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The Human D_2 Dopamine Receptor Synergizes with the A_{2A} Adenosine Receptor to Stimulate Adenylyl Cyclase in PC12 Cells

Oliver Kudlacek[1], Herwig Just[1], Vladimir M Korkhov[1], Nina Vartian[1], Markus Klinger[1], Halyna Pankevych[1], Qiong Yang[1], Christian Nanoff[1], Michael Freissmuth*,[1] and Stefan Boehm[1]

[1]*Institute of Pharmacology, University of Vienna, A-1090 Wien, Austria*

The adenosine A_{2A} receptor and the dopamine D_2 receptor are prototypically coupled to G_s and G_i/G_o, respectively. In striatal intermediate spiny neurons, these receptors are colocalized in dendritic spines and act as mutual antagonists. This antagonism has been proposed to occur at the level of the receptors or of receptor–G protein coupling. We tested this model in PC12 cells which endogenously express A_{2A} receptors. The human D_2 receptor was introduced into PC12 cells by stable transfection. A_{2A}-agonist-mediated inhibition of D_2 agonist binding was absent in PC12 cell membranes but present in HEK293 cells transfected as a control. However, in the resulting PC12 cell lines, the action of the D_2 agonist quinpirole depended on the expression level of the D_2 receptor: at low and high receptor levels, the A_{2A}-agonist-induced elevation of cAMP was enhanced and inhibited, respectively. Forskolin-stimulated cAMP formation was invariably inhibited by quinpirole. The effects of quinpirole were abolished by pretreatment with pertussis toxin. A_{2A}-receptor-mediated cAMP formation was inhibited by other G_i/G_o-coupled receptors that were either endogenously present (P_{2Y12}-like receptor for ADP) or stably expressed after transfection (A_1 adenosine, metabotropic glutamate receptor-7A). Similarly, voltage activated Ca^{2+} channels were inhibited by the endogenous P_{2Y} receptor and by the heterologously expressed A_1 receptor but not by the D_2 receptor. These data indicate functional segregation of signaling components. Our observations are thus compatible with the proposed model that D_2 and A_{2A} receptors are closely associated, but they highlight the fact that this interaction can also support synergism.

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INTRODUCTION

In the central nervous system, adenosine and dopamine act as neuromodulators that bind to G-protein-coupled receptors and impinge on fast synaptic transmission by inhibitory and excitatory amino acids. Both the G_s/G_{olf}-coupled A_{2A} adenosine receptor and the G_i/G_o-coupled D_2 dopamine receptor are enriched in the corpus striatum and in mesolimbic areas, such as the nucleus accumbens and the lateral septum (Svenningsson *et al*, 1999). In these brain regions, the two receptors are mostly colocalized, and various types of interactions between A_{2A} and D_2 receptor ligands reveal a functional link between these two proteins: (i) Blockage of D_2 receptors (eg by neuroleptic drugs or by targeted knockout of the gene) or depletion of dopamine impairs locomotion, and this dysfunction can be ameliorated by the administration of A_{2A} antagonists (see Aoyama *et al*, 2000; Grondin *et al*, 1999; earlier references in Richardson *et al*, 1997). (ii) Activation of A_{2A} receptors induces catalepsy (Ferré *et al*, 1991a) as do D_2 antagonists. Conversely, catalepsy induced by blockage of D_2 receptors is reversed by A_{2A} antagonists (Hauber *et al*, 2001). (iii) The induction of the immediate-early gene c-fos by D_2 antagonists is attenuated by blockage of A_{2A} receptors; this effect is confined to neurons where the receptors are coexpressed (Pinna *et al*, 1999). Taken together, these results indicate that there is a functional antagonism between A_{2A} and D_2 receptors (Ferré *et al*, 1997). In line with these experimental data, both retrospective epidemiological evidence (Fredholm *et al*, 1999; Benedetti *et al*, 2000 and references therein) as well as prospective cohort studies (Ross *et al*, 2000; Ascherio *et al*, 2001) indicate that blockage of A_{2A} receptors by caffeine intake reduces the risk of people to develop Parkinson's disease. Similarly, the adenosine uptake inhibitor dipyridamol enhances the antipsychotic activity of the D_2 antagonist haloperidol

*Correspondence: Dr M Freissmuth, Institute of Pharmacology, University of Vienna, Waehringer Str.13A, A-1090 Wien, Austria, Tel: +43 1 4277 64171, Fax: +43 1 4277 9641,
E-mail: michael.freissmuth@univie.ac.at
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(Akhondzadeh *et al*, 2000). Thus, A_{2A} receptor ligands have been recognized as novel therapeutic agents for illnesses typically treated with D_2 receptor ligands.

The functional antagonism between D_2 and A_{2A} receptors has been proposed to reflect the ability of the A_{2A} receptor to impair high-affinity agonist binding to the D_2 receptor by a membrane-delimited process. This effect was originally observed in rat striatal membranes (Ferré *et al*, 1991b), and subsequently also documented upon heterologous expression of the receptors in fibroblast cell lines (Ltk-murine fibroblasts in Dasgupta *et al*, 1996; Chinese hamster ovary cells in Kull *et al*, 1999). Agonist occupancy of the A_{2A} receptor blunted high-affinity agonist binding to the D_2 receptor in the absence of GTP; hence, the effect was proposed to be accounted for by an interaction at the level of the receptors or of receptor–G protein complexes. While this model explains the antagonistic action of the A_{2A} adenosine receptor, several observations, however, cannot be accounted for by simple reciprocal antagonism at the level of these two receptors. Most importantly, mice lacking A_{2A} receptors display a hypodopaminergic phenotype (Dassesse *et al*, 2001); similarly, stimulation of the striatal A_{2A} receptor fails to elevate cAMP in mice that are deficient in D_2 receptors (Zahniser *et al*, 2000). These findings may reflect adaptive changes that occur during development to cope with the targeted deletion of the respective genes. Alternatively, they may indicate that the interaction between the A_{2A} receptor and the D_2 receptor is not solely mutually antagonistic. In the present work, we employed the neuroendocrine cell line PC12 to examine effector regulation by the combined stimulation of the A_{2A} adenosine and D_2 dopamine receptor. PC12 cells can be differentiated into a neuronal phenotype by nerve growth factor. Our results demonstrate that D_2 receptors can synergize with A_{2A} receptors to stimulate cAMP accumulation, a property that is not shared by other G_i/G_o-coupled receptors.

MATERIAL AND METHODS

Materials

PC12 cells were obtained from the European Collection of Cell Cultures (ECACC; Salisbury, UK). Collagen was from Biomedical Technologies Inc. (Stoughton, MA, USA). Human β-nerve growth factor (NGF) was from R&D Systems Inc. (Wiesbaden, Germany). Adenosine deaminase was from Roche Molecular Biochemicals (Mannheim, Germany). Optimem medium, L-glutamine, penicillin G, streptomycin, horse serum, fetal calf serum (FCS), and G418 (geneticin) were from Life Technologies (Grand Island, NY). Centrifuge tubes and tissue culture plates were from Greiner (Vienna, Austria) and from Corning Costar (Acton, MA). [^{3}H]adenine, [^{3}H]CCPA (CCPA, 2-chloro-CPA), [^{3}H]ZM241385 (ZM241385, 4-(2-[7-amino-2-(2-furyl)[1,2,3]triazolo[2,3-*a*] [1,3,5]triazin-5-ylamino]ethyl)phenol), and [^{125}I]OH-PIPAT (OH-PIPAT, (Rs)-*trans*-8-hydroxy-2-[*N*-propyl-*N*-(3′-iodo-2′-propenyl) amino]tetralin) were from New England Nuclear (Boston, MA), and [^{125}I]epidepride was from the Austrian Research Centre (Seibersdorf, Austria). Forskolin, RO201724 (DL-4-(3-butoxy-4-methoxybenzoyl)-2-imidazolodione), xanthine amine congener (XAC), haloperidol, and pertussis toxin were purchased from Sigma Chemical Co. (St Louis, MO), and CGS21680 (2-[*p*-(2-carboxyethyl)phenethylamino]-5′-*N*-ethylcarboxamidoadenosine) from Tocris (Bristol, UK). Affinity-purified rabbit antiserum against rap1/Krev-1 was from Santa Cruz Biotechnology (Santa Cruz, CA). Immunofluorescence was performed with anti-c-myc antibody (Santa Cruz Biotechnology), anti-FLAG antibody M2 (Stratagene, La Jolla, CA), anti-HA antibody 16B12 (kind gift of E Ogris, Vienna Biocenter), Cy3-coupled goat anti-rabbit (Amersham, Buckinghamshire, UK), and FITC-coupled goat anti-mouse (Jackson, West Grove, PA).

