

Activation of Adenosine A₁ and A_{2A} Receptors Modulates Dopamine D₂ Receptor-Induced Responses in Stably Transfected Human Neuroblastoma Cells

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Abstract: Adenosine can influence dopaminergic neurotransmission in the basal ganglia via postsynaptic interaction between adenosine A_{2A} and dopamine D₂ receptors. We have used a human neuroblastoma cell line (SH-SY5Y) that was found to express constitutively moderate levels of adenosine A₁ and A_{2A} receptors (~100 fmol/mg of protein) to investigate the interactions of A_{2A}/D₂ receptors, at a cellular level. After transfection with human D_{2L} receptor cDNA, SH-SY5Y cells expressed between 500 and 1,100 fmol of D₂ receptors/mg of protein. In membrane preparations, stimulation of adenosine A_{2A} receptors decreased the affinity of dopamine D₂ receptors for dopamine. In intact cells, the calcium concentration elevation induced by KCl treatment was moderate, and dopamine had no effect on either resting intracellular free Ca²⁺ concentration ([Ca²⁺]_i) or KCl-induced responses. In contrast, pretreatment with adenosine deaminase for 2 days dramatically increased the elevation of [Ca²⁺]_i evoked by KCl, which then was totally reversed by dopamine. The effects induced by 48-h adenosine inactivation were mimicked by application of adenosine A₁ antagonists and could not be further reversed by acute activation of either A₁ or A_{2A} receptors. Acute application of the selective A₂ receptor agonist CGS-21680 counteracted the D₂ receptor-induced [Ca²⁺]_i responses. The present study shows that SH-SY5Y cells are endowed with functional adenosine A_{2A} and A₁ receptors and that A_{2A} receptors exert an antagonistic acute effect on dopamine D₂ receptor-mediated functions. In contrast, A₁ receptors induce a tonic modulatory role on these dopamine functions. **Key Words:** Intracellular calcium—Adenosine receptors—Dopamine receptors—Stable transfection—Basal ganglia—Adenosine deaminase.

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1993). Adenosine exerts its effects on neuronal activity via four G protein-coupled receptors: A₁, A_{2A}, A_{2B}, and A₃ (for review, see Fredholm et al., 1994). Although adenosine A₁ and A_{2B} receptors are widely distributed in the brain, A_{2A} receptor distribution is restricted to dopamine-innervated regions such as the striatum, nucleus accumbens, and olfactory tubercle (Alexander and Redington, 1989; Dixon et al., 1996; Ferré et al., 1997). From these anatomical data, it was proposed that dopamine and adenosine receptors may interact in multiple brain functions (for review, see Ferré et al., 1997). In agreement with this hypothesis, large numbers of studies have demonstrated that adenosine can interact with dopaminergic neurotransmission in the basal ganglia. It was suggested that adenosine receptor agonists inhibit, whereas antagonists potentiate, the function of dopamine in this region (Ferré et al., 1992).

Dopamine receptors are subdivided into D₁-like (D₁ and D₅) and D₂-like (D_{2S}, D_{2L}, D₃, and D₄) receptors (for reviews, see Gingrich and Caron, 1993; Cardinaud et al., 1998; Missale et al., 1998). In striatopallidal neurons, the antagonistic adenosine–dopamine interaction seems to be mediated by postsynaptic A_{2A} receptors colocalized with D₂ dopamine receptors (Schiffmann et al., 1991; Ferré et al., 1993a). Behavioral studies have shown that A_{2A} receptor stimulation inhibits D₂-mediated activation in mice and that D₂ receptor stimulation inhibits A_{2A}-

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Abbreviations used: ADA, adenosine deaminase; [Ca²⁺]_i, intracellular free Ca²⁺ concentration; CGS-21680, 2-*p*-(2-carboxyethyl)phenethylamino-5'-*N*-ethylcarboxyamido-adenosine; CPT, 8-cyclopentyl-1,3-dimethylxanthine; DPCPX, 1,3-dipropyl-8-cyclopentylxanthine; R-PIA, *N*⁶-(*R*)-(2-phenylisopropyl)adenosine.

The purine nucleoside adenosine has been proposed to act as a neuromodulator in the CNS (Fredholm et al.,

induced catalepsy in the rat (Ferré et al., 1991a). Biochemical studies have also demonstrated the existence of an antagonistic A_{2A} - D_2 receptor interaction at the level of the membrane, by which the stimulation of the A_{2A} receptor decreases the affinity of D_2 receptors and affects the signal transduction from the D_2 receptor to the G protein (Ferré et al., 1991b, 1993b).

In previous cell cultures, we have shown that A_{2A} can down-regulate D_2 receptor transduction (Yang et al., 1995; Dasgupta et al., 1996) through a decrease in receptor affinity for dopamine agonists. We explore here A_{2A} - D_2 interaction further in the human neuroblastoma cell line SH-SY5Y, which has been reported to express constitutively adenosine A_{2A} and A_1 receptor mRNA (Peterfreund et al., 1996, 1997) and have a very low density of dopamine D_2 receptors (Farooqui, 1994). After transfection with human dopamine D_2 receptor cDNA (D_{2L} isoform), the basal intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$) and dopamine-induced changes to $[Ca^{2+}]_i$ were measured on cells maintained in a culture medium containing or lacking adenosine. Using specific adenosine receptor antagonists, the present study demonstrates the existence of a dual modulation exerted by adenosine on dopamine D_2 receptor function: a short-term A_{2A} receptor-mediated effect and a tonic A_1 receptor-mediated effect.

