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Effector coupling of stably transfected human A_3 adenosine receptors in CHO cells

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Abstract

CHO cells stably transfected with adenosine receptors are widely utilized models for binding and functional studies. The effector coupling of human A_3 adenosine receptors expressed in such a cellular model was characterized. Inhibition of adenylyl cyclase via a pertussis toxin-sensitive G protein was confirmed and exhibited a pharmacological profile in accordance with agonist binding data. The agonist potency was dependent on the assay system utilized to measure cyclase inhibition. Agonists were more potent in a cell-based assay than in experiments where cyclase inhibition was measured in a membrane preparation suggesting that receptor–effector coupling might be more efficient in intact cells. In addition to the modulation of cyclase activity, stimulation of A_3 receptors elicited a Ca^{2+} response in CHO cells with agonist potencies corresponding to the values for the whole cell cAMP assay. The Ca^{2+} signal was completely eliminated by pertussis toxin treatment suggesting that it is mediated via βγ release from a heterotrimeric G protein of the $G_{i/o}$ family. These results show that cAMP and Ca^{2+} signaling characteristics of the A_3 adenosine receptor are comparable to the ones found for the A_1 subtype.

Keywords: Adenosine; Adenosine receptor; A_3 effector coupling; Ca^{2+} signal; Second messenger

1. Introduction

Adenosine modulates the physiological function of many organ systems acting upon four subtypes of G protein-coupled receptors. They are distinguished as A_1, A_{2A}, A_{2B} and A_3 and regulate the activity of adenylyl cyclase [1]. The activation of additional effector systems like PLC [2–4] or K^+ channels [5] has been shown in particular for A_1 and A_3 adenosine receptors. Recently, it has been shown that adenosine receptors may also stimulate mitogen-activated protein kinases like ERK1/2 [6]. It has been confirmed that all four human receptors may do so in CHO cells transfected with the respective subtype [7].

Cellular systems transfected with the individual human receptor subtypes [8] have proven to be very useful models for the development of subtype-selective ligands [9–12] as well as for functional studies [6,7,13]. In particular for their use as functional models, it is important to fully characterize the effector coupling pathways in CHO cells transfected with the receptor under investigation. Recently, a lot of attention has been focused on the A_3 adenosine receptor because it may serve as a novel drug target for the treatment of asthma, neurodegenerative disorders and chronic inflammatory diseases [14–16]. In this study, we characterize A_3 receptor-mediated inhibition of adenylyl cyclase and in addition we provide data for the activation of a Ca^{2+} signal in CHO cells via the human adenosine receptor subtype. We confirm that A_3 adenosine receptors stably transfected into CHO cells trigger a Ca^{2+} signal and show that this signal is exclusively dependent on a pertussis toxin-sensitive G protein suggesting a βγ-mediated pathway.

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Abbreviations: AB-MECA, N^6-4-aminobenzyladenosine-5′-*N*-methyluronamide; CCPA, 2-chloro-N^6-cyclopentyladenosine; CGS 21680, 2-[*p*-(2-carboxyethyl)phenylethylamino]-adenosine-5′-*N*-ethyluronamide; Cl-IB-MECA, 2-chloro-N^6-3-iodobenzyladenosine-5′-*N*-methyluronamide; CPA, N^6-cyclopentyladenosine; IAB-MECA, N^6-4-amino-3-iodobenzyladenosine-5'-*N*-methyluronamide; NECA, 5′-*N*-ethylcarboxamidoadenosine; PENECA, 2-phenylethynyl-adenosine-5′-*N*-ethyluronamide; PHPNECA, 2-(3-hydroxy-3-phenyl)propyn-1-yl-adenosine-5′-*N*-ethyluronamide; PIA, N^6-(2-phenylisopropyl)adenosine; XAC, xanthine amine congener, 8-[4-[[[[(2-aminoethyl)amino]carbonyl]methyl]oxy]phenyl]-1,3-dipropylxanthine.

