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A_{2a}/D_2 RECEPTOR INTERACTIONS ARE NOT OBSERVED IN COS-7 CELLS TRANSIENTLY TRANSFECTED WITH DOPAMINE D_2 AND ADENOSINE A_{2a} RECEPTOR cDNA

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Abstract—The rat D_2 receptor and the dog A_{2a} receptor subcloned into the pXM vector were transiently transfected into COS-7 cells using the DEAE-dextran method. The transfected cells expressed approx. 200 fmol D_2 receptors/mg protein and approx. 5 pmol/mg protein of the A_{2a} receptor as judged by binding experiments with [^{3}H]raclopride [or [^{3}H]-*N*-propyl-apomorphine (NPA)] and [^{3}H]-CGS 21680, respectively. The high affinity K_D values were 0.43 and 19 nM for D_2 and A_{2a} receptors, respectively, in agreement with results obtained from other cells and tissues. The non-selective adenosine receptor agonist NECA stimulated cAMP accumulation both in non-transfected and transfected COS-7 cells with only a slight difference in potency, suggesting that most of the stimulation is due to activation of A_{2b} receptors known to be present on virtually every cell. The two A_{2a} selective agonists CGS 21680 and CV-1808 were essentially inactive in transfected COS-7 cells, but were very active in PC-12 cells known to possess functional A_{2a} receptors. Dopamine did not decrease cAMP accumulation in the transfected COS-7 cells. CGS 21680 (30 nM) did not affect the binding characteristics of D_2 receptors in the co-transfected COS-7 cells in contrast to the increased K_H, K_L and R_H values found previously in rat striatal membranes after CGS 21680 treatment. The present findings indicate that transiently transfected A_{2a} and D_2 receptors in COS-7 cells have normal binding properties, but couple poorly to adenylyl cyclase, despite the presence of G_s protein and adenylyl cyclase in these cells. Our results also demonstrate that the previously reported interactions between A_{2a} receptors and D_2 receptors do not occur when only the receptor proteins are expressed in COS-7 cells, suggesting that the two receptor molecules do not interact directly to influence binding characteristics.

Key words: D_2 receptor; A_{2a} receptor; cAMP; COS-7 cells; co-transfection; receptor–receptor interaction; PC-12 cells

Adenosine A_{2a} receptors [1] are co-localized with DA‖ D_2 receptors [2] in a subgroup of medium-sized spiny neurons in the rat striatum [3, 4]. In a series of articles we have obtained evidence for the existence of antagonistic intramembrane A_{2a}/D_2 receptor interactions in the neostriatum [5–7]. Studies on rat striatal membrane preparations demonstrate that activation of A_{2a} receptors reduces the affinity of high and low affinity D_2 receptors [7]. Based on these and other results it has been postulated that the neuroleptic-like actions of adenosine agonists and the psychostimulant actions of caffeine are related to this antagonistic receptor–receptor interaction [6].

The mechanism underlying the A_{2a}/D_2 receptor interaction is unknown, but several possibilities exist [6]. The simplest explanation is that the receptors themselves interact via a dimerization process [8, 9]. Evidence for such a direct interaction between two receptors of the G-protein coupled type has been obtained using co-transfection in COS-7 cells [10]. The present *in vitro* experiments therefore aimed to examine if a similar direct interaction between A_{2a} and D_2 receptors occurs. To this purpose COS-7 cells were transiently transfected with dopamine D_2 and adenosine A_{2a} receptors. The binding characteristics of the D_2 receptors were studied in competition and saturation experiments, using the D_2 antagonist [^{3}H]raclopride [11] and the D_2 agonist [^{3}H]-NPA as radioligands, respectively [5], and the binding characteristics of the A_{2a} receptors were analysed in saturation experiments using the A_{2a} agonist radioligand [^{3}H]-CGS 21680 [12, 13]. Despite a high density of both D_2 and A_{2a} receptors with a high affinity for their respective radioligands, the adenosine A_2 agonist CGS 21680 failed to modulate the binding characteristics of the D_2 receptors.

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‖ Abbreviations: ADA, adenosine deaminase; DMEM, Dulbecco's modified Eagle's medium; FCS, fetal calf serum; Gpp(NH)p, guanyl-imidophosphate; NECA, 5′-*N*-ethylcarboxamido adenosine; NPA, *N*-propyl-apomorphine; PCA, perchloric acid; DEAE-dextran, diethyl-aminoethyl-dextran; DA, dopamine; CHO, Chinese hamster ovary.

