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# Reciprocal Interactions between Adenosine $A_{2A}$ and Dopamine $D_2$ Receptors in Chinese Hamster Ovary Cells Co-transfected with the Two Receptors

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**ABSTRACT.** Human adenosine $A_{2A}$ and rat dopamine $D_2$ receptors ($A_{2A}$ and $D_2$ receptors) were co-transfected in Chinese hamster ovary (CHO) cells to study the interactions between two receptors that are co-localized in striatopallidal γ-aminobutyric acid-(GABA)ergic neurons. Membranes from transfected cells showed a high density of $D_2$ (3.6 pmol per mg protein) and $A_{2A}$ receptors (0.56 pmol per mg protein). The $D_2$ receptors were functional: an agonist, quinpirole, could stimulate GTPγS binding and reduce stimulated adenylyl cyclase activity. The $A_{2A}$ receptor agonist 2-*p*-(2-carboxyethyl)phenethylamino-5′-*N*-ethylcarboxamidoadenosine (CGS 21680) decreased high-affinity binding of the agonist dopamine at $D_2$ receptors. Activation of adenosine $A_{2A}$ receptors shifted the dose–response curve for quinpirole on adenosine 3′,5′-cyclic monophosphate (cAMP) to the right. However, CGS 21680 did not affect dopamine $D_2$ receptor-induced GTPγS binding, but did cause a concentration-dependent increase in cAMP accumulation. The maximal cAMP response was decreased by the $D_2$ agonist quinpirole in a concentration-dependent manner, but there was no change in $EC_{50}$ and no effect in cells transfected only with adenosine $A_{2A}$ receptors. $A_{2A}$ receptor activation also increased phosphorylation of cAMP response element-binding protein and expression of c-*fos* mRNA. These effects were also strongly counteracted by quinpirole. These results show that the antagonistic actions between adenosine $A_{2A}$ and dopamine $D_2$ receptors noted previously *in vivo* can also be observed in CHO cells where the two receptors are co-transfected. Thus, no brain cell-specific factors are required for such interactions. Furthermore, the interaction at the second messenger level and beyond may be quantitatively more important than $A_{2A}$ receptor-mediated inhibition of high affinity $D_2$ agonist binding to the receptor. BIOCHEM PHARMACOL **58**;6: 1035–1045, 1999. © 1999 Elsevier Science Inc.

**KEY WORDS.** adenosine $A_{2A}$ receptors; dopamine $D_2$ receptors; receptor interaction; CHO cells; G-proteins; transfection; receptor binding; cyclic AMP

There are four cloned and pharmacologically characterized adenosine receptors, i.e. $A_1$, $A_{2A}$, $A_{2B}$, and $A_3$ [1, 2], and of these, the first two have a sufficiently high affinity for the endogenous ligand to be activated by low physiological levels of adenosine [3]. Whereas adenosine $A_1$ receptors are widely distributed in, for example, the central nervous system, the $A_{2A}$ receptor‖ is particularly abundant in the dopamine-rich basal ganglia structures of the brain [see Refs. 3 and 4]. By far the highest density of both the receptor and the corresponding mRNA is found in the striatopallidal γ-aminobutyric acid-(GABA)ergic neurons [4–8]. These neurons are also characterized by possessing a high density of dopamine $D_2$ receptors [for review see Refs. 9–11]. Thus, adenosine $A_{2A}$ and dopamine $D_2$ receptors are extensively co-localized and furthermore, there is excellent evidence that they are functionally antagonistic [see Refs. 12, 13, 4]. Several possible explanations for such interactions exist. It has been shown that agonists at the adenosine $A_{2A}$ receptor can reduce the affinity of dopamine at the dopamine $D_2$ receptor. One possible mechanism that could explain this functionally important antagonism is a direct receptor–receptor interaction. Indeed, it has been shown that activation of $A_{2A}$ receptors decreases the affinity of $D_2$ receptor agonists in membranes prepared from rat striatum [14, 15]. Such an interaction could also be demonstrated

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‖ *Abbreviations:* $A_{2A}$ receptor, adenosine $A_{2A}$ receptor; CHO, Chinese hamster ovary cells; cAMP, adenosine 3′,5′-cyclic monophosphate; $D_2$ receptor, dopamine $D_2$ receptor NECA, 5′-*N*-ethylcarboxamidoadenosine; CGS 21680, 2-*p*-(2-carboxyethyl)phenethylamino-5′-*N*-ethylcarboxamidoadenosine; SCH 58261, 7-(2-phenylethyl)-5-amino-2-(2-furyl)-pyrazolo[4,3-e]-1,2,4-triazolo[1,5-c]pyrimidine; α-MEM, α-minimum essential medium; Gpp(NH)p, 5′-guanylylimido diphosphate; and CREB, cAMP response element-binding protein.

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between canine $A_{2A}$ receptors and rat $D_2$ receptors transiently transfected into COS cells [16] or permanently transfected into a fibroblast cell line [17]. However, in these earlier studies, the interaction could only be detected in binding experiments and not at the functional level, possibly due to poor coupling of the receptors. In particular, signaling via $A_{2A}$ receptors could not be detected.

There is also evidence that dopamine, acting at $D_2$ receptors, activates $G_i$-proteins and thereby opposes the effects of adenosine acting at $A_{2A}$ receptors to stimulate $G_s$ signaling [4, 13]. The ability of a dopamine $D_2$ receptor agonist to counteract the functional effects of adenosine $A_{2A}$ receptor stimulation through $G_s$ and cAMP has been demonstrated in striatal slices *in vitro* [see Ref. 18]. Moreover, there is additional evidence from studies done *in vivo*. Using measurements of the expression of immediate early genes, such as c-*fos*, it was found that either a dopamine $D_2$ receptor agonist or an adenosine $A_{2A}$ receptor antagonist could reduce the expression of mRNA for immediate early genes in striatopallidal neurons [19–21] and that a dopamine $D_2$ receptor antagonist or a disruption of the dopaminergic innervation increased it [22, 23]. These results are compatible with the view that adenosine provides tonic activation of signals that lead to immediate early gene expression in these cells and that dopamine acting on $D_2$ receptors counteracts the effects of adenosine. However, the complicated neuronal circuitry in the intact striatum opens many alternative explanations.

To limit the degree of complexity, we used CHO cells which overexpress $A_{2A}$ receptors and which have been shown to give detectable and reproducible functional responses [24], and overexpressed rat $D_2$ receptors in the same cells in order to examine the ability of adenosine $A_{2A}$ receptors to modify $D_2$ receptor agonist binding and signaling. On the one hand, we examined if activation of adenosine $A_{2A}$ receptors has important effects on the signals generated by dopamine $D_2$ receptor activation. Conversely, we investigated if adenosine analogues acting on adenosine $A_{2A}$ receptors and drugs that act on dopamine $D_2$ receptors directly interact to regulate the production of cAMP and also important downstream signals such as c-*fos* mRNA expression.

