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Adenosine A_{2A} receptor dynamics studied with the novel fluorescent agonist Alexa488-APEC

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Abstract

G protein-coupled receptors, such as the adenosine A_{2A} receptor, are dynamic proteins, which undergo agonist-dependent redistribution from the cell surface to intracellular membranous compartments, such as endosomes. In order to study the kinetics of adenosine A_{2A} receptor redistribution in living cells, we synthesized a novel fluorescent agonist, Alexa488-APEC. Alexa488-APEC binds to adenosine A_{2A} ($K_i = 149 \pm 27$ nM) as well as A₃ receptors ($K_i = 240 \pm 160$ nM) but not to adenosine A₁ receptors. Further, we characterized the dose-dependent increase in Alexa488-APEC-induced cAMP production as well as cAMP response element binding (CREB) protein phosphorylation, verifying the ligand's functionality at adenosine A_{2A} but not A_{2B} receptors. In live cell imaging studies, Alexa488-APEC induced adenosine A_{2A} receptor internalization, which was blocked by the competitive reversible antagonist ZM 241385 and hyperosmolaric sucrose. Further, internalized adenosine A_{2A} receptors co-localized with clathrin and Rab5, indicating that agonist stimulation promotes adenosine A_{2A} receptor uptake through a clathrin-dependent mechanism to Rab5-positive endosomes. The basic characterization of Alexa488-APEC provided here showed that it provides a useful tool for tracing adenosine A_{2A} receptors *in vitro*.

Keywords

G protein-coupled receptor; endocytosis; cyclic AMP; CREB; nucleoside; Rab5; clathrin

1. Introduction

Adenosine A_{2A} receptors belong to the family of G protein-coupled receptors (Fredholm et al., 2003). This member of the adenosine receptor family, which consists of adenosine A₁, A_{2A}, A_{2B}, and A₃ receptors (Fredholm et al., 2001), is coupled to stimulatory G proteins of the G_s family (Furlong et al., 1992). Thus, receptor stimulation leads to an increase in cAMP

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and phosphorylation of the cAMP-regulated element binding protein (CREB) (Kull et al., 1999; Schulte et al., 2003a). Physiologically, adenosine A_{2A} receptors are involved, e.g., in the regulation of sleep-wakefulness, locomotion and activity, platelet function, and T cell-dependent responses (Ledent et al., 1997; Jacobson et al., 2006; Fredholm et al., 2007; Huang et al., 2007).

It is widely appreciated that G protein-coupled receptors, such as the adenosine A_{2A} receptor, are dynamic proteins, which traffic throughout membranous compartments of the cell, depending on the receptor's maturity or activity status (Conn et al., 2007; Xu et al., 2007). Stimulation of receptors exposed on the cell surface can induce receptor desensitization and, consequently, to receptor internalization through different endocytotic pathways leading primarily to a reduction in receptor numbers on the cell surface (Wolfe et al., 2007). However, recent evidence emphasizes that the internalized receptors localized in endosomes can also direct specific signaling by providing a scaffold for active signaling components (Dewire et al., 2007). In the case of the adenosine A_{2A} receptor, previously described patterns of receptor desensitization indicate that agonist-stimulated adenosine A_{2A} receptors are rapidly and transiently phosphorylated and desensitized (Palmer et al., 1994). Further investigation showed that a C-terminal threonine (Thr298) is essential for agonist-induced receptor phosphorylation and short-term desensitization (Palmer et al., 1997). This is a site of phosphorylation through G protein-coupled receptor kinase-2 (GRK2), which is known to regulate agonist-induced desensitization of adenosine A_{2A} receptors (Mundell et al., 1997, 1998b). However, short-term adenosine A_{2A} receptor desensitization appears to depend mainly on agonist-induced receptor phosphorylation and not on clathrin-based endocytosis, since hyperosmotic sucrose or concavalin A treatment did not inhibit agonist-induced desensitization of the adenosine A_{2A} receptor (Mundell et al., 1998a). Receptor distribution and internalization upon agonist treatment have not been characterized yet. Many studies addressing receptor dynamics use fluorescently tagged receptors to trace agonist-induced endocytosis. However, since this approach requires cell transfection or genetic modification, it is not easily applicable in complex cellular systems or *in vivo*. Thus, it is advantageous to detect receptor trafficking by tracing fluorescently labeled agonists, a methodology that has been employed using both agonist and antagonist as well as small molecule and peptide ligands in various receptor systems (Daly et al., 2003; Middleton et al., 2005). In order to study adenosine A_{2A} receptor dynamics in cell culture, we set out to synthesize a novel fluorescent adenosine A_{2A} receptor agonist, Alexa488-APEC, which we used here to trace adenosine A_{2A} receptors transiently expressed in immortalized cell lines over time. We show that this ligand is a moderately selective and functional adenosine A_{2A} receptor agonist suitable for live-cell imaging of stimulation-dependent receptor dynamics. Further, we characterize the internalization pathway of adenosine A_{2A} receptors as clathrin-dependent uptake into Rab5-positive endosomes.

