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RAPID COMMUNICATION

Autocrine activation of adenosine A_1 receptors blocks D_{1A} but not D_{1B} dopamine receptor desensitization

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Abstract

Adenosine is known to modulate dopamine responses in several brain areas. Here, we show that tonic activation of adenosine receptors is able to impede desensitization of D_1 dopamine receptors. As measured by cAMP accumulation in transfected COS-7 cells, long-term exposure to dopamine agonists promoted desensitization of D_{1B} receptor but not that of D_{1A} receptor. The inability of D_{1A} receptor to desensitize was a result of the adenosine present in culture medium acting through activation of adenosine A1 receptors. Cell incubation with either adenosine deaminase, CGS-15943, a generic adenosine receptor antagonist, or the A_1 antagonist DPCPX restored the long-term desensitization time-course of D_{1A} receptors. In Ltk cells stably expressing A1 adenosine receptors and D_{1A} dopamine receptors, pre-treatment of cells with *R*(–)-PIA, a full A1 receptor agonist, did not significantly inhibit the acute increase in cAMP levels induced by D1 receptor agonists, but blocked desensitization of D_{1A} receptors. However, simultaneous activation of A_1 and D_{1A} receptors promoted a delayed D_{1A} receptor desensitization. This suggests that functional interaction between A1 and D_{1A} receptors may depend on the activation kinetics of components regulating D1 receptor responses, acting differentially on D_{1A} and D_{1B} receptors.

Keywords: ischemia, neuromodulation, Parkinson's disease, receptor interactions.

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In the central nervous system, the purine nucleoside adenosine is a product of cell metabolism that is increased upon imbalance between energy supply and demand. Emphasis has been put on the beneficial role that adenosine plays to protect excitable tissues from the damage produced in pathological situations such as ischemia, inflammation or epilepsy (reviewed in Linden 2001). However, a general property of adenosine is to modulate neuronal responses and the efficiency of synaptic transmission in specific brain areas, in response to the metabolic status of the nervous system (Brundege and Dunwiddie 1997). In particular, adenosine has been reported to reinforce the action of $GABA_B$ receptors (Sodickson and Bean 1998) or to inhibit several effects of dopamine in the cortex and basal ganglia (reviewed in Ferré *et al*. 1997).

The modulatory effects of adenosine on dopamine systems have been investigated for their relevance to human pathology such as schizophrenia and Parkinson's disease. These effects are caused by interactions between the adenosine and dopamine receptors. Adenosine actions are mediated by A_1, A_{2A}, A_{2B} and A_3 receptors. The A_1 and A_3 receptors couple to the $G_{i/o}$ heterotrimeric G proteins and activate K^+ channels or phospholipase C (PLCβ), whereas A_{2A} and A_{2B} receptors activate adenylyl cyclase through $G_{s/olf}$ proteins (Linden 2001). Dopamine acts on two classes of receptors, D_1 and D_2, consisting of three subtypes in most vertebrate species (D_{1A}/D_1 and D_{1B}/D_5, D_{1C} in the D_1 receptor class and D_2, D_3, D_4 in the D_2 class). Similar to A_2 adenosine receptors, D_1 receptor subtypes activate adenylyl cyclase and the production of cAMP through $G_{s/olf}$ proteins. The D_2 dopamine receptors mostly inhibit Ca^{2+} channels, and activate K^+ channels through $G_{i/o}$ protein in addition to the inhibition of adenylyl cyclase (Missale *et al*. 1998).

Modulation of dopamine response by adenosine has been shown to occur in brain areas such as basal ganglia, prefrontal cortex or hippocampus, where high levels of D_{1A}/D_1, D_{1B}/D_5 or D_2 receptors are coexpressed with A_1 and A_{2A} receptors. Thus, in the basal ganglia, *in situ* hybridization experiments indicated that A_1 adenosine and D_1 dopamine receptors are colocalized in medium-size neurones of the striatum belonging to direct striatonigral pathway (Ferré *et al*. 1996). Antagonistic interactions between A_1 receptors and D_1 receptors are illustrated by the inhibition promoted by A_1 receptor agonists on the motor activation induced by D_1 receptor agonists in the striatum (Ferré *et al*. 1994). Mechanisms of this inhibition may involve an A_1 receptor-induced decrease in binding affinity of agonists for D_1 dopamine receptor, as shown in membrane preparations of rat striatum, and in mouse fibroblast cells stably expressing A_1 and D_1 receptors (Ferré *et al*. 1997, 1998).