MATERIALS AND METHODS

Cell cultures and transfection

Cells from the the human neuroblastoma cell line SH-SY5Y were cotransfected with human dopamine D_{2L} receptor cDNA, which was subcloned into the expression vector Plxsn (a gift from C. Owman) using the calcium phosphate precipitation method (described in detail by Dasgupta et al., 1996). D_{2L} receptor expression was verified by northern blot and radioligand binding techniques (see below). SH-SY5Y cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% (vol/vol) heat-inactivated fetal calf serum, penicillin (50 μ g/ml), streptomycin (50 μ g/ml), and 2 mM L-glutamine. For transfected clones, the selector geneticin G-418 (0.6 mg/ml; Amersham) was present in the culture medium, and cells were incubated at 37°C in humidified 5% CO₂/95% air. For measurements of intracellular calcium levels, cells were plated onto glass coverslips at $\sim 10^6$ cells per dish. In some experiments, cells were treated with 10 U/ml adenosine deaminase (ADA; Boehringer Mannheim, Indianapolis, IN, U.S.A.), 10 μ M 8-cyclopentyl-1,3-dimethylxanthine (CPT), or 2 μ M 1,3-dipropyl-8-cyclopentylxanthine (DPCPX; RBI, France) for 48 h. Care was taken to avoid many passages (less than five and 10 passages), which could induce a genetic drift.

Analysis of RNA

Northern blots (20 μ g of total RNA per lane) were denatured in a 2.1 M formaldehyde/50% formamide solution by heating for 2 min at 95°C, then separated by electrophoresis on a 1.0% agarose/2.2 M formaldehyde gel, and finally transferred to a nitrocellulose membrane. Blots were hybridized with ³²P-labeled dopamine D_{2L} receptor cDNA by the nick translation method. Following hybridization the membrane was washed and exposed at -70°C to Kodak XAR-5 film with an intensifying screen.

Membrane preparation

SH-SY5Y cells were harvested with a cell scraper and washed twice by centrifugation with phosphate-buffered saline. The cells were then sonicated (30 min) in ice-cold Tris-HCl buffer (50 mM, pH 7.4) containing 5 IU/ml ADA. The homogenate was centrifuged at 1,800 g for 10 min at 4°C, and the pellet was discarded. The supernatant was preincubated for 30 min at 37°C to activate the ADA and to remove endogenous adenosine and then centrifuged at 40,000 g for 40 min at 4°C. The membrane pellet was then resuspended by sonication in the incubation buffer without ADA (final protein concentration, ~ 0.2 mg/ml). In the experiments with [³H]SCH-23390 and [³H]raclopride, the incubation buffer was Tris-HCl (50 mM, pH 7.4) containing 120 mM NaCl, 5 mM KCl, 2 mM CaCl₂, 1 mM MgCl₂, and 1 mM EDTA. In the experiments with [³H]DPCPX and ³H-labeled 2-*p*-(2-carboxyethyl)phenethylamino-5'-*N*-ethylcarboxyamidoadenosine (CGS-21680), the incubation buffer was Tris-HCl (50 mM, pH 7.4) containing 2 mM and 10 mM MgCl₂, respectively.

Radioligand binding experiments

Saturation experiments with the D_1 receptor antagonist [³H]SCH-23390 were done with 10 concentrations (0.2–6.0 nM) of [³H]SCH-23390 (70.3 Ci/mmol; NEN, Boston, MA, U.S.A.) by incubation for 15 min at 37°C. Nonspecific binding was defined as binding in the presence of 100 mM dopamine. Saturation experiments with [³H]raclopride were done with 10 concentrations (1–35 nM) of [³H]raclopride (80 Ci/mmol; NEN) by incubation for 30 min at room temperature. Nonspecific binding was defined as binding in the presence of 1 mM dopamine. Saturation experiments with the A_1 receptor antagonist [³H]DPCPX were done with 10 concentrations (0.6–27.7 nM) of [³H]DPCPX (120.0 Ci/mmol; NEN) by incubation for 2 h at room temperature. Nonspecific binding was defined as that occurring in the presence of the adenosine A_1 receptor agonist *N*⁶-cyclohexyladenosine (40 mM). Saturation experiments of [³H]CGS-21680 were done with 10 concentrations of the A_{2A} agonist [³H]CGS-21680 (30 Ci/mmol; NEN) by incubation for 90 min at room temperature. Nonspecific binding was defined as binding in the presence of 100 μ M 2-chloroadenosine. Competition experiments with dopamine versus [³H]raclopride (90 Ci/mmol; NEN) were performed by incubation with 20 concentrations (10 pM–1 mM) of dopamine and 2 nM [³H]raclopride for 30 min at room temperature in the presence or absence of the adenosine receptor agonist CGS-21680 (RBI). The incubation was stopped by fast filtration through glass fiber filters (GF/B; Whatman) and washing three times with 5 ml of ice-cold Tris-HCl (50 mM, pH 7.4) using an automatic cell harvester (Brandel). The radioactivity on the filters was measured by liquid scintillation spectrometry. Data from saturation experiments were analyzed by nonlinear regression analysis (GraphPad) for the determination of values for the dissociation constant (K_D) and the number of receptors (B_{max}). Data from competition experiments were analyzed by nonlinear regression analysis for the determination of the K_D values of high- and low-affinity binding sites (K_H and K_L values, respectively) and the proportion of high-affinity binding sites (R_H values). R_H and logarithmically transformed K_H and K_L values were analyzed by Student's paired *t* test. Protein content determinations were performed using bovine serum albumin as the standard.

