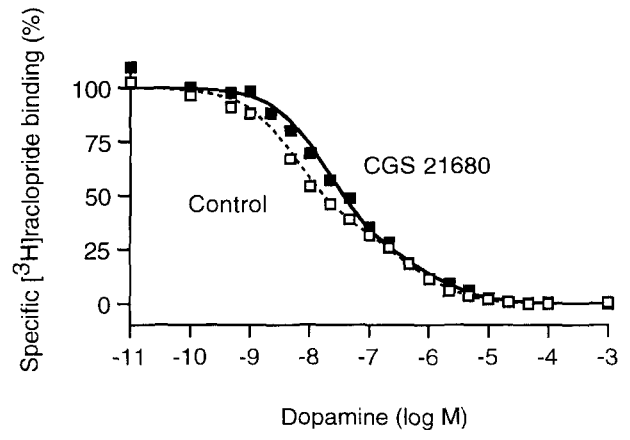

Fig. 3. Representative competitive inhibition curves illustrating the effect of the adenosine A_{2A} receptor agonist CGS 21680 (100 nM) on dopamine-induced inhibition of [^{3}H]raclopride binding in membrane preparations from A_{2A}-D_2 cells (clone 7). K_H and K_L values were estimated to 12.6 and 726.6 nM under normal conditions and 38.5 and 1673.5 nM in the presence of CGS 21680. R_H values were 53.8 and 68.1%, respectively.

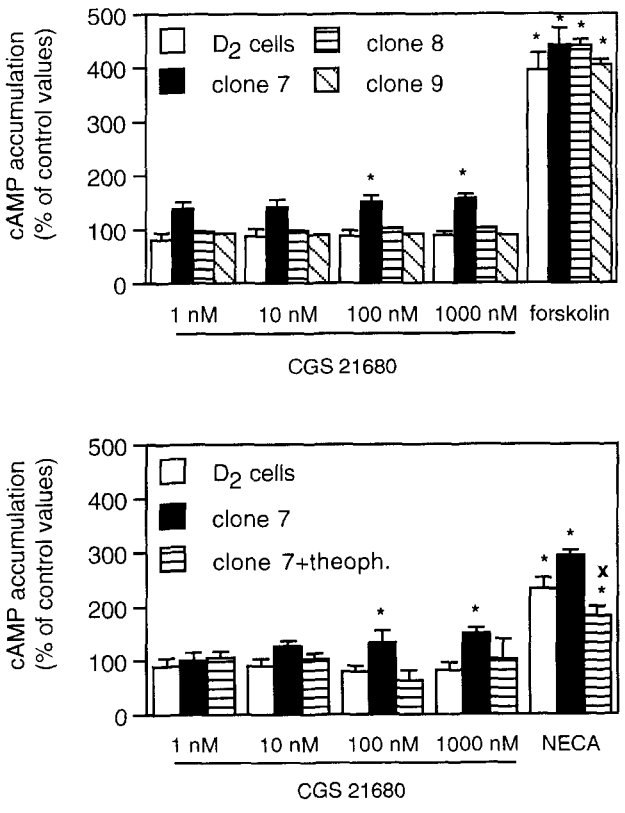

Fig. 4. Upper panel: cAMP accumulation stimulated by the adenosine A_{2A} receptor agonist CGS 21680 (1 nM–1 μM) and forskolin 30 μM in D_2 cells and in A_{2A}-D_2 cells (clones 7, 8 and 9). Control values for D_2 cells, clone 7, clone 8 and clone 9 were (in means ± S.E.M.) 0.50 ± 0.04, 0.27 ± 0.01, 0.56 ± 0.01 and 0.59 ± 0.02 pmol/50 μl, respectively. Lower panel: cAMP accumulation stimulated by CGS 21680 (1 nM–1 μM) and the non-selective adenosine receptor agonist NECA (1 μM) in D_2 cells and in A_{2A}-D_2 cells (clone 7), in the absence and presence of the adenosine receptor antagonist theophylline (30 μM). Control values for D_2 cells and clone 7 were (in means ± S.E.M.) 0.43 ± 0.04 and 0.36 ± 0.02 pmol/50 μl, respectively. Results are expressed as means ± S.E.M. of percentage of control values (n = 6–7/experiment). * Significant difference ($P < 0.05$) compared to control values. × Significant difference compared to clone 7 without theophylline ($P < 0.05$). No significant differences were found in the control values of clone 7 with and without theophylline.