An additional and novel mechanism of adenosine-mediated modulation of D_1 receptor activity is presented here. We show that upon tonic activation of adenosine A_1 receptors, the desensitization of D_{1A} dopamine receptor is strongly inhibited, an effect that does not occur for the D_{1B} dopamine receptor subtype.

Materials and methods

Cell culture and transfection

COS-7 cells were grown in DMEM with 4.5 g/L glucose (Life Technologies) supplemented with 10% calf fetal serum (Dutscher) and

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Abbreviations used: DMEM, Dulbecco's modified Eagle's medium; DMPX, 3,7-dimethyl-1-propargylxanthine; DPCPX, 8-cyclopentyl-1,3-dipropylxanthine; GTP, guanosine tri-phosphate; IBMX, 3-isobutyl-1-methylxanthine; PBS, phosphate-buffered saline; PKA, protein kinase A; PLC, phospholipase C.

2 mM glutamine (Life Technologies), at 37°C in 5% CO_2/95% air. Cells ($2–3 \times 10^6$ cells) were transfected with 10 μg DNA, in 300 μL of culture medium by electroporation using a Bio-Rad Gene Pulser II apparatus (950 μF, 220 V, 25–30 ms). After electroporation, 100 U/mL penicillin/streptomycin (Life Technologies) was added to the culture medium. The D_{1A} (D_1) and D_{1B} (D_5) dopamine receptors used for this study have been described in Cardinaud *et al*. (1997).

Ltk-cells stably expressing the human D_{1A} (D_1) dopamine and A_1 adenosine receptors (a kind gift of S. Ferré; Ferré *et al*. 1998) were grown in DMEM with 4.5 g/L glucose supplemented with 10% fetal calf serum, 0.11 mg/mL sodium pyruvate (Life Technologies), 100 U/mL penicillin/streptomycin, 300 μg/mL hygromycin (Life Technologies), 200 μg/mL neomycin (Promega) and 2 mM glutamine, at 37°C in 5% CO_2/95% air.

In some experiments, fetal calf serum was treated with activated charcoal Dextran Sulfate (Sigma-Aldrich) for 30 min at 56°C as previously described (Guivarc'h *et al*. 1998).

Pharmacological saturation binding assay

After electroporation, cells were grown to confluence in 150-mm diameter dishes, resuspended in binding buffer (50 mM Tris HCl, pH 7.4, 120 mM NaCl, 5 mM KCl, 1.5 mM $CaCl_2$, 5 mM $MgCl_2$, 5 mM EDTA-Na_2), homogenized, and membranes were collected by centrifugation for 25 min at 10 000 *g*. Membranes corresponding to 50 μg proteins were mixed with increasing concentrations of [^{3}H]SCH-23390 (from 3×10^{-11} M to 2×10^{-9} M) and non-specific binding was determined in presence of 10 μM (+)-butaclamol-HCl (Sigma-Aldrich). The mixture was incubated for 90 min at room temperature (~25°C) and filtered through a GF/C 25-mm diameter filter (Whatmann) with a Millipore (Bedford) apparatus. The filter was washed twice (50 mM Tris-HCl, pH 7.4) and radioactivity was measured in a scintillation LKB beta counter.