Intracellular calcium measurements

$[Ca^{2+}]_i$ was measured by dual-emission microspectrofluorometry using indo-1 as the intracellular fluorescent calcium

TABLE 1. Binding parameters from saturation experiments obtained with the dopamine D_1 antagonist [3 H]SCH-23390, the D_2 antagonist [3 H]raclopride, the A_1 antagonist [3 H]DPCPX, and the A_{2A} agonist [3 H]CGS-21680 in membrane preparations from SH-SY5Y cells, both nontransfected (control) and transfected with human dopamine D_{2L} receptor cDNA (clones 16 and 20)

	Control	Clone S16	Clone S20
[3 H]SCH-23390	0	ND	ND
[3 H]Raclopride	23.0 \pm 8.2 (3.0 \pm 1.0)	1,125.3 \pm 60.4 (1.7 \pm 0.2)	525.7 \pm 32.4 (1.5 \pm 0.3)
[3 H]DPCPX	135.2 \pm 13.1 (3.8 \pm 0.7)	164.5 \pm 6.8 (4.2 \pm 0.4)	146.6 \pm 9.0 (2.9 \pm 0.5)
[3 H]CGS-21680	256.4 \pm 34.2 (66.4 \pm 8.3)	213.3 \pm 43.2 (56.3 \pm 19.2)	242.7 \pm 58.3 (72.5 \pm 13.6)

Data are mean \pm SEM values for B_{\max} (in fmol/mg of protein) and, in parentheses, K_D (in nM) from four independent experiments. ND, not determined.

probe (Grynkiewicz et al., 1985). Cultures of SH-SY5Y cells were loaded for 30 min at room temperature with 5 mM indo-1 pentaacetoxymethyl ester (Molecular Probes, Eugene, OR, U.S.A.) in a solution containing 120 mM NaCl, 3 mM KCl, 1.5 mM CaCl_2 , 0.5 mM MgCl_2 , 10 mM glucose, and 10 mM HEPES, adjusted to pH 7.4 (310 mOsm). At the end of the incubation the coverslips were mounted on the recording chamber of an inverted microscope (Nikon Diaphot, Japan) fitted with epifluorescence (40 \times glycerol immersion fluorescence objective) and equipped for microfluorometry (Phocal System, Cambridge, U.K.). The recording medium contained 120 mM NaCl, 3 mM KCl, 5 mM CaCl_2 , 0.5 mM MgCl_2 , 10 mM glucose, and 10 mM HEPES, adjusted to pH 7.4 (310 mOsm). For the assays, excitation light was provided at 355 nm by a 75-W xenon arc lamp. Emitted fluorescence signals were passed through a pinhole diaphragm and, after splitting by a dichroic mirror, were detected simultaneously by two photomultipliers at 405 and 490 nm in a dual-channel photon counting mode. The recording field (a cluster of two to five cells) was delimited by an iris diaphragm positioned along the optical pathway inside the microscope. Quantitative $[\text{Ca}^{2+}]_i$ values were estimated from indo-1 fluorescence by a ratio method and were calculated according to the calcium equation of Grynkiewicz et al. (1985), with a K_D of 250 nM. The baseline was recorded for 3 min. Dopamine (Sigma, France), the dopamine D_2 receptor antagonist sulpiride, the adenosine A_1 receptor agonist N^6 -(R)-(2-phenylisopropyl)adenosine (R -PIA), the adenosine A_1 receptor antagonists CPT and DPCPX, the adenosine A_{2A} receptor agonist CGS-21680 (all from RBI) and ADA (Boehringer, U.S.A.) were prepared in bath solution and directly applied to the recording chamber.

Data were analyzed by one-way ANOVA, and significant group differences at a level of $p < 0.05$ were assessed by Student's t test post hoc comparisons. Results are expressed as mean \pm SEM values.

RESULTS

Saturation experiments with labeled dopamine and adenosine antagonists

Two clones, S16 and S20, showing high dopamine D_{2L} receptor mRNA expression by northern blot (data not shown) were chosen for radioligand binding experiments (Table 1). In nontransfected cells no specific

[3 H]SCH-23390 binding was obtained, and a very low density of [3 H]raclopride binding sites was found, indicating the absence of dopamine D_1 receptors and a low expression of dopamine D_2 receptors in SH-SY5Y cells. In contrast, a high density of [3 H]raclopride binding sites was obtained in the transfected clones, especially in clone S16 (Table 1). [3 H]DPCPX and [3 H]CGS-21680 saturation experiments also demonstrated the existence of a moderate density of both adenosine A_1 and A_{2A} receptors.

Competition experiments on dopamine D_2 receptors

In membrane preparations from clone S16, competitive inhibition experiments of dopamine versus the dopamine D_2 receptor antagonist [3 H]raclopride always showed a better fit for two binding sites rather than one (data not shown; see also Ferré et al., 1991b). Compared with control membrane preparations, the adenosine A_{2A} receptor agonist CGS-21680 increased K_H values by about four times (from 44.7 ± 17.8 to 199.6 ± 32 nM in the absence and presence of 100 nM CGS-21680, respectively; $p < 0.01$; $n = 6$), but K_L (~ 1.75 μM) and R_H ($\sim 40\%$) values showed no significant change.