## MATERIALS AND METHODS

### *Chemicals*

Adenosine deaminase was obtained from Boehringer Mannheim. Cyclic AMP, bacitracin, BSA, 1-10 phenanthroline, GDP, Gpp(NH)p, dithiothreitol, 2-chloroadenosine, NECA, and forskolin were purchased from Sigma. Dopamine, quinpirole, and CGS 21680 were obtained from Research Biochemicals International. SCH 58261 was a gift from Dr. E. Ongini, Schering-Plough Research Institute, Milan, Italy. [$^3$H]CGS 21680, [$^3$H]raclopride, and [$^3$H]cAMP were obtained from Du Pont NEN. [$^{35}$S]GTPγS was from New England Nuclear. Antibodies against phospho-CREB were from Upstate Biotechnology; secondary antibodies were from Jackson Immunoresearch. Oligonucleotide primers were synthesized by Promega, Scandinavian Diagnostic Services.

### *Transfection of $A_{2A}$ Receptor cDNA and $D_2$ Receptor cDNA into CHO Cells*

Chinese hamster ovary cells (CHO-K1 cells; CCL61, American Type Culture Collection) were transfected with plasmid DNA (human adenosine $A_{2A}$ receptor cDNA cloned into the BamH1 site of pcDMB-X) for stable expression [25]. The human $A_{2A}$ receptor was cloned from a cDNA library of a human B-cell lymphoblast cell line (GM03299, NIGMS Human Genetic Mutant Cell Repository). The cloning and characterization are described elsewhere [24, 25]. For double expression of receptors, a rat dopamine $D_{2(short)}$ receptor plasmid (2.47 kb cDNA fragment cloned into the EcoR1 sites of pZem 228R) (gift from M. G. Caron) was similarly transfected into the CHO cells along with the pHyg plasmid [26] to allow for additional selection with hygromycin (25 μg mL$^{-1}$). Monoclonal cell lines expressing one or both receptors were obtained by limiting dilution. Expression of the corresponding mRNA was determined by Northern blot hybridization using $^{32}$P-labeled full-length DNA probes from the corresponding receptors. Non-transfected cells were used as controls in both instances.

### *Culture of CHO Cells*

CHO cells transfected with human $A_{2A}$ receptor cDNA were grown adherent and maintained in α-MEM without nucleosides, containing 10% fetal bovine serum, penicillin (50 U mL$^{-1}$), streptomycin (50 μg mL$^{-1}$), L-glutamine (2 mM), and geneticin (GIBCO; 500 μg mL$^{-1}$) at 37° in 5% $CO_2$/95% air. Cells transfected with both rat $D_2$ receptor and human $A_{2A}$ receptor cDNA were grown in the same medium with addition of hygromycin (Calbiochem; 25 μg mL$^{-1}$).

### *Radioligand-Binding Experiments*

Membranes were obtained as described elsewhere [17] and preincubated for 30 min at 37° with adenosine deaminase (10 U mL$^{-1}$). The membranes used in the experiments with the dopamine $D_2$ receptor antagonist [$^3$H]raclopride were resuspended by sonication in an incubation buffer: Tris–HCl buffer (50 mM, pH 7.4) containing 120 mM NaCl, 5 mM KCl, 5 mM $MgCl_2$, 1 mM $CaCl_2$, and 1 mM EDTA. In saturation experiments, 10 concentrations (1–35 nM) of [$^3$H]raclopride (3.0 TBq mmol$^{-1}$, NEN) were incubated for 30 min at room temperature. Non-specific binding was defined as binding in the presence of dopamine (1 mM). The membranes used in the experiments with the adenosine $A_{2A}$ agonist radioligand [$^3$H]CGS 21680 were resuspended by sonication in an incubation buffer: Tris–HCl buffer (50 mM, pH 7.4) containing 10 mM $MgCl_2$. In

saturation experiments, 10 concentrations (2 to 90 nM) of [$^3$H]CGS 21680 (1.5 TBq mmol$^{-1}$; NEN) were incubated for 90 min at room temperature. Non-specific binding was defined as binding in the presence of 100 μM 2-chloroadenosine.

Competition experiments with dopamine versus [$^3$H]raclopride were performed by incubation with 20 concentrations (10 pM to 1 mM) of dopamine and 2 nM [$^3$H]raclopride for 30 min at room temperature in the presence and absence of the $A_{2A}$ agonist CGS 21680 (30 or 100 nM). The incubation was stopped by washing the membranes three times with 5 mL ice-cold Tris buffer over Whatman GF/B filters (Millipore). The radioactivity content of the filters was measured by liquid scintillation spectrometry. Data from saturation experiments were analyzed by non-linear regression analysis for determination of dissociation constants ($K_d$) and the number of receptors ($B_{max}$). Data from competition experiments were analyzed for the determination of $K_H$ and $K_L$ (the estimated $K_d$ values at the high- and low-affinity states, respectively) and $R_H$ (the relative amount of receptor in the high-affinity state) values, using the non-linear regression procedures in GraphPad Prism. Non-specific binding was calculated by extrapolation of the displacement curve. To achieve homogeneity of variance and allow parametric statistical hypothesis testing, $K_H$ and $K_L$ values were logarithmically transformed before analysis using Student's paired *t*-test.

### *Binding of [$^{35}$S]GTPγS*

Membranes used in [$^{35}$S]GTPγS binding experiments were obtained in the same way as in the radioligand experiments. In order to investigate if quinpirole could induce [$^{35}$S]GTPγS binding, we incubated the membrane with increasing concentrations of the nucleotide GDP in the presence of one high concentration of quinpirole (100 nM). To investigate if activation or inactivation of adenosine $A_{2A}$ receptors could modulate quinpirole-induced [$^{35}$S]GTPγS binding, we incubated the membranes with increasing concentrations of quinpirole (1 nM to 30 μM) in the presence or absence of CGS 21680 (100 nM) or SCH 58261 (20 nM). The binding experiment was performed in 96-well plates at 30°. The incubation mixture contained, in a final volume of 300 μL, 50 mM Tris–HCl pH 7.4, 1 mM EDTA, 5 mM $MgCl_2$, 1 μM 1–10 phenanthroline, 0.1% BSA, 0.03% bacitracin, 10 μM or 10 nM GDP, 1 mM dithiothreitol, 100 mM NaCl, 0.1 nM [$^{35}$S]GTPγS, and 50 μg membrane protein. Non-specific binding was measured in the presence of 0.1 mM Gpp(NH)p. Incubations were terminated by rapid filtration of samples through glass fiber filters (Whatman GF/B) that had been previously soaked in 50 mM Tris–HCl, 1 mM EDTA, 5 mM $MgCl_2$, and 100 mM NaCl. After filtration, the samples were washed once with 6 mL of the same buffer. All the binding data were analyzed by non-linear regression with the software GraphPad Prism, version 2.00.