Cell Culture and Transfection

PC12 cells were plated onto collagen-coated culture dishes (Biomedical Technologies Inc., Stoughton, MA, USA), propagated in Opti-Mem medium containing 10% (vol/vol) horse serum, 5% (vol/vol) FCS, 0.2 mM L-glutamine, 25 000 U/l penicillin G, and 25 mg/l streptomycin. Media for culture of stably transfected cells were supplemented with 0.2 mg/ml geneticin (G418) in order to maintain the selection pressure. Transfection of PC12 cells and of HEK293 was performed using lipofection (TransFast®, Promega, Madison, WI) and $CaPO_4$-precipitation, respectively. The plasmids employed encoded the wild type and epitope-tagged versions of the human D_2 dopamine (Bofill-Cardona *et al*, 2000), the FLAG-tagged metabotropic glutamate receptor-7A (mGluR7A) (ElFar *et al*, 2001), the c-myc-tagged human A_{2A} adenosine receptor (Klinger *et al*, 2002), and the HA-tagged A_1 adenosine receptor; the latter was generated by PCR using a primer that extended the N-terminus by the nine amino acids (YPYDVPDYA) that represent the major epitope of influenza hemaglutinin. The integrity of the coding sequence was verified by fluorescent sequencing. After 48 h, geneticin (G418; 0.8 mg/ml) was added to the media to initiate selection for stably transfected cells. Receptor expression was checked by binding ([^{3}H]CCPA for the A_1 receptor; [^{125}I]epidepride for the D_2 receptor) or by immunofluorescence (in particular mGluR7A because a suitable radioligand is not available). Nontransfected PC12 cells did not contain detectable amounts of A_1 and D_2 receptors (detection limit <10 fmol/mg) and did not respond to any of the receptor agonists (A_1 = CPA or R-PIA, D_2 = quinpirole, mGluR7A = L-AP4) employed. (CPA, N6-cyclopentyladenosine;L-AP4 = L-2-amino-4-phosphonobutanoic acid.)

Prior to the recording of I_{Ca}, PC12 cells were detached from culture dishes and replated at low density. In order to induce neuronal differentiation, PC12 cells were exposed to recombinant human NGF (50 ng/ml) for 5–6 days.

Membrane Preparation and Receptor Binding

Cells were harvested and membranes were prepared as previously described (Waldhoer *et al*, 1998). The final membrane pellet was resuspended in buffer (25 mM Hepes.NaOH, pH 7.5, 1 mM EDTA, 2 mM $MgCl_2$) at a protein concentration of 8–10 mg/ml and stored in aliquots at -80°C. Binding of [^{3}H]CCPA was carried out in 80 µl buffer (25 mM Tris-HCl, pH 8, 1 mM EDTA, 5 mM $MgCl_2$)

containing 8 mg/ml adenosine deaminase and cell membranes (8–10 μg/assay). Nonspecific binding was determined in the presence of 10 μM CPA. Binding of [^{3}H]ZM241385 and of [^{125}I]epidepride was done in 200 μl and 40 μl of the same buffer, respectively, containing cell membranes (60–70 μg and 8–10 μg/assay, respectively); nonspecific binding was determined in the presence of 10 μM XAC (A_{2A} antagonist binding) or of 3 μM sulpiride (D_2 antagonist binding). For binding of [^{125}I]OH-PIPAT, the assay volume was 50 μl and the membrane protein concentration was 15–20 μg/assay. Nonspecific binding was determined in the presence of 5 μM haloperidol. All binding experiments were done at 25°C for 90 min and stopped by filtration over glass fiber filters.

Accumulation of cAMP

The adenine nucleotide pool of PC12 cells was metabolically prelabeled for 16 h with [^{3}H]adenine (1 μCi/well) in Optimem containing 1% (vol/vol) horse serum and 1 U/ml adenosine deaminase as outlined previously (Kudlacek *et al*, 2001). After the preincubation, fresh medium was added that contained adenosine deaminase (1 U/ml) and 100 μM RO201724; 30 min later, cAMP formation was stimulated by the A_{2A} selective agonist CGS21680 (1 μM, if not otherwise indicated) or by 25 μM forskolin for 15 min in the absence and presence of agonists for the G_i/G_o-coupled receptors (quinpirole, ADPβS, ADP, CPA, L-AP4; see figures for concentrations). Assays were performed in triplicate. The formation of [^{3}H]cAMP was determined according to Salomon (1991).

Pull-Down Assay for the Determination of Rap1-Activation

The GST fusion protein comprising the rap1-binding domain of ralGDS (ral-RBD, Franke *et al*, 1997) was expressed in *E. coli* (strain BL21DE3) and used to trap GTP-loaded rap1 as described (Seidel *et al*, 1999). Cells were prepared for the assay in a similar way as for accumulation of cAMP (serum withdrawal for 16 h in the presence of adenosine deaminase followed by replacement by fresh medium containing adenosine deaminase). Subsequently cells were incubated with CGS21680 or vehicle for 5 min. The amount of recovered GTP-liganded rap1 was quantified by scanning immunoblots using a BioRad GelDoc1000 and Molecular Analyst software.

Electrophysiology

Voltage-activated Ca^{2+} currents were determined as described before (Vartian and Boehm, 2001). Prior to the recording of I_{Ca}, PC12 cells were detached from culture dishes and replated at low density. Whole-cell currents were recorded at room temperature (20–24°C) from PC12 cells 24–48 h after replating at low density, using an Axopatch 200B amplifier and the Pclamp 6.0 hard- and software (Axon Instruments, Foster City, CA, USA). Currents were low-pass filtered at 5 kHz, digitized at 50 kHz, and stored on an IBM-compatible computer. Traces were analyzed off-line by the Clampfit program (Axon). Patch electrodes were pulled (Flaming-Brown puller, Sutter Instruments, Novato, CA, USA) from borosilicate glass capillaries (Science Products, Frankfurt/Main, Germany) and filled with a solution consisting of (mM) CsCl (130), tetraethylammonium chloride (20), $CaCl_2$ (0.24), glucose (10), HEPES (10), EGTA (5), Mg-ATP (2), and Li-GTP (2), adjusted to pH 7.3 with KOH, to yield tip resistances of 2–3 MΩ. The external bathing solution consisted of (mM) NaCl (120), tetraethylammonium chloride (20), KCl (3), $MgCl_2$ (2), $CaCl_2$ (5), glucose (20), HEPES (10), adjusted to pH 7.3 with KOH. Drugs were applied via a DAD-12 drug application device (Adams & List, Westbury, NY, USA), which permits a complete exchange of solutions surrounding the cells under investigation within less than 100 ms (see Vartian and Boehm, 2001). To evaluate the effects of receptor agonists, PC12 cells were exposed to these agents for at least 30 s. Unless otherwise indicated, whole-cell Ca^{2+} currents were elicited by 30 ms depolarizations from a holding potential of −80 mV to 0 mV at a frequency of 4 min^{-1}.