Treatment with ADA modulates the dopamine-induced responses

In total, 89 cells from clone S16 were recorded in a 5 mM Ca^{2+} -containing saline solution. They showed a basal $[\text{Ca}^{2+}]_i$ level of 121 ± 18 nM, which remained stable during the course of these experiments. Of 55 tested cells, there was no response after bath application of the dopamine D_2 receptor agonist quinpirole (up to 10 μM) or of the adenosine A_{2A} receptor agonist CGS-21680 (up to 10 μM). However, bath application of 50–100 mM KCl triggered a small but consistent increase in $[\text{Ca}^{2+}]_i$ (see Fig. 1A and C). These latter responses were exclusively due to calcium entry because when external calcium was removed and 1 mM EGTA was added, KCl was ineffective ($n = 27$; data not shown). Bath application of dopamine or quinpirole (1 μM), during the KCl treatment, revealed a slight re-

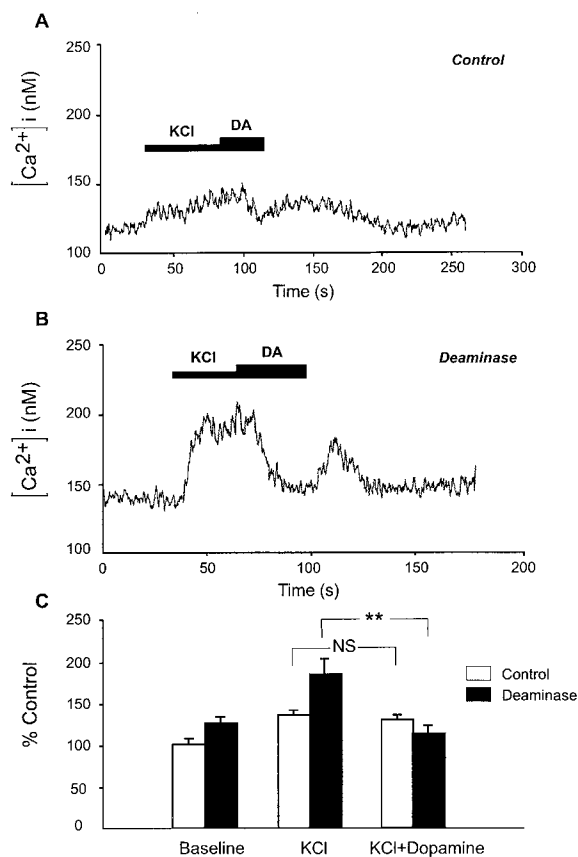


FIG. 1. Effects of ADA on KCl augmentation and dopamine (DA) inhibition of calcium signals in the human neuroblastoma SH-SY5Y cell line stably cotransfected with the long form of the human DA D₂ receptor. [Ca²⁺]_i responses were measured with the spectrofluorometry technique using indo-1-pentaacetoxy-methyl ester. **A:** Under control conditions for cell culture (clone 16), a slight response was evoked by KCl (100 mM) application. **B:** Cells incubated with ADA (10 U/ml) for 48 h responded to application of both KCl and DA (1 μM). **C:** Summary graph shows the percent increase of [Ca²⁺]_i after several treatments. Values are normalized to the resting [Ca²⁺]_i level measured in control conditions. Data are mean ± SEM (bars) values from 18 experiments. Statistical analysis of data was done using the mean comparison test: ***p* < 0.005; NS, nonsignificant.

sponse to dopamine D₂ receptor activation, but this effect was not significant; Ca²⁺ responses of 136 ± 6% with KCl and 129 ± 7% in the presence of KCl plus dopamine when compared with resting [Ca²⁺]_i levels (*n* = 18; Fig. 1C). Similarly, application of CGS-21680 never induced a significant calcium response (*n* = 10; data not shown).

We thus decided to investigate further the reason why the expression of dopamine D₂ receptors was not accompanied by physiological responses. Previous studies in other cell lines have shown that endogenous adenosine, through its action on adenosine A₁ receptors, exerts a tonic inhibitory effect on calcium transport (Zapata et al., 1997). Because responses induced by both KCl and dopamine were small under our standard culture conditions, we hypothesized that activation of adenosine receptors by endogenous adenosine surrounding the cells

could have both decreased the excitability of the cells and their sensitivity to dopamine. Indeed, by reducing the level of adenosine surrounding cultured cells with addition of ADA (10 U/ml) in the culture medium for 48 h, the KCl-triggered [Ca²⁺]_i rise and its reduction induced by dopamine were enhanced. It is also noteworthy that a comparison of resting [Ca²⁺]_i values between nontreated and ADA-treated cells revealed higher values when cells were grown in the absence of adenosine (an increase of 26 ± 8% in nine treated cells with ADA). A typical example of the effects of pretreatment of ADA on [Ca²⁺]_i elevations evoked by application of 100 mM KCl and the dopamine-induced response is shown in Fig. 1B. The magnitude of calcium transients triggered by KCl was 184 ± 20% of control (*n* = 9) after such a pretreatment compared with only 112 ± 10% in the presence of KCl with dopamine (1 μM; *n* = 9; Fig. 1C). Thus, to obtain consistent dopamine-induced responses throughout this study, we used cells systematically pretreated with ADA for 48 h.