### *Measurement of cAMP Accumulation*

CHO cells co-transfected with adenosine $A_{2A}$ and dopamine $D_2$ receptors were sown out in 12-well plates (200,000 cells per well) and were allowed to grow for 36–48 hr. The cells were then washed twice with HEPES-buffered (20 mM) α-MEM and incubated at 37° for 10 min in 0.9 mL of HEPES-buffered α-MEM. The drugs to be tested were added in 0.1 mL of medium and the cells were incubated for a further 10 min. No phosphodiesterase inhibitor was used. The reactions were terminated by the addition of perchloric acid to a final concentration of 0.4 M. After 1 hr at 4°, the acidified cell suspensions were transferred to tubes and neutralized with 4 M KOH/1 M Tris–HCl. The cAMP content in the samples was determined using a competitive radioligand-binding assay [27]. Dose–response curves were analyzed using the GraphPad InPlot program. For statistical comparisons, 95% confidence intervals were used.

### *Protein Analysis by Western Blotting*

For phospho-CREB assays, the cells were starved overnight in 1% bovine serum and stimulated with CGS 21680 (1 μM or 100 nM) and/or quinpirole (1 μM) for 15 min in serum-free medium. The stimulation was stopped by addition of boiling Laemmli sample buffer. The samples were run on a 12% polyacrylamide, denaturing gel, blotted onto a membrane (Immobilon), and non-specific binding blocked by an incubation in Tris-buffered saline (TBS; 50 mM Tris–HCl, pH 7.5, 150 mM NaCl) containing Tween 20 (0.5%) and 3% dry milk. Membranes were subsequently incubated with phospho-CREB antibody overnight at 4°. Antibody binding was detected by a secondary antibody (horseradish peroxidase-conjugated anti-rabbit) and an enhanced chemiluminescence system (Amersham).

### *Immunofluorescence*

Cells were starved overnight in 1% bovine serum and treated with CGS 21680 (1 μM) and/or quinpirole (1 μM) for 15 min. After stimulation, the cells were fixed for 10 min with 4% paraformaldehyde in phosphate buffer (125 mM, pH 7.4). Incubation with the primary antibody was performed at 4° overnight in PBS, pH 7.4, with 0.3% Triton X-100, supplemented with 15% goat serum. The cells were then rinsed in the same buffer without serum several times and incubated with the secondary antibody for 1 hr at room temperature. After several rinses, the cells were mounted in a mixture of glycerol/PBS (3:1) containing 0.1% phenylenediamine in order to retard fading. All micrographs were taken with black-and-white T-max film (Kodak). Background labeling was estimated by comparison with parallel preparations that had not been incubated with the primary antibody.

### In Situ *Hybridization*

Cells were plated in Lab-Tech glass chamber slides and allowed to grow for 36 hr. Then, the cells were treated with CGS 21680 (100 nM) and/or quinpirole (10 nM) for 1 hr and fixed for 10 min with 4% paraformaldehyde in phosphate buffer (125 mM, pH 7.4).

Hybridization was performed as described elsewhere [28]. The 48 mer probe for c-*fos*, complementary to rat c-*fos* mRNA encoding amino acids 137–152 of the c-Fos protein [29], was synthesized by Scandinavian Gene Synthesis. The specificity of the probe has been tested earlier [28, 30]. After hybridization, the sections were dipped in NTB-3 emulsion (Kodak) and exposed for 4–7 weeks. After the emulsion had been developed, sections were lightly counter-stained in cresyl violet (0.5%).

## RESULTS

### *Saturation Binding Experiments*

The expression of dopamine $D_2$ and adenosine $A_{2A}$ receptors was determined using radioligand-binding experiments (Fig. 1). The cells showed high specific binding of the dopamine $D_2$ receptor antagonist radioligand [$^3$H]raclopride ($B_{max}$ 3600 ± 140 fmol mg$^{-1}$ protein; $K_d$ 7.4 ± 0.6 nM; mean ± SEM, N = 3) (Fig. 1) with a better fit for one binding site than for two sites (data not shown). Non-specific binding was less than 10% at concentrations of [$^3$H]raclopride close to the $K_d$ value. The adenosine $A_{2A}$ receptor agonist radioligand [$^3$H]CGS 21680 (Fig. 1) showed a smaller number of binding sites ($B_{max}$ 558 ± 29 fmol mg$^{-1}$ protein; mean ± SEM, N = 4) with a $K_d$ of 59.3 ± 8.4 nM. For unknown reasons, it is difficult to express $A_{2A}$ receptors at higher density [see Ref. 25]. The binding curve showed better fit for one binding site than for two binding sites (data not shown). Non-specific binding was about 40% at concentrations of [$^3$H]CGS 21680 close to the $K_d$ value.

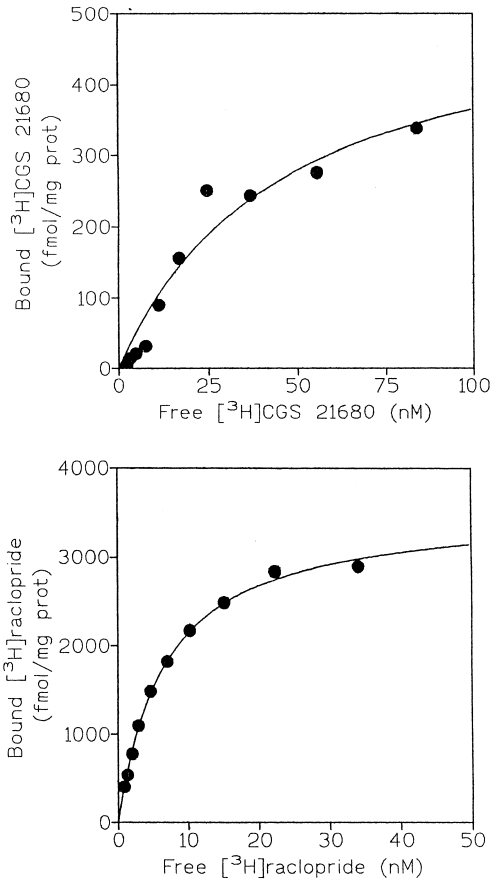



**FIG. 1. Expression of adenosine $A_{2A}$ and dopamine $D_2$ receptors in co-transfected CHO cells. Upper graph: Representative binding isotherm of specific [$^3$H]CGS 21680 binding in membrane preparations from CHO cells co-transfected with $A_{2A}$ and $D_2$ receptor cDNAs. $B_{max}$ and $K_d$ values in this representative experiment were 532 fmol mg$^{-1}$ protein and 45.1 nM, respectively. Lower graph: Representative binding isotherm of specific [$^3$H]raclopride binding in membrane preparations from CHO cells co-transfected with $A_{2A}$ and $D_2$ receptor cDNAs. $B_{max}$ and $K_d$ values in this representative experiment were 3560 fmol mg$^{-1}$ protein and 6.7 nM, respectively.**