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2. Materials and methods

2.1. Materials

[α-^{32}P]ATP was from Du Pont NEN. Adenosine receptor agonists and antagonists were from RBI with the exception of Cl-IB-MECA which was provided by RBI as part of the NIMH Chemical Synthesis Program and PENECA and PHPNECA which were synthesized by G. Cristalli and pharmacologically characterized as described recently [9]. The fura-2/AM was purchased from Calbiochem. The RIA kit for cAMP measurements was from Beckman Coulter. Cell culture media and fetal calf serum were purchased from PanSystems. Penicillin (100 unit/mL), streptomycin (100 μg/mL), L-glutamine and G-418 were from Gibco-Life Technologies. All other materials were from sources as described earlier [8,17,18].

2.2. Cell culture and membrane preparation

CHO cells stably transfected with human A_3 receptors were grown adherently and maintained in Dulbecco's Modified Eagles Medium with nutrient mixture F12 (DMEM/F12) without nucleosides, containing 10% fetal calf serum, penicillin (100 unit/mL), streptomycin (100 μg/mL), L-glutamine (2 mM) and Geneticin (G-418, 0.2 mg/mL; A_{2B}, 0.5 mg/mL) at 37° in 5% CO_2/95% air as described earlier [8].

Crude membranes were prepared from fresh or frozen cells as described recently [8]. In brief, cells were suspended and homogenized in ice-cold hypotonic buffer (5 mM Tris–HCl, 2 mM EDTA, pH 7.4), the homogenate was spun for 10 min (4°) at 1000 *g* and the crude membrane fraction was sedimented from the supernatant for 30 min at 100,000 *g*. For binding experiments, the membranes were resuspended in 50 mM Tris–HCl, 10 mM $MgCl_2$, 1 mM EDTA, pH 8.25, frozen in liquid nitrogen at a protein concentration of 1–3 mg/mL and stored at −80°. For the measurement of adenylyl cyclase activity crude membranes were prepared with only one centrifugation step. The homogenate from fresh cells was sedimented for 30 min at 54,000 *g* and the resulting pellet was resuspended in 50 mM Tris–HCl pH 7.4 for immediate use [8].

2.3. Adenylyl cyclase activity and cAMP levels

The procedure followed the protocol described previously [9,17] with minor modifications. Membranes were incubated for 20 min at 37° in an incubation mixture containing about 150,000 cpm of [α-^{32}P]ATP. All other ingredients were the same as described [17] except EGTA and NaCl which were omitted. The IC_{50}-values for the inhibition of forskolin-stimulated (10 μM) adenylyl cyclase were calculated with the Hill equation. Hill coefficients in all experiments were near unity.

The levels of cAMP accumulation in intact cells was determined with a commercial RIA kit following the instructions of the manufacturer.

Pertussis toxin treatment of cells was carried out as described by Freund *et al.* [3].

2.4. Measurement of intracellular Ca^{2+} levels

Concentrations of free intracellular Ca^{2+} was measured utilizing the fluorescence indicator fura-2/AM as described by Abd Alla *et al.* [19].

3. Results

The A_3 adenosine receptor-mediated inhibition of adenylyl cyclase activity was determined with a series of adenosine receptor agonists with different receptor selectivity. Table 1 summarizes the K_i-values for these compounds which are sorted according to increasing potency. For comparison, K_i-values determined previously in binding experiments (see Table 1) are listed as well. Table 1 shows that the rank order of potencies for both parameters correspond well. The discrepancy between functional and binding data for agonists is a common observation for inhibitory adenosine receptors. Pertussis toxin treatment of the cells completely abolishes the agonist effect on adenylyl cyclase activity (data not shown).

The agonist-mediated cyclase inhibition was antagonized by XAC, which is a nonselective xanthine antagonist at human adenosine receptors. Schild analysis of the concentration-dependent right shift of the agonist-effect curve resulted in a K_B-value of 370 nM (95% confidence interval, 130–1050 nM; N = 3). Fig. 1 shows the data from a representative experiment.