Levels of D₂ receptor expression do not correlate with responses induced by D₂ agonists

To investigate the potential relationship between levels of transfected D₂ receptors with calcium responses triggered by D₂ agonists, we compared dopamine effects on the two distinct clones, S16 and S20, that express different amounts of dopamine D₂ receptors (~1,100 and 500 raclopride binding sites/mg of protein, respectively). As shown from an experiment performed with the two clones (Fig. 2A and B), both KCl- and dopamine-induced responses were similar. The increase of [Ca²⁺]_i following application of 100 mM KCl was 99 ± 18 (*n* = 9) and 88 ± 17% (*n* = 7) for clones S16 and S20, respectively (Fig. 2C). Similarly, the magnitude of the inhibition induced by application of 1 μM dopamine was not significantly different between S16 and S20 cells (Fig. 2C): Respective [Ca²⁺]_i levels were 119 ± 8 (*n* = 9) and 112 ± 9% (*n* = 7) of baseline in the presence of KCl plus dopamine. Because there was no difference using S16 or S20, all subsequent calcium experiments were performed on SH-SY5Y cells isolated from clone S16.

Acute transient activation of A₁ or A_{2A} receptors does not counteract the effects of chronic absence of adenosine

To determine if the effects of ADA are secondary to either the acute or chronic removal of the activation of adenosine receptors, we tested whether a 1-min application of the adenosine A₁ receptor agonist *R*-PIA or the adenosine A_{2A} receptor agonist CGS-21680 could restore the effects of adenosine on cells pretreated with ADA. Application of *R*-PIA (10 μM) modified neither basal [Ca²⁺]_i [control, 100 ± 8% (*n* = 8); *R*-PIA, 110 ± 5% (*n* = 8)] nor the KCl-induced [control, 183 ± 20% (*n* = 8); *R*-PIA, 176 ± 11% (*n* = 8)] responses. Similarly, previous application of 1 μM CGS-21680 did not affect responses to KCl or dopamine (Fig. 3). Calcium

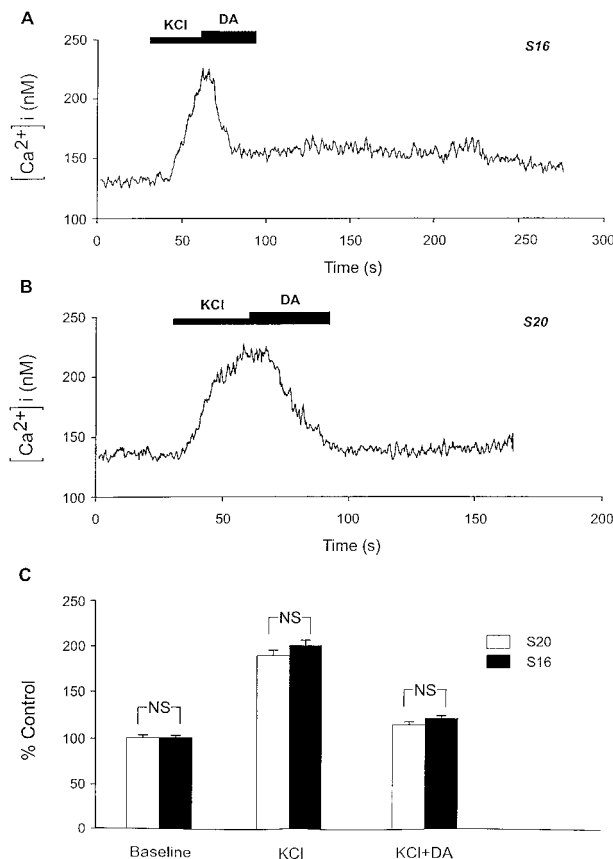


FIG. 2. The level of expression of dopamine (DA) D_2 receptors does not correlate with the amplitude of DA responses. SH-SY5Y cells from the S16 clone (A) or the S20 clone (B) were pretreated with 10 U/ml ADA for 48 h and then subjected to application of KCl (100 mM) and DA (1 μ M). S16 cells contained ~1,100 fmol of D_{2L} /mg of protein; S20 cells contained ~500 fmol of D_{2L} /mg of protein. C: Summary graph shows results expressed as percentages of control values measured from the two clones. Data are mean \pm SEM (bars) values ($n = 9$ experiments). NS, nonsignificant.

concentration rises induced by KCl were 107 ± 12 ($n = 6$) and $99 \pm 11\%$ ($n = 6$) with and without CGS-21680, respectively. Subsequent application of dopamine after KCl treatment brought the $[Ca^{2+}]_i$ levels to 130 ± 6 ($n = 6$) and $121 \pm 14\%$ ($n = 6$) of baseline in the presence and the absence of CGS-21680, respectively (Fig. 3B). Thus, from these experiments, it can be concluded that effects induced by adenosine removal during 48 h cannot be counteracted by an acute 1-min activation of either A_1 or A_{2A} receptors.

Chronic blockade of adenosine A_1 receptors mimics the results obtained with ADA

Evidence that endogenous adenosine is chronically acting at the A_1 receptor was provided by the observation that pretreatment of cells during a 48-h period with the adenosine A_1 antagonist CPT gave similar effects to those obtained with ADA (Fig. 4). The present experiments ($n = 10$) were carried out with a culture medium

in which ADA was changed by CPT (10 μ M). Resting calcium levels were 126 ± 15 nM. These levels rose by 82% (229 ± 22 nM) with KCl, and dopamine was able to reduce significantly the KCl effect (183 ± 13 nM; Fig. 4B). Because at this concentration CPT could act on both A_1 and A_2 receptors, we also have used a more specific A_1 receptor antagonist, DPCPX. It is interesting that DPCPX (2 μ M) caused effects similar to those of CPT (95% increase of resting calcium level induced by KCl and 31% decrease induced by dopamine; $n = 8$).