Desensitization and cAMP accumulation assay

Cells were grown to confluence in 24-well plates and washed with fresh culture medium before the 'desensitization mix' was added to the cells and incubated for various periods of time at 37°C. The 'desensitization mix' contained the D_1 receptor agonists (Sigma; see Results for details) with antioxidant (0.1% ascorbic acid; Sigma) and propranolol (10 μM; Sigma) to block the stimulation of β-adrenoreceptors by dopamine. Treatments with adenosine deaminase (10 U/mL; Roche), or adenosine receptor antagonists (Sigma; see Results for details) were performed by adding the compounds 30 min before dopamine agonist in the culture media, and they were present all along the desensitization protocol. Agonist exposure was stopped by washing the cells twice at 4°C with culture medium containing 0.5 mM IBMX (3-isobutyl-1-methylxanthine; Sigma) to prevent cAMP degradation (see Results for details). Finally, cyclic AMP was allowed to accumulate for 15 min with the stimulation compounds and IBMX. The reaction was stopped by incubating the cells with 0.1 M HCl for at least 20 min to extract cAMP. The quantity of cAMP was assayed following the Nordstedt and Fredholm technique (Nordstedt and Fredholm 1990).

Statistics

Results are expressed as means ± SEM. Statistical significance of the results for the different experimental conditions used to study the desensitization time course was assessed by two-way ANOVA followed by a Tukey's honestly significant difference (HSD) test, and the level of statistical significance was defined as $p < 0.01$ for all the different experimental conditions. Other results were analysed by one-way ANOVA followed by the Student's Newman–Keuls' test: $p < 0.01$ was considered significant.

Results and discussion

Tonic adenosine production specifically blocks D_{1A} dopamine receptor desensitization in transfected COS-7 cells

COS-7 cells were transfected with the D_{1A} receptor subtype, and treated with saturating concentrations of dopamine or apomorphine (10 μM). As compared with non-stimulated levels, cAMP levels were increased about five-fold upon stimulation of the receptor with agonist (data not shown) and remained essentially unchanged for 12 h (Fig. 1a), evidencing the lack of desensitization of the receptor response upon prolonged treatment with agonists.

In contrast, in cells expressing the D_{1B} receptors, a 47% decrease in cAMP accumulation levels (as compared with control levels) occurred within the first 30 min of receptor stimulation, reaching 55% after 12 h of apomorphine treatment (Fig. 1b). These results closely resembled those described for the human D_{1B}/D_5 receptor transfected in COS-7 cells (Jarvie *et al*. 1993). The lack of agonist-induced desensitization appeared to be specifically restricted to the D_{1A} receptor. This differs from other studies where a significant decrease in D_{1A} receptor-dependent stimulation of adenylate cyclase was reported to occur within the first 30 min of exposure to agonists, and lasted for up to 10 h (Olson and Schimmer 1992; Lewis *et al*. 1998). Such differences may be a result of the fact that cells other than COS-7 cells were used in the previous studies. However, given the normal desensitization profile of D_{1B} receptors, this possibility appeared to be unlikely. Instead, it seemed plausible that the regulation of D_{1A} and D_{1B} receptors was differentially sensitive to some component(s) of the culture medium.

This possibility was examined by treating the culture serum with dextran-coated activated charcoal to remove hormones and other small biologically active molecules. Under these conditions, a desensitized response of the D_{1A} receptor was observed as indexed by a 26% decrease in maximal cAMP accumulation after 5 min of agonist stimulation, and lasted for the remaining 12 h of agonist exposure (Fig. 1a). However, the