### *Functional Assay*

In order to investigate if the expressed $A_{2A}$ and $D_2$ receptors were functional, we examined their ability to affect cAMP accumulation. As seen in Fig. 2, the adenosine $A_{2A}$-selective agonist CGS 21680 stimulated cAMP accumulation in a concentration-dependent manner in cells transfected with adenosine $A_{2A}$ receptors, but not in untransfected cells. Another adenosine analogue, NECA, which is able to stimulate both adenosine $A_{2A}$ and $A_{2B}$ receptors, also caused a very modest stimulation at high concentrations in the non-transfected cells. There was no difference between cells transfected only with $A_{2A}$ receptors and cells transfected with both $A_{2A}$ and $D_2$ receptors (Fig. 2), indicating that the presence of the $D_2$ receptor does not alter the signals mediated by the $A_{2A}$ receptor. Quinpirole caused a concentration-dependent inhibition not only of the cAMP accumulation afforded by CGS 21680, but also of that induced by forskolin (3 μM) in cells transfected with the dopamine $D_2$ receptor (Fig. 3). The results also show that the cAMP stimulation afforded by forskolin was lowered by addition of the $A_{2A}$ receptor antagonist SCH 58261 (20 nM), indicating that in the cultures there is a degree of activation of the $A_{2A}$ receptor, possibly via endogenous adenosine. Addition of adenosine deaminase (2 U/mL) lowered the basal level of cAMP by 20 percent, supporting that assumption (data not shown). The decrease in cAMP accumulation afforded by 20 nM SCH 58261 was more pronounced than that afforded by adenosine deaminase. A possible explanation could be that adenosine has a much higher affinity for $A_{2A}$ receptors (about 0.1 μM) than for adenosine deaminase (higher than 10 μM). Together, these results show that the CHO cells we used expressed high levels of functional adenosine $A_{2A}$ and dopamine $D_2$ receptors.

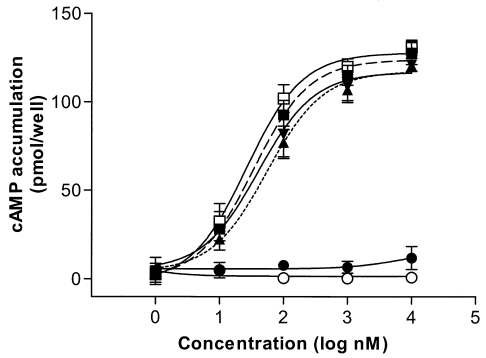



**FIG. 2. Comparison of the dose–response curves of cAMP accumulation induced by a non-selective (NECA) or $A_{2A}$ receptor-selective (CGS 21680) agonist in three types of CHO cells: control cells, cells transfected with human adenosine $A_{2A}$ receptor cDNA, and CHO cells co-transfected with human adenosine $A_{2A}$ receptor cDNA and rat dopamine $D_2$ receptor cDNA. Results are means of two experiments performed in duplicate and are presented as pmol cAMP per well. (■) NECA $A_{2A}$-$D_2$ cells, (▲) CGS 21680 $A_{2A}$-$D_2$ cells, (□) NECA $A_{2A}$ cells, (▼) CGS 21680 $A_{2A}$ cells, (●) NECA control cells, and (○) CGS 21680 control cells.**

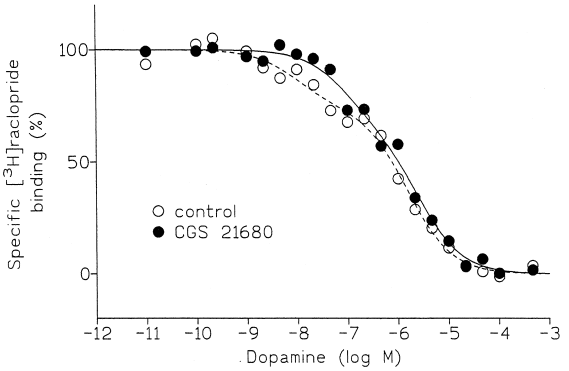



**FIG. 4. Representative competitive inhibition curves illustrating the effect of the adenosine $A_{2A}$ receptor agonist CGS 21680 (100 nM) on dopamine-induced inhibition of [$^3$H]raclopride binding in membrane preparations from CHO cells co-transfected with $A_{2A}$ and $D_2$ receptor cDNAs. In this particular experiment, $K_H$ (high-affinity) and $K_L$ (low-affinity) values were estimated to be 5.1 and 775.6 nM under normal conditions and 47.5 and 1348.2 nM in the presence of CGS 21680. $R_H$ values were 25.9 and 34.3%, respectively. The average values of five experiments are given in Table 1.**

### *Competition Binding Experiments*

Dopamine displaced bound [$^3$H]raclopride in membrane preparations from CHO cells transfected with both adenosine $A_{2A}$ and dopamine $D_2$ receptors (Fig. 4). The displacement curves were fitted better using two rather than one binding site. In cells not treated with the adenosine $A_{2A}$ agonist CGS 21680, more than 80% of the agonist binding showed an estimated $K_d$ value at low affinity ($K_L$) of 1.3 μM and about 18% showed an estimated $K_d$ value at high affinity ($K_H$) of 12 nM; Table 1). When CGS 21680 (30 or 100 nM) was added to membrane preparations of CHO cells, the $K_H$ values were significantly increased, but the $K_L$ values were unchanged. The proportion of receptors in the high-affinity state was increased by the higher concentration of CGS 21680 (100 nM; Table 1).