I_{Ca} was quantified by measuring peak current amplitudes during the depolarization to 0 mV. To account for time-dependent changes in Ca^{2+} current amplitudes, drug effects were evaluated by evoking currents in the presence of test drugs (*B*) and by comparing them to control currents recorded before (*A*) and after (washout, *C*) the application of the drugs, according to the equations: $200*B/(A+C) = \%$ of control current, or $100-(200*B/[A+C]) = \%$ inhibition (see Vartian and Boehm, 2001). Significance of differences between single data points was evaluted by the Mann–Whitney test.

If not otherwise indicated, all experiments were carried out at least three times and means ± SD are reported.

RESULTS

Heterologous Expression of G Protein-Coupled Receptors in PC12 Cells

The rat pheochromocytoma cell line PC12 can be differentiated to adopt a neuron-like phenotype. In addition, PC12 cells endogenously express A_{2A} adenosine receptors. We therefore used this cell line as a model system to study the interaction of A_{2A} and D_2 receptors. The latter was introduced by stable transfection (PC12-D_2), because we were unable to detect appreciable levels of D_2 receptors in PC12 cell lines from two different sources (data not shown). After stable transfection, cell clones were isolated that expressed D_2 receptors at levels ranging from 20 to 1500 fmol/mg. A representative saturation experiment is shown in Figure 1a, where membranes from a clone that expressed 60 fmol/mg were incubated with the high-affinity D_2 antagonist [^{125}I]epidepride ($K_D = 25 \pm 5$ pM). The estimated B_{max} values covered the range of density of D_2 dopamine receptors labeled by [^{125}I]epidepride in striatal membranes (120–160 fmol/mg; Beindl *et al*, 1996). The expression level of the endogenous A_{2a} adenosine receptor in these cells was determined with an A_{2A}-selective antagonist; a B_{max} of 1.6 ± 0.2 pmol/mg was estimated from saturation isotherms for the binding of [^{3}H]ZM241385 to membranes of PC12-D_2 cells (Figure 1c). This was reasonably similar to the amount of receptors detected in parallel in striatal membranes ($B_{max} = 0.9 \pm 0.1$ pmol/mg; data not shown).

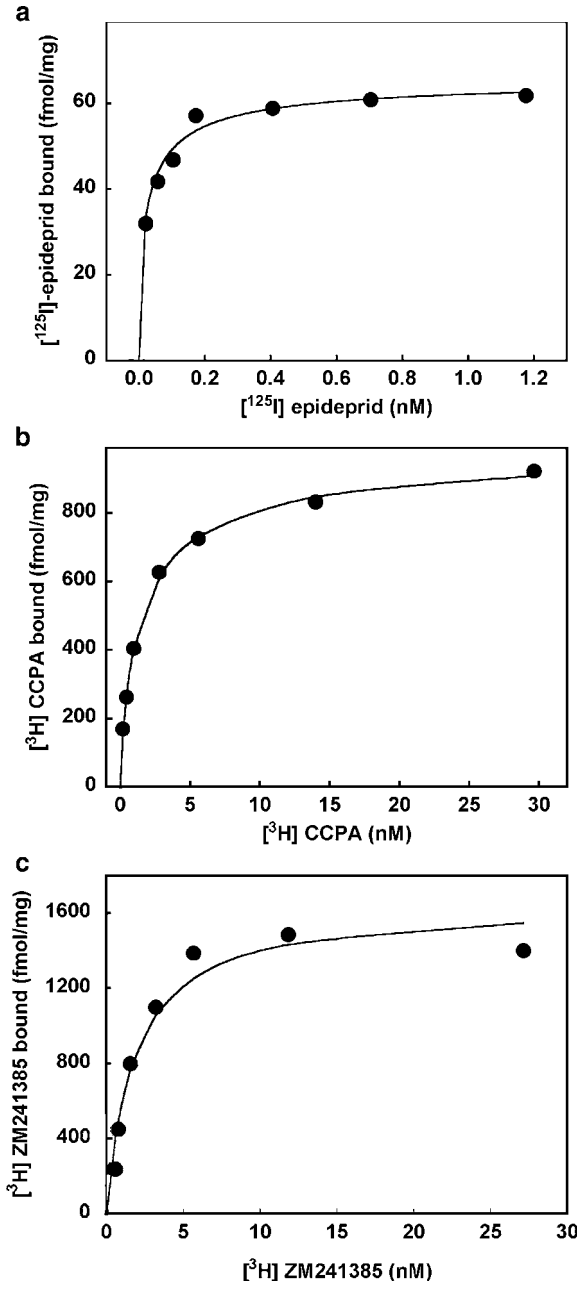

Figure 1 Radioligand binding to membranes from PC12 cells stably transfected with plasmids driving the expression of D_2 dopamine (a, c) and A_1 adenosine (b) receptors. Binding of the D_2 antagonist [^{125}I]epidepride (a) and of the A_1 agonist [^{3}H]CCPA (b) was determined using membranes (10 μg/assay) prepared from stably transfected cells. Endogenous A_{2A} adenosine receptor expression level was determined with the A_{2A} antagonist [^{3}H]ZM241385 (c) in membranes (70 μg/assay) prepared from stably transfected PC12 cells expressing the D_2 receptor. Assay conditions were as outlined under 'Materials and methods'. Data are from a representative experiment that was carried out in duplicate and reproduced three times.

In order to carry out control experiments (see below), two additional $G_{i/o}$-coupled receptors, namely the A_1 adenosine receptor and the mGluR7A, were expressed in PC 12 cells and stable cell lines were generated. The expression level of the A_1 adenosine receptor was determined by binding of the high-affinity A_1 agonist [^{3}H]CCPA to membranes of PC12 cells; a cell clone was selected that expressed about 1 pmol/mg (Figure 1b); that is, the membrane levels of this receptor was within the range at which this receptor is typically expressed in brain membranes of most species, including man (Jockers *et al*, 1994). Cell lines were also generated that expressed the group III mGluR7A. A suitable radioligand is not available for this receptor. Hence, the expression of this receptor was monitored by staining the cells for membrane-associated immunoreactivity. The rationale for employing mGluR7A is the fact that this receptor is specifically targeted to presynaptic specializations (Shigemoto *et al*, 1996; Stowell and Craig, 1999). Hence, the use of mGluR7A allowed to gauge the capacity of PC12 cells to support a spatial segregation of a receptor by sorting and targeting. Upon NGF-induced differentiation of PC12 cells, mGluR7A was indeed delivered to the tips of the extending neurites (data not shown).