Table 1
Competitive-inhibition experiments of dopamine versus [^{3}H]raclopride on membrane preparations from Ltk$^-$ cells containing both dopamine D_2 and adenosine A_{2A} receptors (A_{2A}-D_2 cells) and from Ltk$^-$ cells only containing D_2 receptors (D_2 cells)

Treatment	K_H (nM)	K_L (μM)	R_H (%)
A_{2A}-D_2 cells: clone 7			
Control	12.5 (4.9–31.6)	0.7 (0.2–2.4)	55.2 ± 3.3
CGS 21680 (10 nM)	16.2 (6.0–43.7)	0.9 (0.3–2.9)	59.7 ± 2.9
CGS 21680 (30 nM)	22.1 (7.9–61.2) [a]	1.0 (0.3–3.6)	64.2 ± 4.6 [a]
CGS 21680 (100 nM)	36.4 (15.8–81.8) [b]	1.5 (0.5–3.9) [a]	68.0 ± 4.2 [b]
CGS 21680 (300 nM)	24.7 (9.3–64.8) [b]	1.3 (0.4–4.5) [a]	62.2 ± 3.6
Control	31.6 (9.7–103.0)	0.9 (0.1–10.6)	57.8 ± 6.9
CGS 21680 (100 nM)	56.6 (17.1–187.5) [a]	2.2 (0.5–14.1) [b]	68.9 ± 7.6 [a]
8-PT (10 μM)	19.8 (3.6–108.4)	1.0 (0.1–11.6)	61.5 ± 8.4
CGS 21680 + 8-PT	24.8 (7.0–87.1)	0.9 (0.1–9.7)	57.7 ± 6.9
A_{2A}-D_2 cells: clone 8			
Control	27.0 (6.2–117.0)	0.4 (0.2–0.7)	47.5 ± 7.6
CGS 21680 (100 nM)	94.6 (64.6–138.7) [a]	2.0 (0.3–12.2) [a]	84.2 ± 3.3 [b]
A_{2A}-D_2 cells: clone 9			
Control	13.0 (3.0–50.2)	0.2 (0.1–6.6)	59.3 ± 3.7
CGS 21680 (100 nM)	86.6 (49.3–151.2) [a]	1.9 (0.2–16.4) [b]	79.5 ± 1.2 [a]
D_2 cells			
Control	11.9 (5.3–22.6)	0.5 (0.1–2.4)	64.3 ± 6.2
CGS 21680 (100 nM)	15.4 (6.3–27.2)	0.6 (0.2–3.2)	62.8 ± 2.6
Control	8.0 (4.1–21.2)	0.6 (0.1–4.6)	65.0 ± 5.0
NECA (1 μM)	5.2 (2.6–19.1)	0.4 (0.1–1.6)	57.6 ± 6.8
Control	10.6 (4.8–25.3)	0.6 (0.1–3.7)	64.8 ± 5.9
Forskolin (30 μM)	9.5 (4.3–20.4)	0.6 (0.2–3.4)	65.9 ± 7.1

K_H and K_L values are expressed as geometric means (antilogarithms of the logarithmically transformed data) and in parentheses the 95% confidence limits of the geometric mean. R_H values are expressed as means ± S.E.M. (n = 4–6 per experiment). [a] $P < 0.05$ and [b] $P < 0.01$ compared to control.

values for those adenosine A_{2A} receptor binding sites were (in mean ± S.E.M.) 103.5 ± 23.2, 125.3 ± 13.4 and 157 ± 30.6 nM, respectively (n = 3–4).