Acute application of an A_{2A} receptor agonist reverses responses evoked by dopamine

Because the brief presence of an agonist of A_{2A} receptors before dopamine treatment did not reduce D_2 -mediated responses, we studied the effect of A_{2A} receptor activation during dopamine-induced responses. A typical example of such an experiment is depicted in Fig. 5A. SH-SY5Y cells were first subjected to depolarization evoked by bath application of KCl (100 mM), and then dopamine reversed the KCl-induced calcium rise. It is interesting that bath application of CGS-21680 (100 nM–1 μ M) during continued exposure to dopamine was able to reverse the effects evoked by activation of D_2

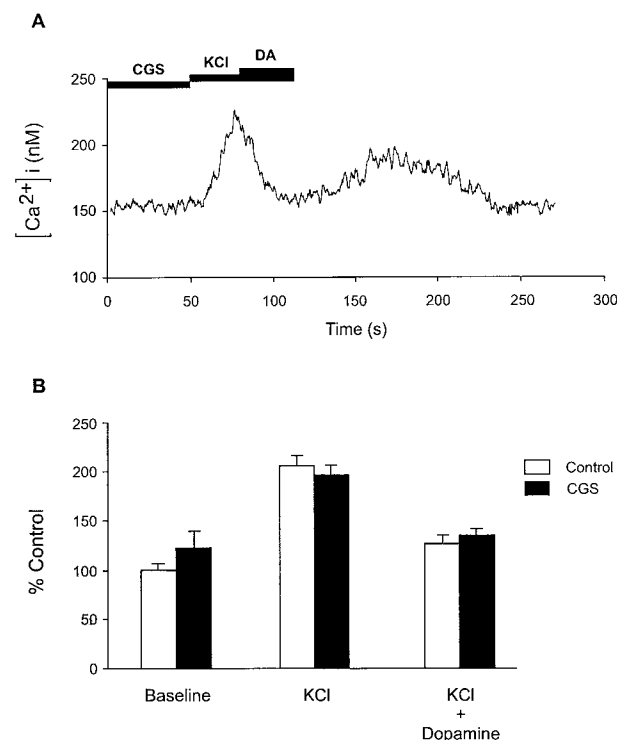


FIG. 3. Effect of the adenosine A_{2A} receptor agonist CGS-21680 (CGS; 1 μ M) pretreatment on $[Ca^{2+}]_i$ response evoked by KCl depolarization and dopamine (DA) inhibition in the S16 clone. Cells were pretreated with 10 U/ml ADA for 48 h. A: A representative experiment shows the time course of $[Ca^{2+}]_i$ level; the presence of CGS for 1 min changes neither the response to the KCl depolarization nor the DA inhibition. B: Summary graph of the percent increase of $[Ca^{2+}]_i$. Data are mean \pm SEM (bars) values ($n = 6$).

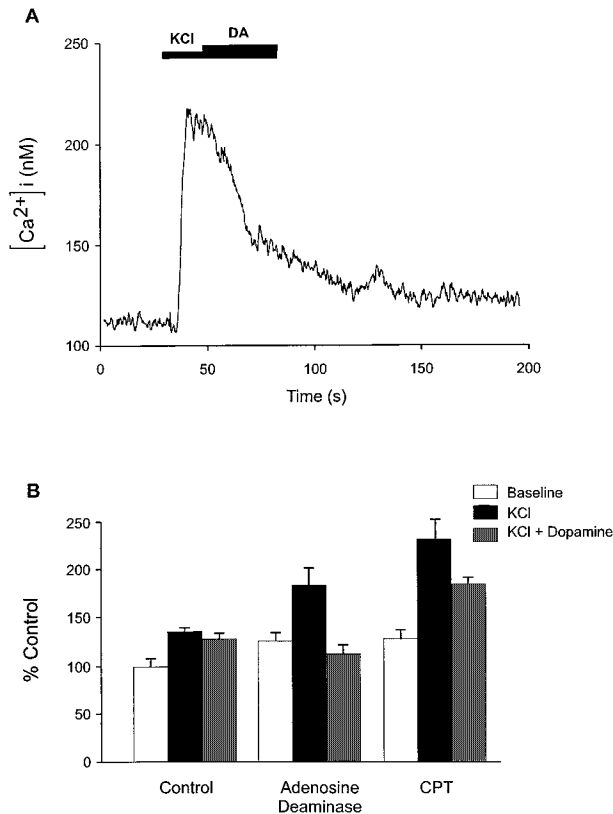


FIG. 4. Pretreatment with the selective adenosine A_1 receptor antagonist CPT mimics effects induced by ADA. SH-SY5Y cells (S16 clone) were incubated with CPT ($10 \mu\text{M}$) for 48 h and then subjected to KCl and dopamine (DA) applications. **A:** This representative experiment illustrates the responsiveness of CPT-pretreated cells to the bath application of KCl (100 mM) and DA ($1 \mu\text{M}$). **B:** Results expressed as percentages of control values. Data are mean \pm SEM (bars) values ($n = 10$ experiments).