Effect of the A_{2A} Adenosine Receptor Agonist CGS21680 on High-Affinity Binding of D_2 Receptor Agonists and Antagonists to Membranes of PC12 cells Heterologously Expressing the D_2 Dopamine Receptor

Stimulation of A_{2A} adenosine receptors impedes high-affinity agonist binding to the D_2 dopamine receptor by blocking the formation of ternary complexes of agonist, receptor, and G protein; this functional antagonism between A_{2A} receptor and D_2 receptor has been reported for various cell systems including striatal membranes (Ferré *et al*, 1991b), Ltk-murine fibroblasts (Dasgupta *et al*, 1996), and Chinese hamster ovary (CHO) cells (Kull *et al*, 1999). We therefore tested the effects of the A_{2A} agonist CGS21680 on the binding of the D_2 agonist [^{125}I]OH-PIPAT to membranes from the PC12 cell clone in which B_{max} for the antagonist [^{125}I]epidepride amounted to $\sim$60 fmol/mg (Figure 2a). In the absence of CGS21680 (open circles, Figure 2a), [^{125}I]OH-PIPAT bound with high affinity to about 40% of the D_2 dopamine receptors ($B_{max} = 26 \pm 5$ f-fmol/mg) that were labeled by the antagonist (cf Figure 1a). Addition of CGS 21680 (closed circles in Figure 2a) did not affect the B_{max} or the K_D (0.65 ± 0.13 and 0.59 ± 0.11 nM in the absence and presence of CGS21680, respectively) for binding of [^{125}I]OH-PIPAT. These findings differ from the observations reported for transfected fibroblast cell lines (Dasgupta *et al*, 1996; Kull *et al*, 1999), where the A_{2A} agonist impedes ternary complex formation by the agonist-liganded D_2 receptor. We therefore also expressed the A_{2A} adenosine receptor and D_2 dopamine receptor in HEK293 cells, a human fibroblast line. As can be seen from Figure 2c, in membranes prepared from these cells, CGS21680 did cause a loss of high-affinity agonist binding; it is evident that the A_{2A} agonist inhibits binding of [^{125}I]OH-PIPAT in a noncompetitive manner ($K_D = 0.72 \pm 0.07$ and 0.91 ± 0.26nM; $B_{max} = 66 \pm 8$ and 39 ± 6 fmol/mg). This loss of ternary complexes (of agonist, receptor, and G protein) is to be expected if A_{2A} receptor stimulation blunts coupling of [^{125}I]OH-PIPAT-liganded D_2 receptor to the G protein (Dasgupta *et al*, 1996; Kull *et al*, 1999). The A_{2A} agonist does not interfere with ligand binding to the D_2-receptor; in fact, when tested on binding of the antagonist [^{125}I]epidepride, addition of 1 μM CGS21680 did not alter binding ($K_D = 18.9 \pm 4$ pM and 16.5 ± 2 pM in the absence and presence of CGS21680 in paired experiments; data not

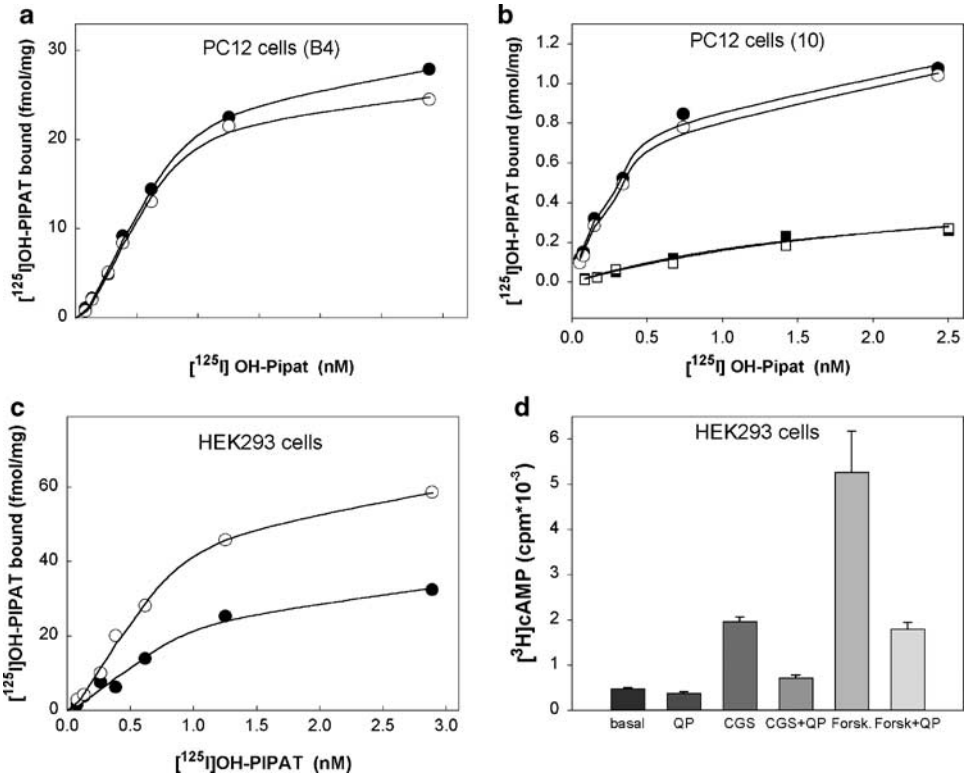

Figure 2 Effects of the A_{2A} adenosine agonist CGS 21680 on D_2 agonist binding to membranes from PC12 cells expressing the D_2 receptor at different levels (a, b) or HEK293 cells (c) expressing the D_2 and the A_{2A} receptor and cAMP accumulation in HEK293 cells (d). Binding of the D_2 agonist [^{125}I]OH-PIPAT was determined in the absence (open circles) and presence (full symbols) of 1 μM CGS21680 or of 100 μM GTPγs (squares in b) using membranes (20–30 μg/assay) prepared from stably transfected PC12 cells (a, b) and HEK293 cells (c). Assay conditions were as outlined under 'Materials and methods'. Data are from representative experiments that were carried out in duplicate and reproduced three times. (d) The adenine nucleotide pool was metabolically labeled by preincubating transfected HEK293 cells with [^{3}H]adenine (1 μCi/ml). Under basal conditions, the cells were maintained in the presence of adenosine deaminase (1 U/ml) and the phosphodiesterase inhibitor RO201724 (100 μM) or stimulated for 15 min with 1 μM CGS21680 (CGS) or 25 μM forskolin (Forsk), 0.1 μM quinpirole (QP), or the combination thereof as indicated.

shown). Finally, it is worth noting that the A_{2A} receptor levels in HEK293 cells were not higher than those observed in PC12 cells ($B_{max} = 0.4$–0.7 pmol/mg; see also Klinger *et al*, 2002). Nevertheless, in PC12 cells the levels of the D_2 receptors may have been too low to be subject to modulation by the A_{2A} agonist. We therefore investigated PC12 cell clones that expressed D_2 receptors at different levels (covering the range of 60 fmol/mg to 1.3 pmol/mg). Figure 2b shows that—even at the highest membrane concentration of D_2 receptor—the A_{2A} agonist did not modulate B_{max} (1.31 ± 0.06 and 1.29 ± 0.05 pM/mg) or the K_D for [^{125}I]OH-PIPAT (0.48 ± 0.05 and 0.53 ± 0.05 nM in the absence and presence of CGS21680, respectively). Finally, we also assessed the ability of the hydrolysis-resistant GTP analogue GTPγS to destabilize the ternary complex in the presence and absence of CGS21680. It is evident that high-affinity agonist binding was suppressed to a similar extent by GTPγS regardless of whether the A_{2A} agonist was present or absent (Figure 2b, open and closed squares). Similar findings were obtained in three other PC12 cell clones (not shown).