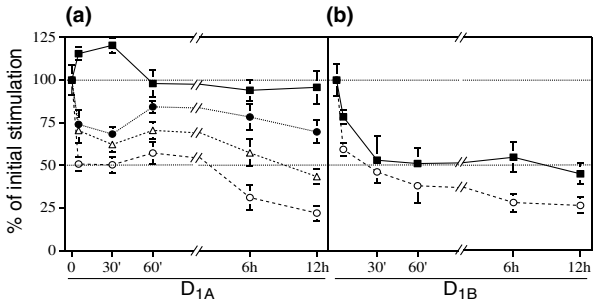

Fig. 1 Influence of adenosine and adenosine receptors on the desensitization time-course of the D_{1A} and D_{1B} receptor subtypes in COS-7 cells. COS-7 cells transfected with D_{1A} (a) or D_{1B} receptor (b) subtypes were treated with 10 μM apomorphine as described in Materials and methods for 5 min, 30 min, 1 h, 6 h and 12 h in basal conditions (■). Subsequent to pre-treatment, cAMP production was assessed by stimulating cells with 10 μM dopamine, 10 μm propranolol and 0.5 mM IBMX for 15 min. D_{1A} receptor-transfected, COS-7 cells were also incubated with charcoal-treated serum (●), adenosine deaminase 10 U/mL (△) and CGS-15943 10 μm (○). The cAMP content was assayed and values were divided by receptor number (B_{max}/fmol protein) expressed in COS-7 cells, per well. This graph is the mean ± SEM of four independent experiments, performed in duplicate. As analysed by two-way ANOVA, each of the desensitization experiments in which adenosine action has been impaired appeared significantly different from the basal conditions ($p < 0.01$). CGS-15943-treated cells (○) are also significantly different from the two other conditions($p < 0.01$)

desensitization response of the D_{1A} receptor remained weak, suggesting that the presence of a compound endogenously produced by the cells and accumulating in the culture medium could block the D_{1A} receptor desensitization.

Among the many possible compounds released by the cells, adenosine was a likely candidate because adenosine/dopamine receptors interactions have been documented (Ferré *et al*. 1998; Gines *et al*. 2000). To deplete the culture medium from adenosine, we added adenosine deaminase (10 U/mL), which degrades adenosine into inosine. In this condition, an enhanced desensitization of the agonist-induced cAMP accumulation occurred in D_{1A} receptor expressing cells (Fig. 1a). Within the first 5 min, the decrease in cAMP accumulation reached 70% of control levels and persisted for 12 h. This effect of adenosine deaminase strongly suggested that adenosine produced by the cells and released into the culture medium, was responsible for the blockage of D_{1A} receptor desensitization.

To test this hypothesis further, we added a potent adenosine receptor antagonist, CGS-15943 (10 μM), to the cell medium. In this condition, a strong, agonist-induced desensitization of D_{1A} receptors occurred (Fig. 1a), characterized by a decrease in agonist-induced cAMP accumulation in the first 5 min reaching 50% of maximal stimulation. Accumulation of cAMP continued to decrease with time, reaching 22% of maximal stimulation after 12 h of agonist exposure. In addition, when treated with dopamine or apomorphine, the maximal levels of cAMP produced in D_{1A} receptor-expressing cells was 1.6-fold higher in the presence of CGS-15943 relative to control conditions (2.24.0 ± 0.12), and 1.42. ± 0.01 pmoles of cAMP/fmole receptor, $n = 4$, respectively). This agreed well with the previous demonstration that A_1 receptor agonists decreased, whereas antagonists increased, the cAMP production stimulated by D_{1A} receptor activation (Gines *et al*. 2000). Again, this effect of adenosine receptors on cAMP production appeared to be specific for D_{1A} receptors, as in D_{1B} receptor-expressing cells, CGS-15943 did not significantly modify the desensitization profile (Fig. 1b).

Taken together, these data indicate firstly that adenosine receptors in COS-7 cells are permanently activated by adenosine released by the cells, and secondly that they attenuate agonist-induced desensitization of D_{1A} but not D_{1B} dopamine receptors. The autocrine role of adenosine previously been observed in GH4 pituitary cells (Zapata *et al*. 1997), neutrophils (Thibault *et al*. 2000) and epithelial cells (Musante *et al*. 1999). These data further support the role of adenosine as a tonic modulator of cell responses.