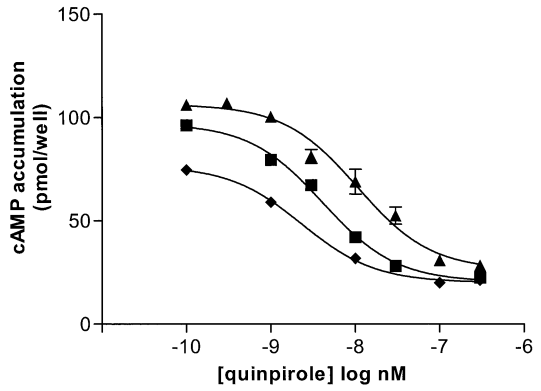



**FIG. 3. Dose–response curve of quinpirole on cAMP accumulation in CHO cells co-transfected with human adenosine $A_{2A}$ receptor cDNA and rat dopamine $D_2$ receptor cDNA. Results are means of two experiments carried out in duplicate and are presented as pmol cAMP per well. (■) Forskolin 3 μM, (◆) forskolin 3 μM + SCH 58261 20 nM, and (▲) CGS 21680 1 μM.**

### *Functional Interaction Experiments*

As already noted, activation of dopamine $D_2$ receptors by quinpirole caused a concentration-dependent inhibition of cAMP accumulation (Fig. 3). The potency of quinpirole was dependent on the degree of stimulation of adenosine $A_{2A}$ receptors: it was highest when $A_{2A}$ receptors were blocked ($EC_{50}$ 2.3 [1.7–3.2] nM) and lowest when they were stimulated by CGS 21680 ($EC_{50}$ 10.61 [7.67–14.66] nM), the $EC_{50}$ value for quinpirole with forskolin alone being 4.08 [3.1–5.3] nM (mean and 95% confidence interval). Since some signaling by $D_2$ receptors may occur via other pathways than inhibition of cAMP, we also examined $D_2$ agonist-induced binding of [$^{35}$S]GTPγS on washed cell membranes. In an initial experiment, the membranes were incubated with increasing concentrations of GDP. At low concentrations of GDP (below 1 nM), GTPγS binding was high but was not significantly modified by quinpirole. At high concentrations of GDP (above 0.1 mM), the binding of [$^{35}$S]GTPγS was very low, and under these conditions it was not possible to detect any effects of quinpirole. The maximal effects of quinpirole were found to occur in the presence of 10 μM GDP (data not shown). At this concentration, the basal specific binding of [$^{35}$S]GTPγS (0.1 nM) was about 6000 cpm per 50 μg protein and the non-specific binding of [$^{35}$S]GTPγS, measured in the presence of Gpp(NH)p (0.1 mM), represented about 10–20% of total binding. Analysis of the dose–response curve for quinpirole (Fig. 5) gave an estimated $EC_{50}$ value of 847 nM (572–1255; 95% confidence interval). Addition of CGS 21680 or SCH 58261 did not significantly change the $EC_{50}$ value (821 nM [405–1668] in the presence of 100 nM CGS

**TABLE 1. Competitive inhibition experiments of dopamine versus [$^3$H]raclopride on membrane preparations from CHO cells containing both dopamine $D_2$ and adenosine $A_{2A}$ receptors**

| Treatment | $K_H$ (nM) | $K_L$ (μM) | $R_H$ (%) |
|---|---|---|---|
| Control | 12.4 (6.8–22.6) | 1.3 (1.1–1.5) | 18.2 ± 3.3 |
| CGS 21680 (30 nM) | 41.1 (9.8–172.2)* | 1.4 (0.8–2.2) | 28.9 ± 6.1 |
| CGS 21680 (100 nM) | 51.4 (13.8–190.9)† | 1.9 (1.4–2.8)* | 32.5 ± 4.9* |

The equilibrium dissociation constants for low-affinity ($K_H$) and high-affinity ($K_L$) states are expressed as geometric means (antilogarithms of the logarithmically transformed data), with the 95% confidence limits of the geometric mean given in parentheses. The proportion of dopamine $D_2$ receptors in the high-affinity state ($R_H$) is expressed as means ± SEM (N = 5 per experiment). The [$^3$H]raclopride binding itself was not affected by CGS 21680 (data not shown).

*$P < 0.05$ and †$P < 0.01$ compared to control, using Student's *t*-test on log-transformed raw data.

21680 and 658 nM [334–1294] in the presence of 20 nM SCH 58261).

### *Reduction by Dopamine $D_2$ Receptor Activation of cAMP Accumulation Induced by Adenosine $A_{2A}$ Receptor Activation*

The dopamine $D_2$ receptor agonist quinpirole reduced the response to adenosine $A_{2A}$ receptor stimulation in a concentration-dependent manner: cells treated with increasing concentrations of CGS 21680 (1 nM to 10 μM) showed progressively smaller responses over the entire concentration–response curve in the presence of increasing concentrations of quinpirole (Fig. 6A). There were no clear differences in the potency of CGS 21680: $EC_{50}$ values for CGS 21680 at 0, 1, and 100 nM quinpirole were, 52 (24–118), 56 (24–136), and 40 (21–77) nM, respectively (means and 95% confidence intervals). By contrast, the maximal responses to CGS 21680 were markedly reduced from 102 (91–112) to 61 (54–67) pmol cAMP per well by 1 nM quinpirole and even further to 31 (30–33) pmol at 100 nM quinpirole. This shows that quinpirole caused an equivalent degree of inhibition over the entire concentration–response curve for CGS 21680. Quinpirole had no effect in cells transfected only with the adenosine $A_{2A}$ receptor (Fig. 6B).

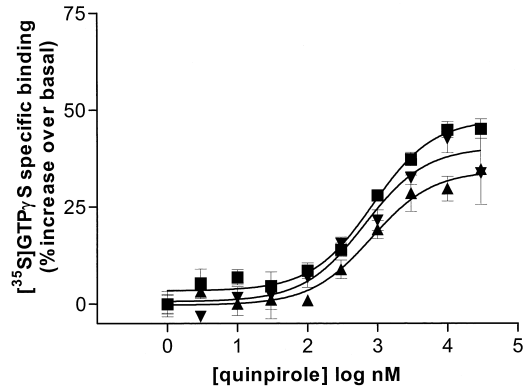



**FIG. 5. Dose–response curves for quinpirole-induced specific [$^{35}$S]GTPγS binding to homogenates of transfected CHO cells (■), in the presence of 100 nM CGS 21680 (▲) and the presence of 20 nM SCH 58261 (▼). Results are expressed as % increase over basal. Means of two experiments with duplicates are shown (10 μM GDP was present in all these experiments.)**

### *Phosphorylation of CREB*

In order to determine if the interactions at the level of the second messenger were reflected in changes at the level of phosphorylation, we used an antibody specific for the phospho-form of CREB. The average optical density of 5 Western blots indicates that the $A_{2A}$ agonist CGS 21680 (100 nM and 1 μM) increased the amount of immunore-