Cyclic AMP Formation of PC12 Cells Heterologously Expressing the D_2 Receptor

PC12 cells are notorious for their genetic instability; accordingly, a substantial variability of individual PC12 cell lines has been observed, if individual cell clones were investigated with respect to their complement of A_{2A} adenosine receptor, $G\alpha_s$ and adenylyl cyclase and with respect to A_{2A}-agonist-mediated cAMP increases (Arslan *et al*, 1999). In the clones employed in the present work, the A_{2A} agonist CGS21680 gave a robust stimulation of cAMP levels ($E_{max} = \sim 4$–8-fold over basal levels; $EC_{50} = 25$–50 nM; see Figure 4c). Activation of the D_2 dopamine receptor was expected to inhibit adenylyl cyclase via G_i/G_o-dependent signaling and to thereby blunt the stimulatory action of the A_{2A} agonist. However, if the D_2 agonist quinpirole was added to PC12 cells that expressed D_2 receptors at ~ 60 fmol/mg in the presence of a saturating concentration of CGS21680 (1 μM), the level of cAMP was further augmented by about 30% (circles in Figure 3a). The EC_{50} of quinpirole for this effect was 4.0 ± 1.2 nM. This potentiation was also seen with a second (independent, stably transfected) cell clone that expressed lower levels of D_2 receptor (20 fmol/mg, closed squares in Figure 3b); conversely, quinpirole did not elicit any effect on PC12 cells that did not express the D_2 dopamine receptor (not shown). Alternatively, cAMP accumulation was triggered by direct activation of adenylyl cyclase in the presence of 25 μM forskolin; this concentration resulted in cAMP levels that were reasonably similar to those triggered by saturating CGS21680 (see Figure 4a and b and Kudlacek *et al*, 2001). Addition of quinpirole inhibited the forskolin-stimulated

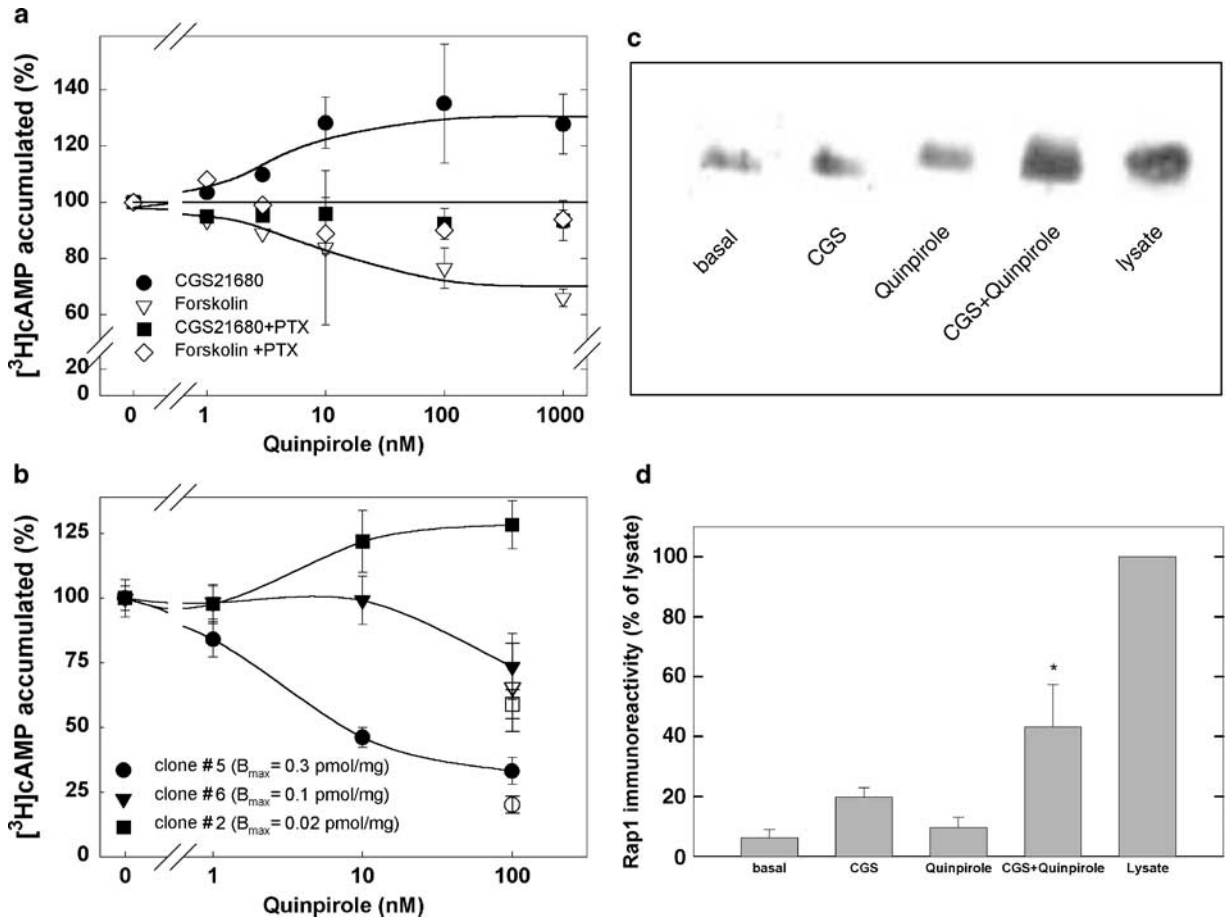

Figure 3 Cyclic AMP accumulation (a, b) and rap1 activation (c, d) by combined stimulation of the A_{2A} and the D_2 receptor in PC12 cells stably expressing the human D_2 receptor. (a) Cells expressing 60 fmol/mg D_2 receptors were maintained for 36 h in medium (circles and triangles) or pretreated for 36 h with 100 ng/ml pertussis toxin (squares and diamonds). In the last 16 h [^{3}H]adenine (1 μCi/ml) was added to metabolically label the adenine nucleotide pool; thereafter, the cells were preincubated for 30 min with RO201724 (100 μM) and adenosine deaminase (1 U/ml) and subsequently stimulated for 15 min with 1 μM CGS21680 (circles and squares) or 25 μM forskolin (triangles and diamonds) and the indicated concentrations of quinpirole. Data are means ± SD from three independent experiments that were performed in triplicate and carried out in parallel. [^{3}H]cAMP accumulation in the absence of quinpirole varied between 8000 and 13 000 cpm in individual experiments and was set at 100%. (b) Assay conditions were the same as in panel a; stably transfected PC12 cells expressing D_2 receptors at ~0.3 (circles), 0.1 (triangles), and 0.02 pmol/mg (squares) were stimulated in the presence of 1 μM CGS12680 (closed symbols) or forskolin (open symbols). (c) PC12 cells were rendered quiescent by serum withdrawal for 16 h in the presence of adenosine deaminase (1 U/ml); 30 min after the addition of fresh medium containing adenosine deaminase (ADA), the cells received vehicle (lane ADA) or were stimulated by 1 μM CGS21680 (CGS), 10 nM quinpirole, or the combination of CGS21680 and quinpirole. GTP-liganded rap1 was trapped by the addition of a fusion protein comprising GST and the rap-binding domain of ral(GDS); 30% aliquots of the material recovered in the pull-down assay were applied to an SDS-polyacrylamide gel, transferred to nitrocellulose and immunoblotted for rap1; in the lane labeled lysate an appropriate amount of the detergent lysate from unstimulated cells was applied to the gel. The bar diagram summarizes the densitometric quantitation ($n = 3$); error bars indicate SD; *$p < 0.05$ quinpirole + CGS21680 *vs* CGS21680 (Student's *t*-test).

cAMP formation by 30–40% (triangles in Figure 3a; open squares in Figure 3b). The EC_{50} (8.7 ± 4.6 nM) was in the same range as that for the stimulating effect. PC12 cells were pretreated with pertussis toxin to block signaling via G_i/G_o. This pretreatment abolished both the ability of quinpirole to enhance cAMP formation in the presence of A_{2A} agonist CGS21680 (squares in Figure 3a) and to reduce forskolin-stimulated cAMP accumulation (diamonds in Figure 3a).