MATERIALS AND METHODS

Transfection of D_2 and A_{2a} receptors into COS-7 cells. The full-length cDNAs coding for the rat short type dopamine D_2 receptor [14] and for the RDC8 dog A_{2a} receptor [15, 16] were subcloned into the plasmid vector pXM [17]. These constructs were transfected into COS-7 cells by the DEAE dextran-chloroquine method [17]. Plasmid DNA (30 μg) was used for each 100 mm plate. A plasmid expressing a β-galactosidase gene (pCH110, Pharmacia, Sweden) was transfected in parallel dishes, and β-galactosidase activity was measured in cytoplasmic extracts as a control for transfection efficiency. Cells were harvested approx. 65 hr after transfection. The culture medium, DMEM containing 4.5 mg/mL of glucose, was supplemented with 10% FCS, 100 U/mL of penicillin, 0.1 mg/mL of streptomycin and 2 mM L-glutamine. (Gibco BRL, Life Technologies, Paisley, U.K.).

Membrane preparation. Cells were removed from culture dishes with a rubber policeman and washed twice in PBS. They were then resuspended in 1 mL of ice-cold homogenization buffer (50 mM Tris, pH 7.4, 7.5 mM $MgCl_2$, 5 mM EDTA) and disrupted by sonication 4 × 10 sec at 1 min intervals at 4°. Unbroken cells and nuclei were sedimented at 1000 *g* for 10 min and discarded. Plasma membranes and the cytosolic fraction were then separated by centrifugation at 30,000 *g* for 60 min. The membrane pellet was resuspended in assay buffer with 3–5 U/mL of ADA (Boehringer Mannheim, Germany) and incubated for 60 min to remove endogenous adenosine.

Binding experiments with dopamine versus [^{3}H]-raclopride. The experiments with [^{3}H]raclopride (72 Ci/mmol; NEN Research Products, Boston, MA, U.S.A.) were performed using 20 concentrations of (50 nM–1 mM) of DA. The binding buffer contained 50 mM Tris pH 7.4, 5 mM $MgCl_2$, 1 mM EDTA and 0.01% L-ascorbic acid [7]. The membranes were incubated for 30 min with 3 nM [^{3}H]raclopride at room temperature in the presence or absence of the A_2 receptor agonist CGS 21680 (30 nM) or the GTP analog Gpp(NH)p (10–100 μM) (Boehringer Mannheim). The incubation was stopped by washing the membranes three times with ice cold binding buffer over Whatman GF/B filters (Millipore). The radioactivity content of the filters was detected by liquid scintillation spectroscopy.

Binding experiments with [^{3}H]-NPA. The experiments with [^{3}H]-NPA (71 Ci/mmol; NEN Research Products) were performed with 10 concentrations of the radioligand (0.5–20 nM) and nonspecific binding was defined as binding in the presence of 1 μM raclopride. The buffer contained 15 mM Tris buffer, 1 mM EDTA, and 0.01% ascorbic acid (for details, see Ref. 5). The membranes were incubated for 30 min at room temperature in the presence or absence of CGS 21680 (30 nM). Filtration and radioactivity determination were performed as described above.

Binding experiments with [^{3}H]CGS 21680. The binding buffer contained 50 mM Tris, pH 7.4 and 2 mM $MgCl_2$. Aliquots (100 μL = 40–50 μg of protein) containing 3 U/mL of ADA were added to wells in a 96 well micro-titre plate together with increasing concentrations of [^{3}H]CGS 21680 (7 points, 0.8–50 nM) to a final volume of 0.3 mL. Nonspecific binding was defined as that occurring in the presence of 100 μM 2-chloroadenosine. Binding was performed at 25° for 2 hr and terminated by filtration over glass fibre filters. Filtration was performed with a Skatron 1719 cell harvester (Skatron AS, Lier, Norway), and the filters were further washed with 5 mL ice-cold assay buffer. Filters were then transferred to scintillation vials (Skatron AS) and after the addition of 3 mL scintillation fluid (OptiPhase "HiSafe" II, LKB, Sweden) counted in a β-counter (1209 Rackbeta, LKB, Sweden). Protein determinations were performed with the Bio-Rad protein assay using BSA as a standard.