In addition to the measurement of adenylyl cyclase activity in a membrane preparation the effect of selected

Table 1
Inhibition of adenylyl cyclase with adenosine receptor agonists[a]

	Adenylyl cyclase IC_{50} (nM)	Binding K_i (nM)
IAB-MECA	140 (70–280)	0.6[b]
PHPNECA	190 (90–400)	0.4[c]
PENECA	1150 (920–1430)	6.2[c]
Cl-IB-MECA	1170 (590–2320)	11[c]
NECA	1760 (1230–2530)	6.2[c]
R-PIA	4550 (2680–7750)	16[b]
AB-MECA	6270 (4350–9040)	22[b]
CPA	11,400 (5,300–24,300)	43[b]
CGS 21680	18,200 (11,500–29,000)	67[b]
CCPA	28,200 (21,300–37,200)	42[b]
S-PIA	37,000 (23,500–58,000)	45[b]

[a] IC_{50}-Values (with 95% confidence limits in parentheses) for A_3 adenosine receptor-mediated inhibition of adenylyl cyclase were determined in a membrane preparation as described in Section 2. For comparison binding data from competition experiments are shown.

[b] Values taken from Klotz *et al.* [9].

[c] Values taken from Klotz *et al.* [8].

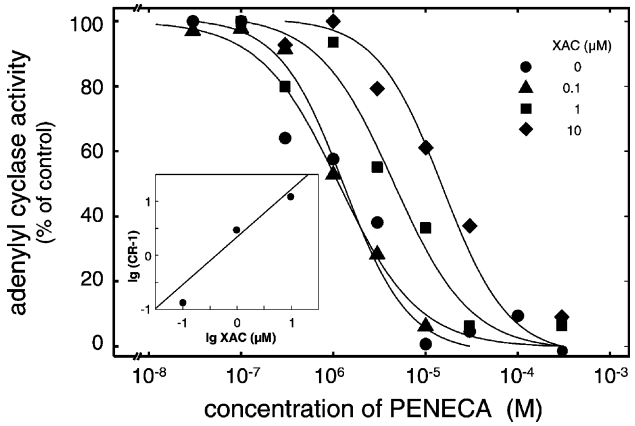

Fig. 1. Antagonism of agonist-mediated inhibition of adenylyl cyclase. The A_3 selective agonist PENECA inhibited adenylyl cyclase activity in a membrane preparation with an IC_{50}-value of 1.1 μM in the experiment shown. Increasing concentrations of the adenosine receptor antagonist XAC shifted the inhibition curve to the right in a concentration-dependent manner. The inset shows a Schild-plot calculated from the concentration ratio (CR) of the shift caused by increasing antagonist concentrations. The XAC inhibition constant from the experiment shown is 598 nM.

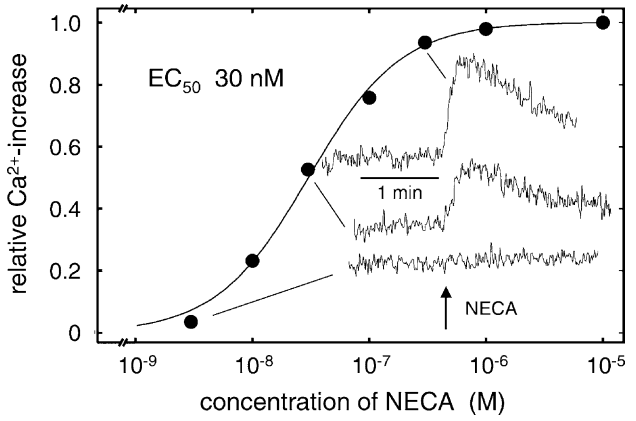

Fig. 2. Agonist-mediated Ca^{2+} signal. Intracellular concentrations of Ca^{2+} were measured after loading the A_3 CHO cells with the fluorescence indicator fura-2/AM. The inset shows original traces from the ratio of fluorescence at 340/380 nM over time. The relative increase in intracellular Ca^{2+} is shown as a function of the concentration of NECA. In the experiment shown an EC_{50}-value of 30 nM was found.

agonists on the accumulation of cAMP was studied in whole cells. The IC_{50}-values from this experimental approach are given in Table 2.