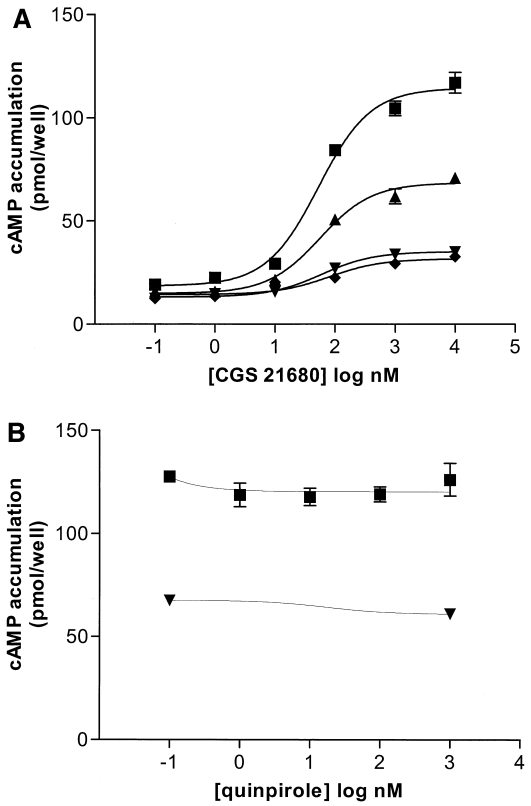



**FIG. 6. Adenosine $A_{2A}$ receptor-mediated increase in cAMP accumulation is counteracted by activation of co-expressed dopamine $D_2$ receptors. (A) Quinpirole effects on CGS 21680-induced dose–response curves in CHO cells co-transfected with human adenosine $A_{2A}$ receptor cDNA and rat dopamine $D_2$ receptor cDNA. Results are means of two experiments conducted in duplicate and are presented as pmol cAMP per well. (■) Control, (▲) quinpirole 1 nM, (▼) quinpirole 10 nM, and (◆) quinpirole 100 nM. (B) Dose–response curve of quinpirole on cAMP accumulation in CHO cells transfected with human adenosine $A_{2A}$ receptor cDNA. (■) Forskolin 10 μM, (▼) CGS 21680 1 μM.**

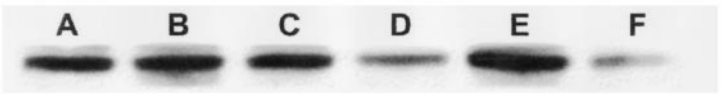



**FIG. 7. Adenosine $A_{2A}$ receptor-mediated increase in CREB-phosphorylation is counteracted by activation of co-expressed dopamine $D_2$ receptors. Western blot with phospho-CREB antibody. (A) Control, (B) quinpirole 1 μM, (C) CGS 21680 100 nM, (D) CGS 21680 100 nM + quinpirole 1 μM, (E) CGS 21680 1 μM, and (F) CGS 21680 1 μM + quinpirole 1 μM. The illustrated Western blot is representative of five experiments showing the same principal finding.**

activity for phospho-CREB (10.6 ± 6.5% and 26 ± 4% of control, respectively), and that quinpirole alone caused a small decrease (20.5 ± 14% of control). The CGS 21680-induced increase was counteracted by 1 μM quinpirole (Fig. 7). The same phospho-CREB antibody can also be used for immunofluorescence. As shown in Fig. 8, CGS 21680 (1 μM) induced an increase in phospho-CREB immunofluorescence detected over nuclei. The CGS 21680-induced increase in phospho-CREB was blocked by 1 μM quinpirole (Fig. 8D).

### *Changes in c-fos mRNA*

To detect changes in the expression of c-*fos* mRNA, we used *in situ* hybridization of $A_{2A}$-$D_2$ transfected cells treated with 100 nM CGS 21680 or 10 nM quinpirole or both. In a defined square, five hundred cells from each treatment were counted and divided in two categories: labeled and not labeled. CGS 21680 (100 nM) caused an increase in the number of labeled cells, which was blocked by 10 nM quinpirole. Quinpirole alone caused a small decrease in the number of labeled cells (Fig. 9).

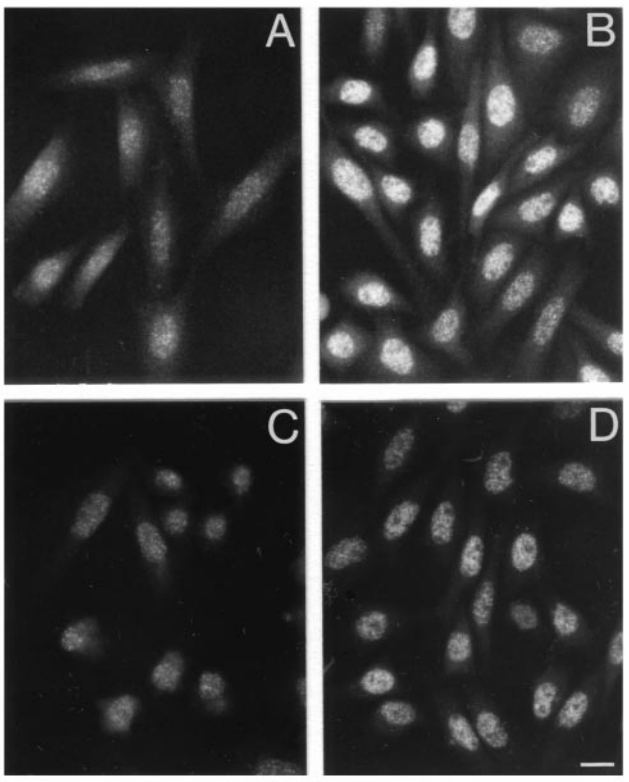
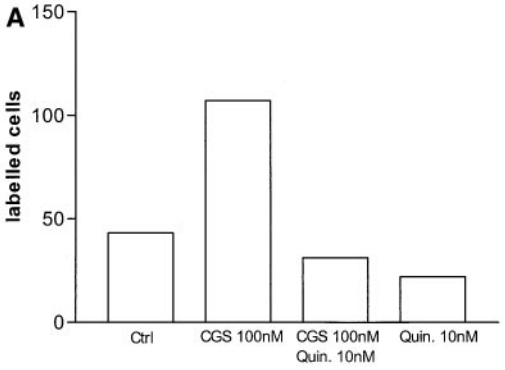



**FIG. 8. Immunofluorescence with phospho-CREB antibody. (A) Control, (B) CGS 21680 1 μM, (C) quinpirole 1 μM, and (D) CGS 21680 1 μM + quinpirole 1 μM (scale bar 10 μm). Typical result from two experiments using the same technique.**