We also examined additional stably transfected PC12 cell clones that expressed higher levels of D_2 receptors, namely ~100 and 300 fmol/mg (as assessed by binding of [^{125}I]epidepride). In PC12 cells carrying higher receptor levels, quinpirole inhibited A_{2A} agonist (closed circles and triangles in Figure 3b) and forskolin-stimulated cAMP accumulation to a roughly equivalent extent (open circles and triangles in Figure 3b). Finally, we also measured the effect of a quinpirole on A_{2A}-agonist-induced cAMP formation in HEK293 cells, in which the level of D_2 receptor expression was reasonably comparable to that seen in PC12 cells (cf Figure 2a and c). In HEK293 cells, stimulation of the D_2 dopamine receptor invariably caused inhibition of A_{2A}-receptor-induced cAMP accumulation. The transfection of HEK293 cells was only transient. However, the transfection efficiency was high (ie substantially >50%); this is also evident from the observation that forskolin-stimulated cAMP accumulation was inhibited by about 75% (right-hand bars in Figure 2d).

Inhibition of cAMP accumulation is the expected response to D_2 dopamine receptors. In the PC12 cell clone that expressed ~60 fmol/mg D_2 receptors, we have there-

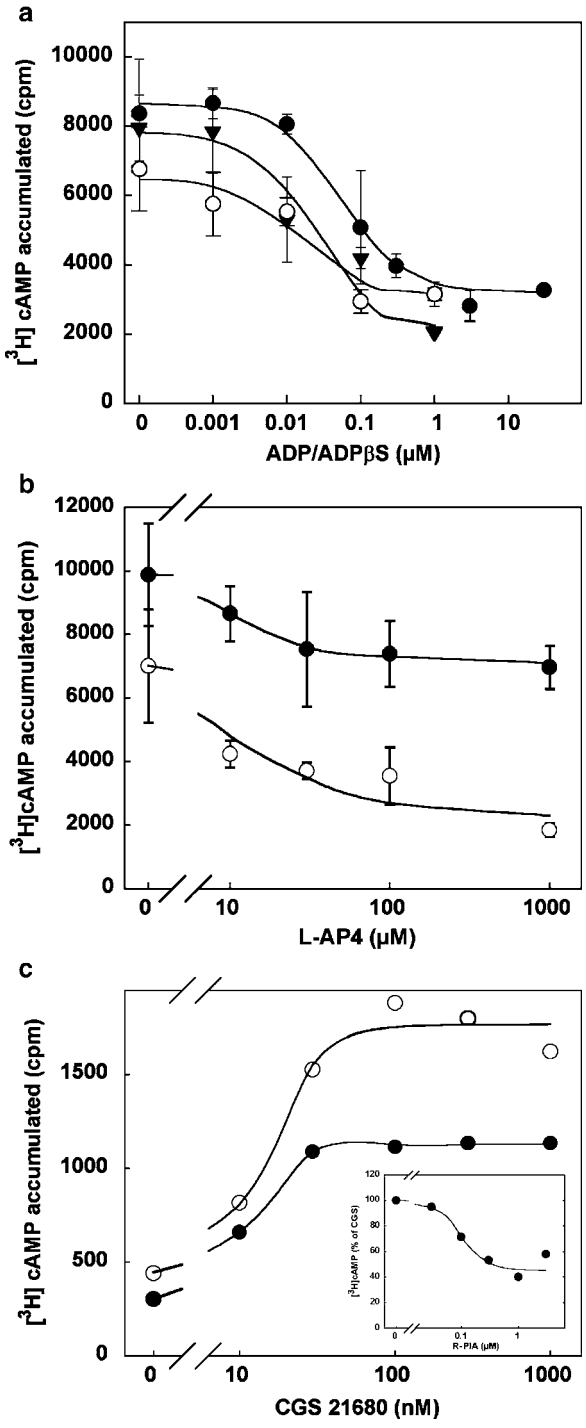

Figure 4 Inhibition of cAMP accumulation in PC12 cells by the endogenously expressed P2y receptor (a), and following stable expression of mGluR7A (b) and of the A_1 adenosine receptor (c). Assay conditions were as outlined in the legend to Figure 3. (a, b) Cells were stimulated with 1 μM CGS21680 (closed symbols) or 25 μM forskolin (open symbols); cAMP accumulation was inhibited by the concomitant addition of the indicated concentrations of ADP (panel a: circles), ADPβS (panel a: triangles) and L-AP4 (panel b: circles). Data are means ± SD ($n = 3$–5). (c) The concentration–response curve for CGS21680 was determined in the absence (open circles) and presence of 20 nM CPA (closed circles); data are from a representative experiment that was reproduced twice. Inset in panel c: Cells were stimulated with 1 μM CGS21680; cAMP accumulation was inhibited by the concomitant addition of the indicated concentrations of R-PIA.

fore employed an additional approach to obtain independent evidence for enhanced cAMP accumulation. This relied on Epac-induced activation of rap1. Epac, the guanine nucleotide exchange factor of the small G protein rap1, is directly controlled by cAMP (de Rooij *et al*, 1998); raising cellular cAMP levels thus leads to exchange of GTP for GDP on rap1 and the resulting GTP-liganded rap1 can be trapped—after cell lysis—by using the Rap-binding domain (RBD) of RalGDS as a bait (Franke *et al*, 1997). As expected, CGS21680 stimulated the accumulation of GTP-loaded rap1 (Figure 3c). In contrast, quinpirole *per se* did not have any appreciable effect (cf control lane *ADA* with lane *quinpirole* in Figure 3c). However, if PC12 cells were challenged with both CGS21680 and quinpirole, GTP-bound rap1 accumulated to levels that significantly exceeded ($p < 0.05$, Student's *t*-test) those seen in the presence of CGS21680 alone (Figure 3c and d). Thus, the synergistic stimulation induced by the D_2 agonist resulted in accumulation of cAMP over a range that was physiologically relevant because it was sensed by an intracellular cAMP-binding effector, namely Epac (de Rooij *et al*, 1998).

Inhibition of cAMP Formation by other G_i/G_o-Coupled Receptors in PC12 Cells

With the exception of the type IX isoform, all membrane-bound forms of mammalian adenylyl cyclase are activated by forskolin, albeit to a variable extent (Sunahara *et al*, 1996; Hanoune and Defer, 2001). The ability of the D_2 receptor to discriminate between forskolin-mediated and A_{2A}-receptor-mediated (ie $G\alpha_s$-mediated) activation of cAMP formation presumably reflects the net effect on enzyme isoforms that are susceptible to inhibition (ie via $G\alpha_i$ and/or $G\alpha_o$ and $G\beta\gamma$) and costimulation (via $G\beta\gamma$). We have tested if other G_i/G_o-coupled receptors also discriminated between forskolin- and A_{2A}-receptor-stimulated cAMP accumulation. PC12 cells endogenously express a P_{2Y} receptor that couples $G\alpha_{i/o}$ and shares many pharmacological characteristics with the $P2y_{12}$ receptor (Unterberger *et al*, 2002). In both, the untransfected parent PC12 cells (Figure 4a) as well as in the PC12 cell lines stably expressing the D_2 receptor (not shown), the physiological agonist ADP and ADPβS, an analog that is more resistant to metabolism (phosphorylation to ATP and dephosphorylation to AMP), inhibited the cAMP accumulation induced by CGS21680 with a potency in the submicromolar range. Forskolin-stimulated activity was inhibited over a similar concentration range of ADPβS and with similar efficacy, that is, by about 40–50%.