Blockage of D_{1A} receptor desensitization depends on A_1 adenosine receptors

To specifically address the question of which class of adenosine receptor may be involved in the impairment of D_{1A} receptors desensitization in COS-7 cells, we treated the cells with specific A_1 or A_2 receptor antagonists. Cell incubation with the adenosine A_2 receptor antagonist, 3,7-dimethyl-1-propargylxanthine (DMPX) had a small but non-significant effect on the responses of D_{1A} receptors to both short-term and long-term agonist exposure (Fig. 2). In contrast, in the presence of the adenosine A_1 receptor antagonist, 8-cyclopentyl-1,3-dipropylxanthine (DPCPX), COS-7 cells expressing the D_{1A} receptors displayed a strong desensitization of dopamine-promoted cAMP accumulation, when treated by apomorphine for 5 min, 30 min, 1 h and 6 h (Fig. 2), as shown in cells treated with the generic adenosine receptor antagonist CGS-15943 (Fig. 2). Thus, the inhibition of D_{1A} receptor desensitization by released adenosine depends mainly on the activation of a receptor of the A_1 class.

The availability of a mouse fibroblast cell line (Ltk-) that stably expresses A_1 and D_{1A} receptors at similar levels (B_{max} values of 4 and 4.6 pmoles/mg of protein, respectively; Ferré *et al*. 1998) gave us the opportunity to analyse further the molecular interactions between these two types of receptors. We first tested the ability of D_{1A} receptors expressed in the Ltk-cell line to desensitize. In these cells, no attenuation of dopamine-induced accumulation of cAMP was noticed for up to 2 h (Fig. 3a), as was observed in COS-7 cells. A similar response was obtained with SKF-38393, a selective D_1 receptor agonist (Fig. 3b). When the cells were challenged for D_1 receptor desensitization in presence of the adenosine receptor antagonist CGS-15943 (10 μM; Fig. 3c), a significant decrease of the D_{1A} receptor-induced cAMP production (41% of maximal stimulation) was observed within the first 15 min, and lasted for the 2 h of our test. Thus, again, the blockade of D_{1A} dopamine receptor desensitization depend on A_1 receptor activation, the only adenosine receptor subtype expressed in this Ltk-cell line (Ferré *et al*. 1998).

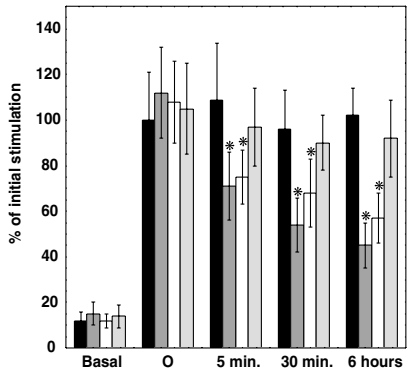

Fig. 2 Inhibition of D_{1A} receptor desensitization by adenosine depends on A_1 receptors. D_{1A} receptor-expressing COS-7 were treated as in Fig. 1 for 5 min, 30 min and 6 h either in basal conditions (black bars) or in presence of the generic adenosine receptor antagonist CGS-15943 (10 μM; dark grey bars), of either the specific A_1 receptor antagonist DPCPX (10 μM, open bars) or the specific A_2 receptor antagonist DMPX (10 μM, light grey bars). Subsequent to pre-treatment, cAMP production was assessed by stimulating cells with 10 μM dopamine, 10 μM propranolol and 0.5 mM IBMX for 15 min. The cAMP content was assayed as in Fig. 1. This graph is the mean ± SEM of three independent experiments, performed in duplicate. Values significantly different from controls ($p < 0.01$) are indicated by asterisks.

To gain additional insight into the mechanism that may account for the A_1 receptor-mediated modulation of D_{1A} receptor desensitization, Ltk-cells were treated with A_1 receptor agonist and D_1 receptor agonist, either alone or in combination. Treatment of cells with *R*(–)-PIA (a potent agonist of A_1 receptors) did not prevent D_1 receptor activation of adenylyl cyclase, but instead blocked agonist-induced desensitization of D_1 receptors, as previously observed (Fig. 3d). Interestingly, when cells were treated simultaneously with the D_1 receptor agonist SKF-38393 and *R*(–)-PIA, desensitization of D_1 receptors was delayed but occurred with a significant amplitude (45.5% of maximal cAMP accumulation; Fig. 3d). Although this result may seem somehow contradictory to our previous data, it has been recently shown that when A_1 and D_1 receptors are coactivated, they do not cluster in the same subcellular compartment. In contrast, when an A_1 receptor agonist was used, D_{1A} and A_1 receptors appeared frequently colocalized in the same membrane clusters (Gines *et al*. 2000). Our interpretation is that, when only A_1 adenosine receptors are stimulated first, modulation of the activity of intracellular regulatory components rendered the D_{1A} receptor refractory to desensitization. Conversely, when A_1 and D_1 receptors are costimulated, the D_1 receptors may be physically isolated from A_1 receptors action, permitting the desensitization process.