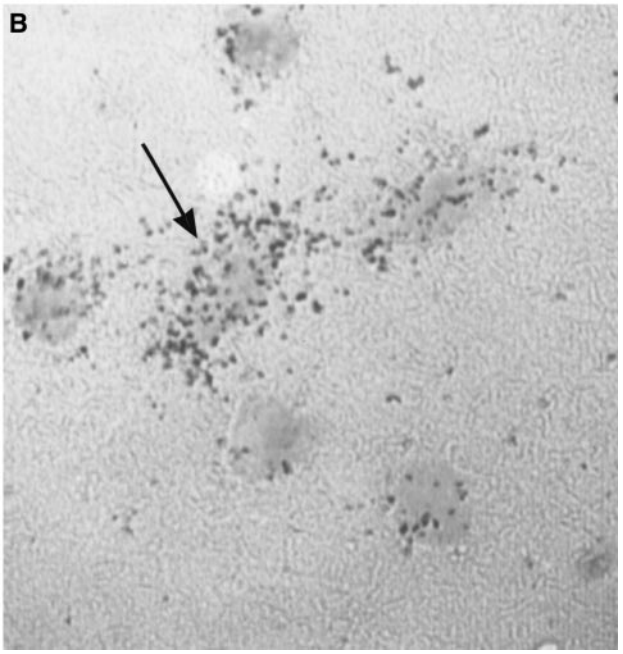




**FIG. 9. Adenosine $A_{2A}$ receptor-mediated increase in c-*fos* mRNA expression is counteracted by activation of co-expressed dopamine $D_2$ receptors. After *in situ* hybridization using a probe specific for c-*fos* mRNA, five hundred cells in a defined square of the chamber from each treatment were counted and divided into two categories: labeled and non-labeled. (A) Histogram showing the number of cells labeled with a probe specific for c-*fos* mRNA. (B) Emulsion autoradiogram showing the c-*fos* mRNA (silver grains) from the *in situ* hybridization experiment. The arrow indicates a labeled cell.**

## DISCUSSION

The present results show reciprocal interactions between adenosine $A_{2A}$ and dopamine $D_2$ receptors co-transfected in CHO cells. We have confirmed that stimulation of adenosine $A_{2A}$ receptors can decrease agonist affinity at dopamine $D_2$ receptors and demonstrate that this is related to a small change in the potency of a dopamine $D_2$ receptor agonist to induce some functional responses. Conversely, we show that activation of dopamine $D_2$ receptors effectively reduces signaling induced by adenosine $A_{2A}$ receptor stimulation.

### *Inhibition of Dopamine $D_2$ Receptor Activation by Adenosine $A_{2A}$ Receptor Stimulation*

It was first observed using membranes prepared from the rat striatum that the binding of dopamine $D_2$ receptor agonists, but not that of antagonists, was reduced by the addition of an agonist that acts on adenosine $A_{2A}$ receptors present in the same membrane preparation [14]. This interaction at the level of binding could also be observed when the long form of the human $D_2$ receptor was stably expressed together with the dog $A_{2A}$ receptor in a fibroblast cell line [17]. Unfortunately, it was difficult in these cells to register functional effects following stimulation of $A_{2A}$ receptors. Hence, functional consequences of the interaction at the level of binding could not be registered. In the present series of experiments, the short form of the rat $D_2$ receptor was transfected together with the human $A_{2A}$ receptor, and interactions at the level of binding and function could be registered. As discussed by Kenakin [31], problems may occur in systems where the recombinant receptor is overexpressed. However, this does not seem to be a concern in the present case. The density of $A_{2A}$ receptors in the transfected CHO cells is, if anything, lower than the number of receptors in naturally occurring cells in the brain. In membranes from the rat striatum that abundantly express native $A_{2A}$ receptors, a $B_{max}$ of close to 1000 fmol per mg protein was found [32]. It is now known that these endogenous $A_{2A}$ receptors are found only in neurons and not in glial cells ($A_{2A}$ receptors have been found on microglial cells [33]; however, these cells are very rare in normal brain) and that only about 50% of the neurons express the receptor [4]. The number of receptors in these naturally expressing cells is thus apparently higher than in the transfected CHO cells, where some 500–600 fmol per mg protein was found. Thus, there is no evidence that the transfected cells express an unnaturally high number of $A_{2A}$ receptors. Similarly, in rat striatal membranes, the $B_{max}$ for [$^3$H]raclopride binding was close to 400 fmol per mg protein [14]. These $D_2$ receptors are found on approximately half of the neurons in this tissue and on occasional nerve terminals, but not on glial cells. Thus, less than 10% of the membranes from this mixed striatal cell population would be expected to harbor $D_2$ receptors. Consequently, the density of $D_2$ receptors on the natural cell membranes is not expected to be materially lower than in the transfected CHO cells.

In the present series of experiments, we have not specifically examined the mechanism behind the interaction at the binding level. Nonetheless, some tentative conclusions may be drawn. The affinity of dopamine receptor agonists is reduced by adenosine $A_{2A}$ receptor stimulation in membranes from rat striatum where the native rat $A_{2A}$ and $D_2$ receptors are co-expressed [14], in fibroblasts where the long form of the human $D_2$ receptor ($D_{2(long)}$) is expressed together with the canine $A_{2A}$ receptor [17], and, finally, in CHO cell membranes expressing the short form of rat $D_2$ receptor together with human $A_{2A}$ receptors (present data). Hence, the interaction is unlikely to be due to cell-specific co-factors, to be species-dependent, or to depend on particular forms of the two receptors. Secondly, the effect of CGS 21680 to reduce high-affinity binding of $D_2$ agonists has previously been shown to be blocked by adenosine $A_{2A}$ receptor antagonists [14, 17]. Hence, it is in all likelihood due to $A_{2A}$ receptor stimulation and not to some other direct action on $D_2$ receptors. Thirdly, we observed the interaction in membrane preparations with no added nucleotides, suggesting that second messenger-dependent or phosphorylation events may not be very important. This conclusion was also recently drawn by Dixon and co-workers [15], who demonstrated the decrease in $D_2$ agonist binding in lysed striatal synaptosomes. In particular, cAMP-dependent processes are probably not involved, since the binding interaction was observed in fibroblasts where the transfected canine $A_{2A}$ receptors did not cause any significant adenylyl cyclase activation [17].

However, in the present study, there were interactions between the two co-transfected receptors at the level of the second messenger, even though the magnitude of the effect of the $A_{2A}$ agonist on the response to the $D_2$ agonist was rather small. A study of the role of adenosine $A_{2A}$ receptor activation on $D_2$ signaling was complicated by the fact that there was evidence for activation even in the absence of exogenous agonist, whereas no such effect was noted in the case of the $D_2$ receptor. Adenosine is elaborated by these (and all other) cells, but dopamine is formed by and released from specialized cells only. Hence, in order to determine the effect of adenosine $A_{2A}$ receptor stimulation, we had to compare the situation in the presence of an agonist with that in the presence of an antagonist. Thus, there was an approximately 3-fold shift in the apparent affinity of the dopamine receptor agonist between a situation where the $A_{2A}$ receptor was completely blocked and a situation where it was almost maximally occupied. Interestingly, this shift in the concentration–response curve had the same magnitude as the decrease in the affinity of dopamine at the high-affinity binding site in the competition experiments.