Culture of PC12 cells. PC12 cells were cultured in DMEM with 4.5 g glucose/mL, supplemented with 10% horse serum, 7.5% fetal calf serum, 2 mM L-glutamine and 0.1 mg/mL penicillin/streptomycin at 37° in a humidified 5% CO_2 95% air incubator. The cells were recultivated two to five times per week. Twenty-four hours before the experiments the cells were recultivated at a cell density of 0.1×10^6/mL. The cells were washed twice in HEPES-buffered DMEM before incubation with drugs. In all incubations the phosphodiesterase inhibitor rolipram was present (30 μM). After 15 min incubation with the indicated drugs, cAMP was extracted with PCA (final concentration 0.4 M). The protein-free supernatant, obtained by centrifugation, was neutralized with 4 M KOH/1 M Tris buffer.

Determination of cAMP in cultured cells. After scraping the cells off the tissue culture dish with a rubber policeman, they were washed twice with PBS and resuspended in DMEM supplemented with 20 mM HEPES, pH 7.4, and 0.1% BSA. Aliquots (0.2 mL = 0.175×10^6 cells) were transferred to test tubes and the indicated drugs, together with 30 μM of the phosphodiesterase inhibitor rolipram, added to a final volume of 0.3 mL. In some experiments 10 μM forskolin was added to stimulate cAMP accumulation. Reactions were terminated after 10 min incubation at 37° by the addition of 50 μL PCA to a final concentration of 0.1 M. Samples were neutralized with 60 μL KOH and the cAMP content in the supernatants determined with a protein binding assay as described [18].

Data analysis. Data from the competition experiments were analysed by the LIGAND program [19]. Specific binding and affinity for high and low affinity binding states, K_H and K_L respectively, were calculated by non-linear iterative least square regression for each curve separately, together with the proportion of receptors in the high affinity state, R_H. The data from the saturation experiments were analysed by non-linear regression of the raw data for the determinations of the K_D and B_{max} values. Linear fitting of Scatchard plots gave similar results.

RESULTS

As shown in Table 1, competition experiments with DA versus [^{3}H]raclopride on COS-7 cells co-transfected with D_2 and A_{2a} receptors demonstrate

Table 1. Effects of CGS 21680 on the ability of DA to compete for [^{3}H]raclopride binding sites in COS-7 membrane preparations from D_2 and A_{2a} receptor co-transfected cells

Binding parameters	Control	CGS 21680 30 nM
K_H(nM)	135 ± 32	120 ± 39
K_L(nM)	7860 ± 4147	5919 ± 1893
R_H(%)	70 ± 7	72 ± 9

The competitive inhibition experiments in the presence and absence of the adenosine A_2 agonist CGS 21680 (30 nM) were performed on membranes prepared from COS-7 cells co-transfected with A_{2a} and D_2 receptor cDNAs.

Means ± SEM are shown for the DA high and low affinity dissociation constants of [^{3}H]raclopride binding sites (K_H and K_L, respectively) as well as for the proportion of D_2 binding sites in the high affinity state (R_H). N = 7 experiments.

Table 2. Effects of CGS 21680 on the binding characteristics of [^{3}H]-NPA in COS-7 membrane preparations from A_{2a} and D_2 receptor co-transfected cells

Binding parameters	Control	CGS 21680 30 nM
K_D (pM)	430 ± 71	436 ± 68
B_{max}(fmol/mg)	188 ± 26	200 ± 30

Saturation experiments were performed in the absence or presence of CGS 21680 (30 nM). The [^{3}H]-NPA concentrations ranged from 0.05 to 2 nM. Means ± SEM are shown. N = 9 experiments.

the existence of both high and low affinity binding sites in the transfected D_2 receptors. Most of the sites are in the high affinity state. In the membranes from the COS-7 cells transfected with both A_{2a} and D_2 receptors the A_2 agonist CGS 21680, at a concentration of 30 nM, an optimal concentration for modulation of D_2 receptors in striatal membranes from rat brain [5], failed to modulate the binding characteristics of the D_2 receptors as shown from the K_H, K_L and R_H values (Table 1).