Next, the A_3 receptor-mediated Ca^{2+} signal was investigated. Agonist-induced increase of free intracellular Ca^{2+} was monitored with the fluorescent Ca^{2+}-indicator fura-2/AM. Fig. 2 shows that NECA caused a concentration-dependent increase in intracellular Ca^{2+} with an EC_{50}-value of 41 (30–55) nM (Table 2). The A_3 selective agonist PENECA caused a similar Ca^{2+} signal with an EC_{50}-value of 12 (7–19) nM (Table 2). The observed signal was not abolished in the absence of extracellular Ca^{2+} or the additional presence of 5 μM EGTA in Ca^{2+}-free medium suggesting that it was caused by Ca^{2+} release from intracellular stores (data not shown). However, the activation of this effector pathway in the transfected CHO cells was completely abolished by treatment of the cells with pertussis toxin (Fig. 3) and might, therefore, be mediated by release of βγ subunits from activated G proteins of the $G_{i/o}$ family. CHO cells transfected with the human A_{2B} adenosine receptor showed no Ca^{2+} signal in response to 10 μM NECA confirming that the increase in intracellular Ca^{2+} was caused by the transfected A_3 adenosine receptor (data not shown).

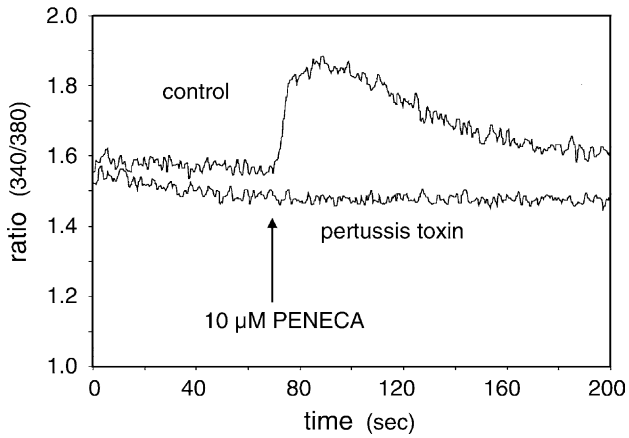

Fig. 3. Pertussis toxin effect on the A_3 receptor-mediated Ca^{2+} signal. The rise of intracellular Ca^{2+} in CHO cells expressing A_3 receptors caused by the A_3 selective agonist PENECA was completely abolished by pertussis toxin treatment. This suggests that G_q is not involved in Ca^{2+} signaling in this model.

Table 2
Effector coupling of A_3 adenosine receptors[a]

	Adenylyl cyclase (IC_{50})		Ca^{2+} signal EC_{50}	Binding K_i (nM)[b]
	AC	cAMP		
NECA	1760 (1230–2530)	53 (35–79)	41 (30–55)	6.2 (3.5–11)
PENECA	1150 (920–1430)	104 (57–189)	12 (7–19)	6.2 (5.1–7.5)

[a] A comparison of the potency of NECA and PENECA in various functional and binding assays is shown. IC_{50}-Values for A_3 adenosine receptor-mediated inhibition of adenylyl cyclase were determined in a membrane preparation (AC) or by the measurement of intracellular cAMP levels (cAMP) as described in Section 2. In addition, EC_{50}-values for the agonist-mediated increase in intracellular Ca^{2+} levels and K_i-values from binding experiments are shown. All data are in nM and are means from 3–5 experiments with 95% confidence limits in parentheses.

[b] Values taken from Klotz *et al.* [9].