We also examined the ability of an adenosine $A_{2A}$ receptor agonist to modify the effect of a $D_2$ agonist at an earlier step in the signaling cascade. Quinpirole was able to

stimulate [$^{35}$S]GTPγS binding, albeit with a rather low potency. Using this assay, we could detect no significant effect of the $A_{2A}$ agonist on the ability of the $D_2$ agonist to activate G-proteins. Thus, two different functional assays in the transfected cells suggest that the $A_{2A}$ receptor-mediated decrease in high-affinity dopamine binding has limited functional consequences. One important difference between the two assays is that much higher concentrations of the dopamine $D_2$ receptor agonist were required in the GTPγS binding assay than in the adenylyl cyclase assay. The reason for this is probably that the ratio of GDP to GTP is markedly different, and that consequently only a small proportion of the receptor-associated G-proteins in the latter assay are actually converted into the activated state. This could mean that the small change in high-affinity receptor binding only results in a measurable change in signaling when there is an optimal ratio between receptor and G-protein. Thus, these data could mean, but certainly do not prove, that the ability of adenosine to decrease the capacity of dopamine to activate $D_2$ receptors is mediated to a small degree by a direct receptor–receptor interaction.

### *Inhibition of $A_{2A}$ Signaling by $D_2$ Receptor Activation*

In the converse set of experiments, we examined inhibition by dopamine of adenosine $A_{2A}$ receptor-mediated signaling. The results also show that, by contrast, dopamine inhibition of adenosine $A_{2A}$ receptor-mediated signaling is very marked and can be observed at several levels of the signaling cascade. Dopamine $D_2$ receptor stimulation could, in a concentration-dependent manner, lower the maximal cAMP response to the $A_{2A}$ agonist, even though the potency of the $A_{2A}$ agonist as determined by the $EC_{50}$ value was essentially unchanged. This could be expected if this interaction depended on the ability of $G_i$-proteins generated by $D_2$ receptor stimulation to antagonize the consequences of an $A_{2A}$ receptor-mediated increase in the stimulatory $G_s$ pathway. The observed functional interaction is, however, not what one would expect from a $D_2$ receptor-mediated decrease in adenosine $A_{2A}$ receptor agonist potency. Thus, these data could mean that the ability of $D_2$ agonists to counteract $A_{2A}$ receptor-mediated responses is largely due to postreceptor effects.

We also found that the phosphorylation of CREB was influenced in a reciprocal manner by the two receptors. Thus, the adenosine $A_{2A}$ agonist CGS 21680 increased the amount of total immunoreactive phosphorylated CREB and in particular increased the expression in nuclei. The explanation is probably that the catalytic subunit of cAMP-dependent protein kinase translocates to the nucleus where it causes the phosphorylation of CREB [34]. There, it binds to CREB-binding protein [35], which leads to increased transcription of several genes. It has been shown that CREB mediates the effects of dopamine via dopamine $D_1$ receptors in the striatum [36]. The stimulatory effect of the $A_{2A}$ agonist was virtually abolished by a maximal dose of the $D_2$ receptor agonist quinpirole. This interaction between $D_2$ and $A_{2A}$ receptors in CHO cells co-transfected with the two receptors is rather similar to that observed in striatal slices using another cAMP-dependent phosphorylation, that of DARPP-32 [37]. This dopamine- and cAMP-regulated phosphoprotein is phosphorylated by both dopamine $D_1$ and adenosine $A_{2A}$ receptor agonists in non-overlapping neuronal populations and via a cAMP-dependent protein kinase [18]. The adenosine $A_{2A}$ receptor-mediated DARPP-32 phosphorylation was completely antagonized by dopamine $D_2$ receptor stimulation, and the effect of the $D_2$ agonist did not require nerve impulses, indicating that it is a direct interaction at the striatopallidal neuron. Preliminary results indicate that CREB phosphorylation in striatal slices is regulated in a manner similar to that reported here for CHO cells.* It is well known that the expression of several immediate early genes, including c-*fos*, can be increased by phospho-CREB, even though phospho-CREB is not the only regulatory factor [38, 39]. It is therefore interesting that the expression of c-*fos* mRNA in the transfected CHO cells was stimulated by the $A_{2A}$ agonist CGS 21680 and that this increase was blocked by the $D_2$ agonist quinpirole. This finding in a non-neuronal cell shows that the interaction between the two receptors at the level of immediate early gene expression can occur in the absence of neuronal contacts between cells. Our findings *in vitro* may be directly compared to previous data obtained *in vivo*. Thus, blockade of $A_{2A}$ receptors mimics the effect of stimulation of dopamine $D_2$ receptors on motor behavior in rodents, both procedures causing a stimulation of locomotion [19, 20, 40] and a marked enhancement of the actions of stimulation of dopamine $D_1$ receptors [21, 41]. Furthermore, there is a clear relationship between the motor stimulation and an inhibition of the expression of several immediate early genes, including c-*fos*, specifically in the striatopallidal neurons that co-express $A_{2A}$ and $D_2$ receptors [19–21].

The present results together with these previous data suggest the following scheme. Under normal physiological conditions, endogenous adenosine exerts a tonic influence on the striatopallidal neurons, causing an increase in cAMP, an activation of CREB phosphorylation, a basal expression of some immediate early genes such as c-*fos*, as well as an increase in neuronal activity. This tonic influence can be counteracted either by dopamine released from the dopaminergic neurons acting on dopamine $D_2$ receptors or by drugs, such as caffeine, that inhibit adenosine $A_{2A}$ receptors. Dopamine $D_2$ receptor stimulation or adenosine $A_{2A}$ receptor blockade result in demonstrable decreases in immediate early gene expression and a decreased activity of the inhibitory striatopallidal neurons. This latter effect is manifested as a decrease in the release of the inhibitory transmitter γ-aminobutyric acid in the output structure, the globus pallidus [42], and in a parallel increase in the

* Svenningsson P, Lindskog M, Fisone G and Fredholm BB, unpublished data.

expression of such immediate early genes that can be used to monitor neuronal activity in that structure [21, 43]. Thus, *in vivo* as well as *in vitro*, the ability of dopamine to inhibit tonic activation of adenosine $A_{2A}$ receptors may be at least as important as the ability of adenosine to alter the activity of dopamine on $D_2$ receptors. This shift in emphasis of the importance of the reciprocal interactions between the two receptors co-expressed in high abundance on the same neurons makes some physiological sense: the level of the neurotransmitter dopamine at the receptor can vary rapidly in response to appropriate stimulation, whereas the levels of the neuromodulator adenosine are altered much more sluggishly.

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