Gpp(NH)p in concentrations of 10–100 μM failed to reduce the proportion of D_2 receptors in the high affinity state (data not shown). Also with [^{3}H]-NPA used as the ligand, membrane preparations from the COS-7 cells co-transfected with A_{2a} and D_2 receptors showed a high density of high affinity D_2 receptor agonist binding sites (Table 2). However, CGS 21680 at a concentration of 30 nM, was unable to alter the B_{max} value or the K_D value of the high affinity [^{3}H]-NPA binding sites.

In A_{2a} and D_2 receptor cotransfected COS-7 cells, a very high density of high affinity binding sites for [^{3}H]-CGS 21680 could also be demonstrated. The binding was best described by a single site model with a K_D value of 19 ± 8 nM and a B_{max} value of 4985 ± 913 fmol/mg (means ± SD of three separate experiments). The pseudo-Hill slope was close to unity. In two experiments, 10 μM dopamine added to the binding buffer had no effect on the K_D or B_{max} values observed (data not shown). No binding of CGS 21680 was found in untransfected cells.

Untransfected COS-7 cells responded to NECA (above 1 μM) with a concentration-dependent increase in cAMP accumulation. The rather low potency of the agonist suggests that the response is due to activation of A_{2b} receptors, known to be present on virtually every cell [1].

In COS-7 cells co-transfected with D_2 and A_{2a} receptors NECA produced a concentration-dependent increase in cAMP accumulation. In contrast CGS 21680, which is selective for A_{2a} receptors, did not cause more than a minimal increase in cAMP accumulation. Another A_{2a} receptor selective agonist, CV 1808, was similarly ineffective (Fig. 1A). The results with the transfected COS-7 cells were thus rather similar to those obtained with the non-transfected COS-7 cells (Fig. 1B). However, at 0.3 μM there was a significant effect of NECA in the transfected but not the non-transfected cells. These results differed markedly from those obtained in PC-12 cells, which are known to possess high amounts of A_{2a} receptors [20] and showed marked increases in cAMP accumulation in response to low concentrations of CGS 21680 and CV1808 (Fig. 1C). In cells cotransfected with D_2 and A_{2a} receptor cDNAs, DA (10 μM) failed to inhibit cAMP formation in the absence or presence of forskolin. (Basal: 14.5 ± 0.8 without; 35.2 ± 1.2 with DA pmol/10^6 cells; forskolin: 80.3 ± 2.2 without; 128.7 ± 0.2 with DA) DA similarly did not significantly reduce production induced by NECA (91.3 ± 1.8 without DA; 86.9 ± 2.1 with DA).

DISCUSSION

The present results demonstrate that in COS-7 cells transiently co-transfected with DA D_2 and adenosine A_{2a} receptor cDNAs, a high expression of D_2 and A_{2a} receptor proteins can be demonstrated in a binding assay. Thus, as demonstrated in the experiments with the radioligands [^{3}H]raclopride and [^{3}H]-NPA, these cells show high densities of D_2 receptor protein. Using the radioligand [^{3}H]-CGS 21680 high densities of high affinity A_{2a} receptors could also be demonstrated in membrane preparations from the co-transfected COS-7 cells. The receptor proteins were probably incorporated in the cell membrane, since the binding assay was carried out on membranes, but the possibility that at least some of the receptors were located on intracellular membranes [21] cannot be discounted.

Despite this there was no evidence for any interaction between A_{2a} and D_2 receptors. This means that the interaction previously observed in membranes from the rat striatum [5, 7] may not be due only to a direct interaction between the receptor proteins (between muscarinic and adrenergic receptors in COS-7 cell membranes in an earlier report [10]).

The transiently transfected D_2 receptor showed both high and low affinity binding sites for DA in agreement with the results on striatal membrane [7]. However, the D_2 receptors appeared to be poorly