3.3. Competition experiments with dopamine versus [^{3}H]raclopride

Competition curves with dopamine versus [^{3}H]raclopride in membrane preparations from A_{2A}-D_2 and D_2 cells showed a better fit for two than for one binding site (data not shown). Different sets of experiments were performed to analyze the effect of different treatments (see Section 2). The adenosine A_{2A} receptor agonist CGS 21680, when added to membrane preparations of clone 7 at concentrations from 10 to 300 nM, significantly, and in a concentration-related way, increased the K_H, K_L and R_H values. The maximal enhancement was found at 100 nM CGS 21680, at which concentration an approximately 3-fold increase in the K_H and a 2-fold increase in the K_L values were observed (Table 1; Fig. 3). The same effect of CGS 21680 (100 nM) was obtained in membrane preparations from clones 8 and 9 (Table 1). The effect of CGS 21680 (100 nM) was counteracted by the adenosine antagonist 8-PT (10 μM), which, by itself did not modify competition curves with dopamine versus [^{3}H]raclopride (Table 1). D_2 cells did not show any significant change in their dopamine D_2 receptor binding characteristics in the presence of CGS 21680 (100 nM), the non-selective adenosine receptor agonist NECA (1 μM) or forskolin (30 μM) (Table 1).

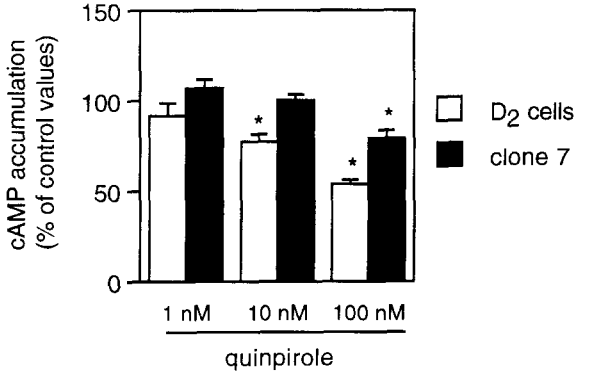

Fig. 5. Forskolin-induced cAMP accumulation in D_2 cells and in A_{2A}-D_2 cells (clone 7), in the presence of the dopamine D_2 receptor agonist quinpirole (1–100 nM). Results are expressed as means ± S.E.M. of percentage of control values (n = 6/experiment). Control values for D_2 cells and clone 7 were 1.98 ± 0.09 and 1.19 ± 0.02 pmol/50 μl, respectively. * Significant difference ($P < 0.05$) compared to control values.

3.4. cAMP accumulation experiments

In clone 7, but not in clone 8, clone 9 or D_2 cells, CGS 21680 induced a small but concentration-dependent increase in cAMP formation (Fig. 4). Forskolin (30 μM) induced a strong and similar cAMP formation in the four

different cell lines (Fig. 4). The CGS 21680-induced cAMP accumulation found in clone 7 was counteracted by the non-selective adenosine receptor antagonist theophylline (Fig. 4). A high concentration of NECA (1 μM) clearly increased cAMP formation in clone 7 and D_2 cells, which was also counteracted by theophylline (Fig. 4). Quinpirole showed a significant but weak counteraction of forskolin-induced cAMP accumulation in clone 7 and D_2 cells, which was significantly weaker in clone 7 (repeated measures ANOVA: significant interaction between cell and quinpirole factors). In clone 7 only a 20% inhibition was observed with the highest concentration of quinpirole (100 nM) (Fig. 5).

4. Discussion

The major finding of the present paper is that we can demonstrate that in stably A_{2A}/D_2 cotransfected fibroblast cells, but not in control cells only containing D_2 (long-form) receptors, the A_{2A} receptor agonist CGS 21680 can increase the K_L, K_H and R_H values of dopamine D_2 binding sites, labelled with [^{3}H]raclopride. These results are very similar to those obtained in striatal membrane preparations (Ferré et al., 1991). However, in the present experiments the most effective concentration of CGS 21680 to modulate dopamine D_2 receptor binding is 100 nM, while in the striatal membrane preparations the peak value is 30 nM. In agreement the K_d values for [^{3}H]CGS 21680 obtained in membrane preparations from cotransfected cells (around 100 nM) are higher than those from the rat striatum (around 1 nM; Jarvis et al., 1989). The lower affinity of the adenosine A_{2A} receptors in cotransfected cell lines could be due to the different cellular environment, different species (dog versus rat adenosine A_{2A} receptors) or a lower coupling to the G-protein, with a predominant number of receptors in the low-affinity state. In fact, a better fit for one binding site in saturation experiments with [^{3}H]CGS 21680 and a low CGS 21680-induced cyclic AMP accumulation was obtained in the cotransfected fibroblasts. The involvement of an adenosine receptor in the effects of CGS 21680 on K_H, K_L and R_H values of dopamine D_2 binding sites is demonstrated by the fact that those effects were fully counteracted by the adenosine receptor antagonist 8-PT.