4. Discussion

Cloning of the human A_3 adenosine receptor revealed its coupling to adenylyl cyclase in an inhibitory manner in analogy to the A_1 subtype [20]. Several reports showed that A_3 receptors also couple to PLC and consequently give rise to an intracellular Ca^{2+} signal [4,21]. This is again in analogy to the signaling pathways described for the A_1 receptor. The pharmacology of human adenosine receptors expressed in CHO cells has been studied in detail [8], however, with the exception of the A_{2B} receptor only limited data are available about the functional effector coupling of adenosine receptors in these cellular models. In this study, we describe that the pharmacological profile of agonist-mediated inhibition of adenylyl cyclase corresponds well to the binding profile of the respective agonists. A consistent discrepancy between functional IC_{50}-values and K_i-values from binding experiments was observed. Similar discrepancies were reported for A_1 adenosine receptors, e.g. in guinea pig and rat heart [5,22]. One possible explanation was that in functional experiments the presence of GTP was required with the consequence of shifting receptors to the low affinity state for agonists. This may only partially explain the differences found for the A_3 receptor in CHO cells, as measuring cAMP levels in a whole cell preparation led to IC_{50}-values for agonists much closer to the respective K_i-values from binding experiments (Table 2). As opposed to binding affinity, the functional potency depends on receptor density and coupling efficiency along the signaling cascade. The receptor–effector coupling might be more efficient in whole cells than in membranes and, thus, explain the discrepant results. However, this cannot be considered to be a general phenomenon as for instance agonist-mediated stimulation of adenylyl cyclase via A_{2B} adenosine receptors results in comparable EC_{50}-values for a membrane-based vs. a whole-cell assay [8]. The importance of the effector coupling for functional potency is nicely documented for A_{2B} receptors transfected into CHO cells which show a 100-fold difference between adenylyl cyclase stimulation and ERK1/2 phosphorylation in the same cell line [7].

The functional potency determined in a cellular model depends on the expression level of the receptor. Although the CHO cells utilized in this study [8] may express more A_3 receptors than are found in a 'real' cell, the expression is not beyond levels found for other adenosine receptor subtypes like A_1 or A_{2A}, e.g. in specific brain areas [23,24]. The data from an artificial system like transfected cells may not correspond to 'true' potencies in a physiological environment. Nevertheless, these data are important to know for the interpretation of results from the widespread use of such artificial systems not only for binding but also for functional studies.

Activation of a Ca^{2+} signal or of phospholipase C in CHO cells stably transfected with human [2] or rat A_1 adenosine receptors [3] has been shown to be pertussis toxin-sensitive suggesting it occurs through release of $\beta\gamma$ subunits from $G_{i/o}$ proteins. This was confirmed in later studies [25,26] and in the past it was generally accepted that pertussis toxin-sensitive activation of PLC is via $G_{\beta\gamma}$ [27]. Our data show that the human A_3 receptor expressed in CHO cells also activates a Ca^{2+} signal. This response is completely abolished by pertussis toxin suggesting that in this system $\beta\gamma$ release might be responsible for Ca^{2+} signaling whereas coupling to G_q does not appear to play a role. Recently, it was shown that the tyrosine kinase Src may serve as an effector for activated α_s and α_i opening up the possibility of triggering a Ca^{2+} signal via PLCγ [28]. At this point, we cannot exclude such a mechanism to be operative in our cellular model. Further investigation will be required to clarify a contribution of A_1 and A_3 receptor-mediated tyrosine kinase activation in Ca^{2+} signaling.

In summary, we have functionally characterized human A_3 adenosine receptors stably expressed in CHO cells and shown that the pattern of effector coupling is similar to what has been described for inhibitory A_1 receptors in a variety of systems. Both signals investigated in this study, inhibition of adenylyl cyclase and the intracellular Ca^{2+} response, are exclusively mediated by activation of pertussis toxin-sensitive G proteins of the $G_{i/o}$ family. This information should help to identify the mechanisms of A_3 receptor-mediated effects investigated in functional studies in this cellular model for the human receptor subtype.

Acknowledgments

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