The discrepancy between the P2y receptor and the D_2 receptor may have arisen from the difference between endogenous and heterologous expression. We have therefore also checked if the A_{2A}-receptor-dependent cAMP accumulation was enhanced or inhibited by two additional, heterologously expressed G_i/G_o-coupled receptors, namely the A_1 adenosine receptor and the mGluR7A. In stably transfected PC12 cells endowed with mGluR7A, the A_{2A} adenosine receptor- and forskolin-induced cAMP formation was inhibited by the group III-specific metabotropic glutamate receptor agonist L-AP4 (Figure 4b). While the effect was seen over a similar concentration range, the inhibition of forskolin-stimulated cAMP accumulation was

more pronounced than that induced by activation of the A_{2A} adenosine receptor (Figure 4b). Similarly, in PC12 cells expressing the A_1 receptor at levels compatible with the endogenous expression in the CNS (~1 pmol/mg, see Figure 1b), the A_1 agonist CPA employed at the A_1-selective concentration of 20 nM blunted the stimulatory effect of the A_{2A} agonist CGS21680 by about 30–40%; this inhibition was also seen with R-PIA, another A_1-selective agonist, and was uniform over the entire concentration–response curve for CGS21680 (Figure 4c). As expected, A_1-selective agonists displayed biphasic concentration–response curves when tested in the presence of forskolin, namely an A_1-receptor-mediated inhibition of cAMP formation at low concentrations (by about 50%) (data not shown) and an A_{2A} adenosine receptor-dependent stimulation at high concentrations (not shown).

Effects of G_i/G_o-Coupled Receptors on Voltage-Activated Ca^{2+} Currents in PC12 Cells

In neuroendocrine cells G_i/G_o-coupled receptors inhibit voltage-activated Ca^{2+} currents, an effect that can also be reproduced in both differentiated and undifferentiated PC12 cells (Vartian and Boehm, 2001). We therefore investigated if quinpirole inhibited Ca^{2+} currents in PC12 cells that stably expressed the D_2 dopamine receptor. In these transfected cells, the P_{2Y} agonists ADP and ADPβS, which were employed as positive controls, decreased the Ca^{2+} current by 50% (Figure 5a and b). In contrast, quinpirole had little or no effect on the Ca^{2+} current even when applied at the very high concentration of 10 µM (Figure 5a and b). Again, we ruled out that this discrepancy between endogenously present P_{2Y} receptor and heterologously introduced D_2 receptor was accounted for by the forced expression; in PC12 cells that heterologously expressed the A_1 adenosine receptor, the receptor agonist CPA decreased the Ca^{2+} current by 30%, an effect that was totally abolished by addition of the adenosine receptor antagonist XAC at 1 µM (Figure 6a and b). XAC did not affect the ADP-mediated inhibition of Ca^{2+} current in PC12 that carried A_1 adenosine receptors. Similarly, the effect of CPA was absent in cells that did not express the A_1 adenosine receptor (not shown). It is worth pointing out that the discrepancy between the A_1 adenosine receptor and the D_2 dopamine receptor can neither be accounted for by the low expression levels of the D_2 dopamine receptor nor by clonal variation. (i) Even in clone 10, in which D_2 receptor levels clearly exceeded those of the A_1 receptor, there was only a very modest inhibition of Ca^{2+} currents (by some 10% at most; see Figure 5b, right-hand panel). (ii) The response to ADP was not significantly different in the two cell lines (~40–50% inhibition in the presence of ADP, cf Figure 5b, right-hand panel, and Figure 6b). Finally, the deficiency was not restricted to the D_2 receptor. Similar to PC12-D2 cells, upon agonist stimulation, mGluR7A failed to inhibit Ca^{2+} currents (Figure 6c and d) although cAMP accumulation was inhibited (cf Figure 4b).

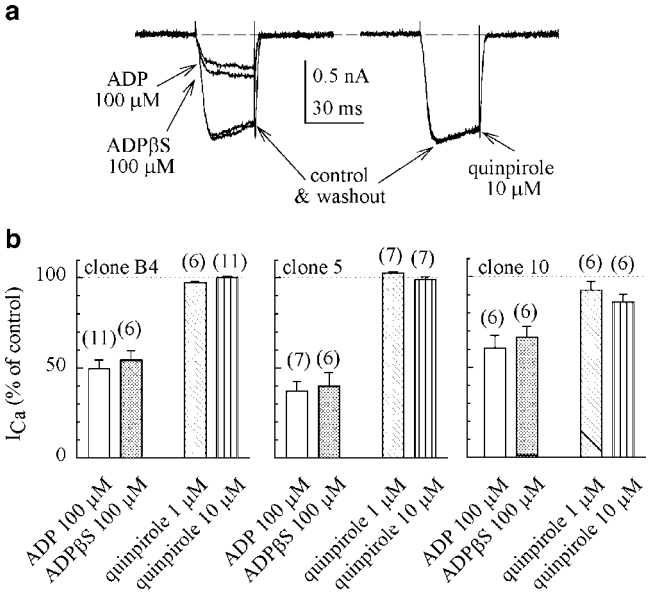

Figure 5 Regulation of voltage-activated Ca^{2+} currents by the endogenously expressed P2y receptor, and following stable expression of the human D_2 dopamine receptor. Differentiated PC12 cells were plated at low density and whole-cell currents were recorded at room temperature (20–24°C); Ca^{2+} currents were elicited by 30 ms depolarizations from a holding potential of −80 to 0 mV before (control), during, and after (washout) application of the indicated receptor ligands. Panel a shows original traces for PC12 cells stably expressing D_2 dopamine receptors (clone B4), as an internal control, the endogenous P2y receptor was activated by ADP and the metabolically stable analog ADPβS. Panel b summarizes the inhibitory effects of ADP, ADPβS, and quinpirole in PC12 cell clones that express the D_2 dopamine receptor at different levels (see Figures 2 and 3). The graphs show peak Ca^{2+} current amplitudes in the presence of these drugs (arithmetic means ± SEM) as a percentage of the amplitudes in their absence. The number of cells investigated is indicated in parentheses.

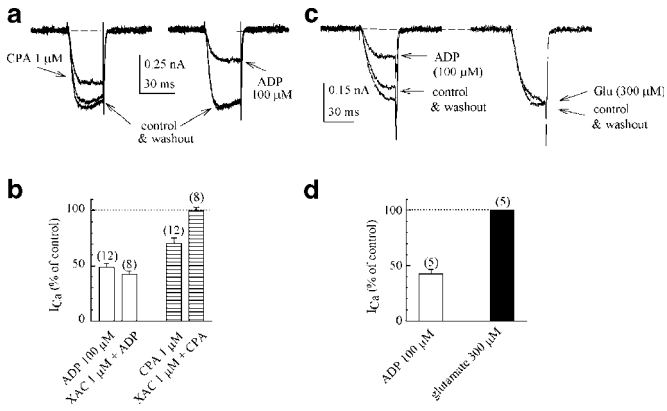

Figure 6 Regulation of voltage-activated Ca^{2+} currents by the endogenously expressed P2y receptor, and following stable expression of the A_1 adenosine receptor (a, b) and the mGLuR7A (c, d). Recording conditions were as in Figure 5. Panels a and c show original traces for PC12 cells heterologously expressing the indicated receptors. Panels b and d summarize the inhibitory effects of ADP, CPA, and glutamate. Panel b also documents the effect of the adenosine receptor antagonist XAC. The graphs show peak Ca^{2+} current amplitudes in the presence of these drugs (arithmetic means ± SEM) as a percentage of the amplitudes in their absence. The number of cells investigated is indicated in parentheses.