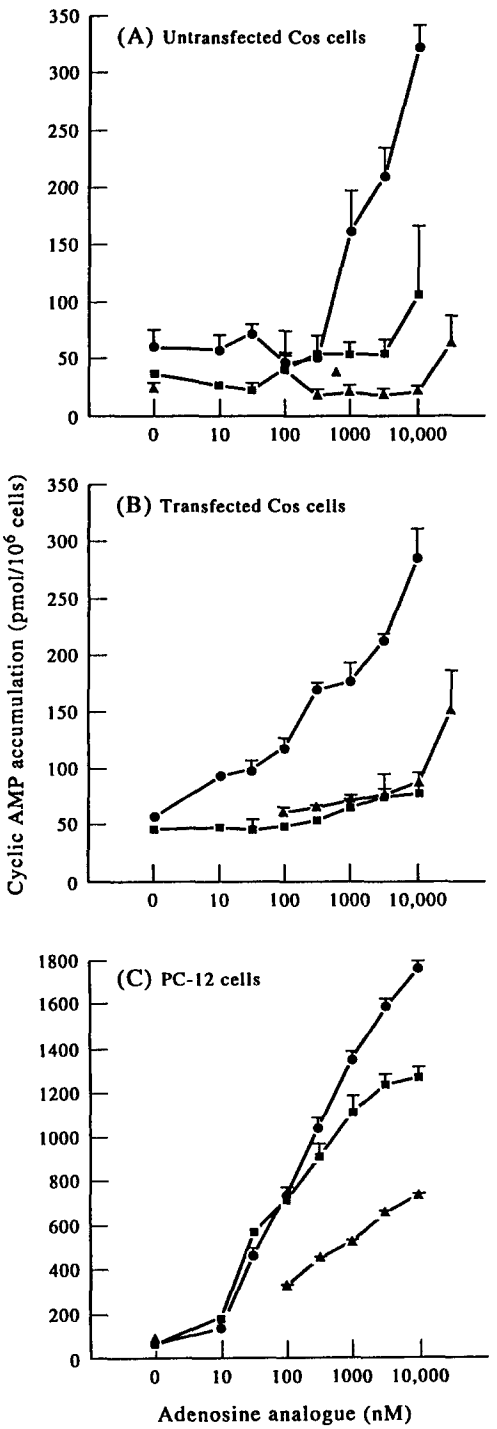

Fig. 1. Effect of NECA (circle), CGS 21680 (square), and CV 1808 (triangle) on cAMP accumulation in untransfected (B) or co-transfected COS-7 cells (A), and in PC-12 cells (C). Means ± SEM (N = 4).

coupled to their G-proteins, since the GTP-analog did not reduce the proportion of D_2 receptors in the high affinity state. Thus, a high affinity state of the receptor that is not sensitive to GTP analog may have been formed [22]. Furthermore, DA did not produce an inhibition of cAMP accumulation, despite its well known inhibitory coupling to adenylate cyclase in brain [23]. This apparent failure is probably not due to the form of the D_2 receptor used, since if anything the short isoform has been reported to be more strongly coupled to adenylyl cyclase than the large isoform [24]. The finding that if anything DA stimulated cAMP production could be due to the fact that COS-7 cells possess a small number of G_s coupled β-adrenoceptors [25] that are stimulated, albeit weakly, by dopamine [26].

The A_{2a} receptors also appeared to be abnormally coupled in the membranes from the COS-7 cells co-transfected with A_{2a} and D_2 receptor cDNAs, since CGS 21680 produced only a minor increase in cAMP formation in these preparations. The latter finding is particularly intriguing, since the untransfected COS-7 cells do possess the machinery to respond to adenosine derivatives with an increase in cAMP. Thus, the cells had already responded before transfection to the non-selective adenosine receptor agonist NECA with an increase in cAMP (Fig. 1B), demonstrating that both G_s and adenylyl cyclase are present. The fact that the cells responded with a cAMP rise to forskolin also supports the presence of adenylyl cyclase. The potency of NECA indicates that the effect is due to the presence of A_{2b} receptors on the untransfected cells [27]. Lower concentrations of NECA (0.5 μM) were ineffective in untransfected cells, but showed a significant response in the transfected cells. This indicates that at least a proportion of the transfected A_{2a} receptors did in fact couple to the signalling machinery, since NECA is more potent on A_{2a} than on A_{2b} receptors [27]. The poor activity of CGS 21680 could be due to the fact that the dog A_{2a} receptor (RDC 8) was used. At the dog A_{2a} receptor CGS 21680 appears to be a weaker agonist than at human or rat A_{2a} receptors, judging from studies on stably expressed receptors in CHO cells (Fuxe, Ahlberg, Owman, Kull & Fredholm, unpublished observations).

The present results therefore suggest that A_{2a} and D_2 receptors do not interact directly at the protein level to alter D_2 receptor binding. Additional membrane components seem to be required to allow an interaction between the two receptors to take place.

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