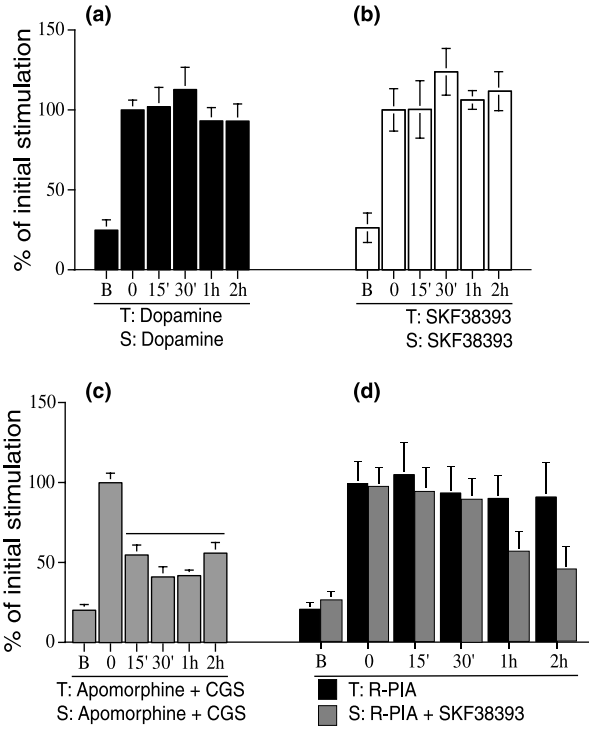

Fig. 3 Human A_1 adenosine and D_{1A} dopamine receptors interaction in Ltk-cells. Ltk-cells stably expressing A_1 adenosine and D_{1A} dopamine receptors were treated with dopamine, SKF-38393, apomorphine + CGS-15943 (10 μM each), or *R*(–)-PIA (100 nM), as indicated on the graph (T). Treatments were maintained for 15 min, 30 min, 1 h and 2 h. The stimulation media contained 10 μM propranolol, 0.5 mM IBMX and 10 μM of the indicated compounds (S). Basal conditions, i.e. non-stimulated cells, were also represented (B). The cAMP content was reported to receptor number (B_{max}/fmol protein) expressed in Ltk-cells, per well. No desensitization of the D_1 receptor occurs in response to agonists (a, dopamine; b, SKF 38393) except when A_1 receptors are blocked (c) or when A_1 and D_1 receptors are simultaneously activated by PIA and SKF-38393 (grey bars) as compared with PIA alone (black bars; d). The graphs are the mean ± SEM of four independent experiments, each performed in duplicate. $p < 0.05$ compared with initial stimulation (0).

To conclude, our data demonstrate that a tonic activation of adenosine A_1 receptors by adenosine released in the culture medium is able to block D_{1A} dopamine receptor desensitization. The possibility that a tonic effect of adenosine on the regulation of D_{1A} receptors may be mediated by A_1 receptors is reinforced by the fact that A_1 receptors do not undergo significant desensitization (Palmer *et al*. 1996). This mode of action of the A_1 receptor is highly selective for the D_{1A} subtype, as the D_{1B} receptor was unable to be similarly regulated under identical experimental conditions. Such concerted interactions between functionally distinct neurotransmitter receptors increasingly appear to be part of a general overall mechanism by which neurones are able to receive and integrate diverse extracellular signals.

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