DISCUSSION

A specific interaction of the A_{2A} adenosine receptor and the D_2 dopamine receptor has repeatedly been evoked to

account for the mutual antagonism that can be observed in striatopallidal neurons (Lindskog *et al*, 1999). This assumption is based on both observations in membranes prepared from the striatum (Ferré *et al*, 1991b) as well as from transfected cells (Dasgupta *et al*, 1996; Kull *et al*, 1999), in which A_{2A} agonists blunted high-affinity agonist binding to D_2 receptors. However, in PC12 cells, we did not detect any appreciable inhibition of D_2 agonist binding. In contrast, our present experiments demonstrate that A_{2A} and D_2 receptors can also synergize in the regulation of their prototypic common effector, adenylyl cyclase. Thus, antagonism of D_2-receptor-mediated responses by activation of A_{2A} receptor (and *vice versa*) is not a universal phenomenon. In fact, a synergistic activation of cAMP-dependent signaling by A_{2A} and D_2 receptors has recently also been reported in NG108-15 cells that stably expressed the D_2 receptor (Yao *et al*, 2002). Our observations also indicate that the expression level of the D_2 receptor is important in determining whether this receptor pair raises cAMP synergistically. Low levels of D_2 receptors favored coactivation while at higher expression levels the A_{2A}-induced cAMP response was inhibited. The concentration of D_2 receptors in PC12 cell membranes was within the range found in various brain areas; nevertheless, it is not possible to extrapolate these numbers and predict the response of D_2 receptor containing neurons because the local concentration at pre- or postsynaptic specializations is not known. Regardless of these limitations, the physiological relevance of our observations is underscored by the recent study of Yao *et al* (2002); this work also demonstrates that D_2 and A_{2A} receptors synergize to activate cAMP-dependent signaling in rat hippocampal neurons and in the nucleus accumbens. The synergism exerted in the latter region apparently supports sustained alcohol consumption (Yao *et al*, 2002).

Synergistic stimulation of adenylyl cyclase activity is to be expected because most isoforms of adenylyl cyclase have the capacity to function as coincidence detectors (Sunahara *et al*, 1996; Hanoune and Defer, 2001); for example, adenylyl cyclase type-I and to a lesser extent type-VIII are synergistically activated by $G\alpha_s$ and Ca^{2+}/calmodulin (Xia and Storm, 1997). Similarly, the activation of adenylyl cyclase type-II, -IV, and -VII by $G\beta\gamma$ is conditional on the presence of activated $G\alpha_s$ (Taussig *et al*, 1994). In PC12 cells, the potentiating effect of the D_2 agonist (and its inhibitory action) was abrogated upon pretreatment of the cells with pertussis toxin. Thus both stimulation and inhibition depended on the presence of G_i or G_o. Most cells express several adenylyl cyclase isoforms (Hanoune and Defer, 2001). This is true for PC12 cells where the type-VI enzyme is abundant but not the only isoform (Chern *et al*, 1995). Adenylyl cyclase type-VI and -V are inhibited by Ca^{2+}. In reconstitution experiments, they are also inhibited by $G\alpha_i$ subunits, but $G\beta\gamma$ has no appreciable effect (Taussig *et al*, 1994; Kudlacek *et al*, 2001). In contrast, D_2-receptor-mediated stimulation was contingent on the formation of active $G\alpha_s$ by the A_{2A} adenosine receptor; forskolin-induced cAMP accumulation was invariably inhibited by the D_2 receptor. For a parsimonious explanation, the synergism is most readily accounted for by assuming that G protein subunits, that is, GTP-liganded $G\alpha_s$ generated by activation of the A_{2A} receptor and $G\beta\gamma$ generated by activation of the D_2 receptor, converge at the level of adenylyl cyclase. Hence, the likely candidate mechanism that underlies the costimulatory action of the D_2 receptor is $G\beta\gamma$-dependent activation of a type-II-like adenylyl cyclase. In fact, protein fragments that scavenge free $G\beta\gamma$ are capable of eliminating the synergism between D_2 and A_{2A} receptors in both NG108-15 cells and neurons (Yao *et al*, 2002).

At low expression levels, D_2 receptors supported synergistic activation of A_{2A}-receptor-dependent cAMP accumulation; the effect was lost at higher expression levels and a robust inhibition was seen. This observation is consistent with the combined action on a type-II- and a type VI-like adenylyl cyclase isoform. The conditional activation of a type-II-like adenylyl cyclase requires concentrations of $G\beta\gamma$ in the low nanomolar range (Taussig *et al*, 1993). In contrast, inhibition of isoforms V and VI by activated $G\alpha_i$ requires concentrations that are about 10-fold higher (Taussig *et al*, 1994). Thus $G\beta\gamma$ generated at low D_2 receptor concentrations may suffice for sensitization of a type-II-like adenylyl cyclase, whereas at high receptor levels the amounts of active $G\alpha_i$ reach the threshold to support inhibition of the abundant type-VI enzyme in PC12 cells.

In the clones that express high receptor numbers, stimulation of cAMP accumulation was to be expected at low occupancy of the receptor by the D_2 agonist. In some experiments, this was indeed the case. However, it was not seen reproducibly. The limits of detection can be calculated by Monte Carlo simulation by using the parameters obtained at high and low receptor expression, that is, 1.3-fold maximum stimulation and maximum inhibition by 60–70%. The synthetic data were generated with a realistic random error of 5–10%. Under these assumptions, an initial stimulation was only detected reliably if EC_{50} (for stimulation of cAMP accumulation) and IC_{50} (for inhibition of cAMP accumulation) differed by >100-fold (for an analogous analysis, see Freissmuth *et al*, 1987). Thus, if receptor occupancy and effect were directly related, the expression levels would have to be increased 100-fold in order to detect the stimulation at low agonist concentrations (in the presence of the ensuing inhibition at higher receptor occupancy). In other words, inhibition would be reliably detected only if D_2 receptor expression levels were raised to >6 pmol/mg.

The synergism was restricted to the pair A_{2A} receptor/D_2 receptor and not seen if the endogenous P_{2Y} receptor or the transfected A_1 receptor and mGluR7A were activated. Obviously, the A_1 adenosine receptor was expressed at levels that far exceeded those at which synergism was seen with the D_2 dopamine receptor. Contrary to the D_2 receptor, mGluR7A only supported inhibition of cAMP formation. Similarly, when present at 1 pmol/mg, the A_1 receptor only inhibited A_{2A}-dependent cAMP accumulation by $\sim 50\%$. In contrast, when present at 0.3 pmol/mg, the D_2 receptor blunted the A_{2A} response by 70%. The most plausible explanation for these discrepancies is to assume that receptors (and effectors) are segregated rather than freely diffusible and that A_{2A} and D_2 receptors are closely associated. In fact, complexes of these two receptors have been observed that are sufficiently stable to allow for coimmunoprecipitation of D_2 receptors with A_{2A} receptors (Hillion *et al*, 2002).

Taken together, our data are consistent with the concept that the D_2 receptor and the A_{2A} receptor form a unique pair when present within the membrane of a given cell (Ferré *et al*, 1991b; Dasgupta *et al*, 1996; Kull *et al*, 1999). However, the interaction between these two receptors does not necessarily result in mutual antagonism. Finally, the emphasis on adenylyl cyclase regulation and on G protein coupling may detract from alternative signaling pathways. Although A_{2A} receptors do not raise cAMP levels in striata of mice that are deficient in D_2 receptors (Zahniser *et al*, 2000), A_{2A} antagonists still partially restore locomotor activity in these animals. This indicates that neither changes in cAMP nor the presence of D_2 receptors are required to account for their beneficial effect on animal models of Parkinson's disease (Aoyama *et al*, 2000; Zahniser *et al*, 2000; Chen *et al*, 2001). In fact, the A_{2A} receptor has the capacity to signal in a manner independent of $G\alpha_s$ (Sexl *et al*, 1997; Seidel *et al*, 1999).

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