receptors. From the summary graph illustrated in Fig. 5B, it can be seen that CGS-21680 did not change the responses to KCl but partially antagonized the action of dopamine: With KCl plus dopamine, calcium levels were $165 \pm 14\%$ ($n = 5$) and $112 \pm 5\%$ ($n = 5$) in CGS-21680-treated and untreated cells, respectively. As proposed earlier, this latter observation favors the existence of an antagonistic interaction between adenosine A_{2A} and dopamine D_2 receptors in the control of $[\text{Ca}^{2+}]_i$ levels. To evaluate other possible mechanisms independent of a direct A_2 - D_2 interaction, we studied possible effects of CGS-21680 on basal cytoplasmic calcium levels and on KCl-evoked responses. The cells were incubated with ADA (10 U/ml) during 48 h, and application of $1 \mu\text{M}$ CGS-21680 did not modify the resting $[\text{Ca}^{2+}]_i$ or the $[\text{Ca}^{2+}]_i$ rise evoked by 100 mM KCl ($n = 8$; data not shown). This observation demonstrates that the reversal of dopamine responses induced by CGS-21680 occurs by activation of adenosine A_{2A} receptors interacting with D_2 receptors rather than a direct action on calcium mobilization.

Activation of D_2 receptors mediates the effects of dopamine

Because the SH-SY5Y cells were transfected with D_2 receptors, all effects of dopamine may be mediated by activation of these receptors. In this respect, when $1 \mu\text{M}$ sulpiride (a specific D_2 receptor antagonist) was simultaneously applied with KCl, dopamine was ineffective in reducing calcium concentration rises (Fig. 6). This result confirms the participation of dopamine D_2 receptors in the modulation of $[\text{Ca}^{2+}]_i$ levels and demonstrates that adenosine receptors were interacting specifically with dopamine D_2 receptors.

DISCUSSION

These experiments were originally conducted to examine the mechanisms underlying the interactions between adenosine A_{2A} receptors and dopamine D_{2L} receptors in a cell type used as a model for the study of the human basal ganglia. Our results demonstrate that adenosine A_{2A} and A_1 receptors can exert an antagonistic

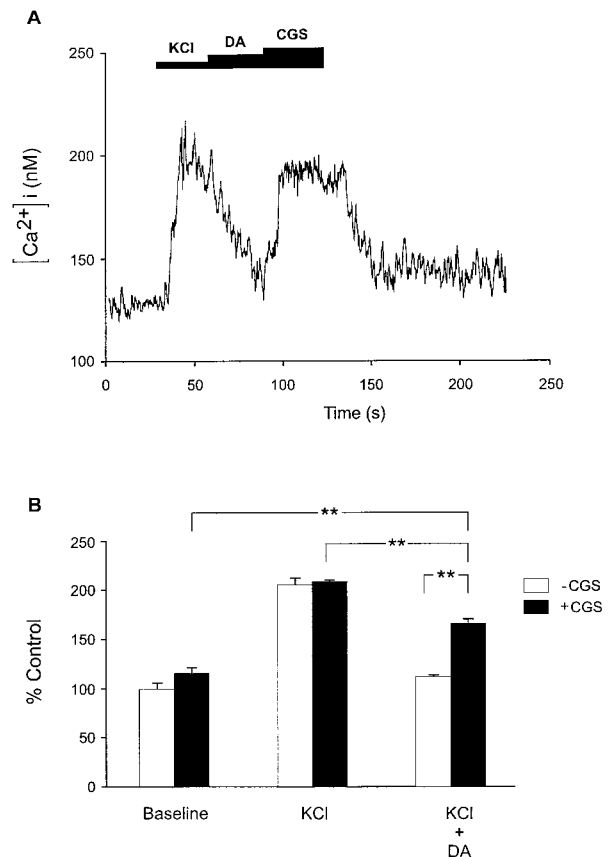


FIG. 5. Effects of the adenosine A_{2A} receptor agonist CGS-21680 (CGS) on the $[\text{Ca}^{2+}]_i$ reduction evoked by dopamine (DA). SH-SY5Y cells were incubated with 10 U/ml ADA for 48 h. **A:** Application of CGS ($1 \mu\text{M}$) immediately reverses the DA-evoked response. **B:** Summary graph shows the percent change in relation to control values. Data are mean \pm SEM (bars) values ($n = 8$ experiments). The antagonistic action of CGS is highly significant: $**p < 0.005$.