Both adenosine A_{2A} and dopamine D_2 receptors belong to the G-protein-linked receptor family and stimulation of adenosine A_{2A} receptors activates and stimulation of dopamine D_2 receptors inhibits adenylyl cyclase by coupling to Gs and Gi proteins, respectively (Collis and Hourani, 1993; Gingrich and Caron, 1993). Therefore, an A_{2A}/D_2 receptor interaction could take place at the adenylyl cyclase level, which could in turn be responsible for the adenosine A_{2A} receptor-mediated regulation of the binding characteristics of dopamine D_2 receptors. In both cotransfected cells and control cells only containing dopamine D_2 receptors, adenylyl cyclase activation could be induced by forskolin and by the non-selective adenosine agonist NECA. Forskolin-induced cAMP accumulation could only be weakly counteracted by dopamine D_2 receptor stimulation with high concentrations of the selective dopamine D_2 agonist quinpirole. The ability of high concentrations of NECA to induce the same degree of cAMP accumulation in cotransfected and control cells is most probably related to the presence of low-affinity adenosine A_{2B} receptors. In fact, adenosine A_{2B} receptor mRNA expression was demonstrated in both fibroblast cell lines. On the other hand, in cotransfected but not in control cells CGS 21680 induced a weak cAMP accumulation. Altogether these results suggest that both adenosine A_{2A} and dopamine D_2 receptors are poorly coupled to adenylyl cyclase in the cotransfected cells, which, therefore, are not suitable for studying the A_{2A}/D_2 receptor interaction at the adenylyl cyclase level. On the other hand, they suggest that adenylyl cyclase is not involved in the adenosine A_{2A} receptor-mediated modulation of the binding characteristics of the dopamine D_2 receptors. In fact, two clones of transfected cells were obtained (clones 8 and 9) showing the adenosine A_{2A} receptor-mediated modulation of dopamine D_2 binding without any significant adenosine A_{2A} receptor-mediated adenylyl cyclase activation. Furthermore, neither forskolin nor NECA, which induced a strong cAMP accumulation, changed the binding characteristics of dopamine D_2 receptors. Finally, we have recently demonstrated in the cotransfected cells (clone 7) the existence of a functional antagonistic interaction between adenosine A_{2A} and dopamine D_2 receptors. Stimulation of adenosine A_{2A} receptors with CGS 21680 dose-dependently inhibited the influx of Ca^{2+} induced by the dopamine D_2 receptor agonist quinpirole (Yang et al., 1995). Those results show that the absence of an A_{2A}/D_2 receptor interaction at the adenylyl cyclase level does not preclude the existence of a functional A_{2A}/D_2 receptor interaction, which could, in fact, be related with the adenosine A_{2A}-mediated modulation of the binding characteristics of dopamine D_2 receptors.

In conclusion, the present experiments show that adenosine A_{2A} receptors antagonistically modulate the binding characteristics of dopamine D_2 (long-form) receptors in a fibroblast cell line stably transfected with dopamine D_2 (long-form) and adenosine A_{2A} receptor cDNAs. The failure in demonstrating this interaction in transiently transfected COS-7 cells (Snaprud et al., 1994) could therefore be due to a relative low number of cells containing both receptors. The present results also show that the mechanism for this type of A_{2A}/D_2 receptor interaction is not related with an adenosine A_{2A}-mediated adenylyl cyclase activation. It is also unlikely that protein phosphorylation is involved, since adenosine A_{2A} receptor-induced protein phosphorylation seems to be mainly mediated by a cAMP-dependent protein kinase (Collis and Hourani, 1993). Furthermore, ATP, which is a substrate for adenylyl

cyclase, was not added in the incubation assay. Finally, the demonstration of an A_{2A}/D_2 interaction with the same characteristics of that previously found in the rat striatum (Ferré et al., 1991) in a very different cellular type and cellular envionment underlines that these intramembrane receptor-receptor interactions (Zoli et al., 1993) could be a generalized functionally important mechanism in mammalian cells.

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