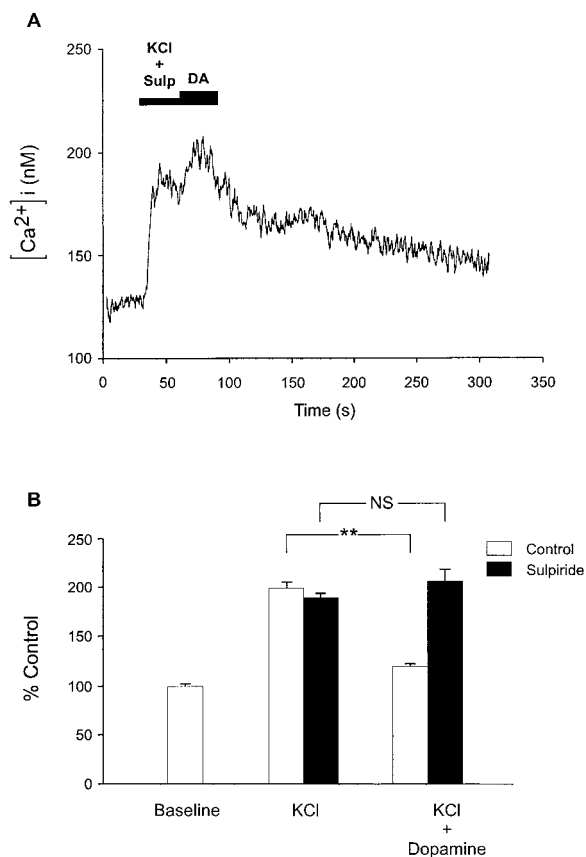


FIG. 6. Dopamine (DA) D_2 receptors mediate the effect induced by DA. **A:** A representative single experiment illustrates the time course of the $[Ca^{2+}]_i$ level. The augmentation evoked by application of KCl (100 mM) was not reduced by subsequent DA (1 μ M) application in the presence of a D_2 receptor antagonist, sulpiride (Sulp; 1 μ M). **B:** Results expressed as percentages of control values. Data are mean \pm SEM (bars) values ($n = 7$ experiments). $**p < 0.01$; NS, nonsignificant.

acute and tonic modulatory role on dopamine D_2 receptor-mediated functions, respectively. Activation of adenosine A_{2A} receptors, as previously described in other models, antagonistically modulates the binding and functional characteristics of dopamine D_2 receptors (Ferré et al., 1991b, 1992, 1993b, 1994; Pollack and Fink, 1995; Yang et al., 1995; Dasgupta et al., 1996; Latini et al., 1996; Mayfield et al., 1996; Lepiku et al., 1997; Fuxe et al., 1998; Rimondini et al., 1998). Activation of adenosine A_1 receptors could down-regulate D_2 receptor signal transduction and/or the effectors modulated by activation of D_2 receptors, i.e., voltage-gated ionic channels controlling cell excitability. These results shed new light on possible interactions between adenosine and dopamine in the basal ganglia because the same kind of effects might well take place in the striopallidal neuron, which, in fact, contains the three different receptors here studied.

In undifferentiated SH-SY5Y cells, pharmacological evidence for the presence of both L- and N-type calcium channels has been provided (Reeve et al., 1994). These

channels are probably the ones involved in the $[Ca^{2+}]_i$ rise evoked by our application of KCl (50–100 mM). Treatment of cell cultures with ADA revealed that the chronic presence of adenosine down-regulates the effect of KCl, suggesting a reduction in the depolarization of the membrane potential (through a reduction in potassium conductances) and/or a direct reduction in calcium conductances. This effect evoked by endogenous adenosine was revealed either using a free adenosine-containing medium or by adding specific A_1 receptor antagonists. Further work is underway to elucidate the specific mechanisms mediated by the chronic activation of A_1 receptors, which results in a down-regulation of the activity of voltage-gated ionic channels involved in the excitability of SH-SY5Y cells.

Of particular interest for the adenosine A_{2A} receptor–dopamine D_2 receptor interaction are the results obtained with acute activation of adenosine A_{2A} responses. The acute application of the specific A_2 receptor agonist CGS-21680 did not exert any significant effect on resting $[Ca^{2+}]_i$ levels, but it counteracted the $[Ca^{2+}]_i$ reduction evoked by activation of D_2 receptors. This is in agreement with our findings reported from a cotransfected mouse fibroblast cell line (Yang et al., 1995) in which activation of A_{2A} receptors did not directly control $[Ca^{2+}]_i$ signals. Although we cannot exclude that the high level of D_2 receptors expressed by transfected cells could have led to changes in the interactions relative to striatal membranes, it is noteworthy that the A_{2A} – D_2 interaction we report here was similar to the one seen in striatal membranes (see Ferré et al., 1991b). The rapid interaction found after acute activation of A_{2A} receptors with D_2 receptors may involve a reduction of the D_2 receptor coupling as indicated from GTP analogue experiments reported in a previous study (Ferré et al., 1993b). There is evidence that the binding characteristics of one G protein-coupled receptor can be altered by the stimulation of another type of G protein-coupled receptor in crude membrane preparations (see Zoli et al., 1993). These intramembrane interactions have been postulated to be direct interactions between the receptor molecules or to involve G proteins or other mobile molecules associated with the membrane (Zoli et al., 1993). In particular, the stimulation of A_{2A} receptors decreases the affinity of the D_2 receptor for the agonist and does not change the affinity for antagonists (Ferré et al., 1991b). The present study shows that the same kind of intramembrane interaction could occur between human adenosine A_{2A} and human dopamine D_{2L} receptors, although other possible mechanisms such as intracellular second messenger cascades should not be discarded.

The chronic effect of A_1 receptor activation was revealed by pretreatment of cell culture with ADA or a specific A_1 receptor antagonist for 48 h. It is well documented that neurotransmitters alter calcium concentration after a short-term exposure (Dolphin, 1990; Anwyl, 1991; Stefani et al., 1996; Zapata et al., 1997), but little is known about chronic modulation. In the present model, the chronic activation of A_1 receptors by endog-

enous adenosine was shown to maintain cells in a quiescent state because both KCl and dopamine were, under these conditions, ineffective in mediating calcium responses. This could represent an aspect of the chronic mechanism by which adenosine reduces excitability of neurons. It needs to be clarified whether the present short- and long-term adenosine effects reflect the involvement of different receptor pools or, alternatively, whether they result from multidirectional effector pathways from the same receptor pool.

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