2. Materials and methods

2.1. Material

[¹²⁵I]N⁶-(4-Amino-3-iodobenzyl)adenosine-5'-N⁶-methyluronamide ([¹²⁵I]-AB-MECA, 2000 Ci/mmol) was purchased from GE Healthcare (Piscataway, NJ USA). [³H] 4-(2-[7-Amino-2-(2-furyl)[1,2,4]triazolo[2,3-a][1,3,5]triazin-5-ylamino]ethyl)phenol ([³H]ZM 241385, 17 Ci/mmol) was purchased from Tocris (Bristol, UK). [³H]2-[p-(2-carboxyethyl)phenylethylamino]-5'-N⁶-ethylcarboxyamido-adenosine ([³H] CGS21680, 47 Ci/mmol), [³H] 2-chloro-N⁶-cyclopentyladenosine ([³H]CCPA, 42.6 Ci/mmol) and adenosine 3', 5' cyclic monophosphate ([2,8-³H]cAMP, 27.4 Ci/mmol) were purchased from Perkin Elmer (Waltham, MA, USA). NECA (5'-N-ethylcarboxamidoadenosine), rabbit anti-FLAG (F7425), and forskolin were purchased from Sigma-Aldrich (St. Louis, MO, USA). ZM214,385 was from Tocris. Alexa Fluor[®] 488 carboxylic acid, succinimidyl ester (mixed isomers) was obtained

from Invitrogen (Carlsbad, CA, USA). 2-[4-(2-aminoethylaminocarbonyl)ethyl]phenylethylamino]-5'-N-ethylcarboxamidoadenosine (APEC) bistrifluoroacetic acid was provided by NIMH Chemical Synthesis and Drug Supply Program. Rabbit anti-Ser133-P-CREB and mouse anti-clathrin (3F133) were from Upstate Biotechnology (Cambridge, UK) and Abcam (Hampshire, UK), respectively. Other chemicals were from standard commercial sources and of analytical grade. Plasmids coding for FLAG (N-terminal)-A_{2A} and A_{2A}-CFP (C-terminal) fusion proteins, both constructs lacking the most C terminal 49 aa, were generous gifts from Dr. C. Hoffmann, Würzburg University, Germany. Rab5-GFP was a gift from Dr. P.D. Stahl, Washington University, School of Medicine, St. Louis, MO, USA (Roberts et al., 1999).

2.2. Synthesis of Alexa488-APEC

Two milligrams of the Alexa Fluor 488 active ester (3.1 μ mol, 16 mM) was dissolved in 0.2 ml of dimethyl sulfoxide (DMSO). APEC bistrifluoroacetic acid salt (2.5 mg, 3.2 μ mol) was added to this solution to achieve a final theoretical concentration of 16 mM, followed by the addition of 5 μ l of triethylamine (35.9 μ mol). The reaction was covered and stirred for 1 h. The product was purified using an Agilent HPLC (Santa Clara, CA, USA) equipped with a Phenomenex Luna 5 μ Reverse Phase C18 analytical column (250 $\times$ 4.6 mm; Torrance, CA, USA), with acetonitrile:water mixtures at a flow rate of 2 ml/min and the following gradient: 0 min, 30% acetonitrile; 6.5 min 50% acetonitrile; 9 min, 90% acetonitrile; 10 min, 30% acetonitrile. The compound eluted at a retention time of 4.2 min. After lyophilization, 2.24 mg (2.12 μ mol) of chromatographically homogeneous product was recovered for a 68% yield. The mass spectrum was taken using a Waters LCT Premier mass spectrometer. The only major peak had a molecular weight of 1056.2614, consistent with the expected molecular weight of the product, Alexa488-APEC, 1056.2616 (C₄₆H₄₆N₁₁O₁₅S₂). For thin layer chromatography Alexa488-APEC solutions were separated on a silica gel 60 thin layer chromatography plate (Merck, Whitehouse Station, NJ, USA) using acetonitrile: water (7:1) as solvent. For further detail on the path of synthesis, structure and analysis by mass spectroscopy, see supplemental material Fig. S1.

2.3. Competitive ligand binding

Competitive radioligand binding assays were done as described previously (Perreira et al., 2005). Human A₁ and A₃ARs were stably expressed in Chinese hamster ovary (CHO) cells, while the human A_{2A}AR was stably expressed in HEK293 cells. Cell membrane suspensions were prepared as described (Gao et al., 2004).

Stock solutions of APEC bistrifluoroacetic acid and Alexa488-APEC in DMSO (5 mM) were prepared and were stable when stored frozen. For the binding determination, each tube contained 50 μ l of the appropriate radioligand (A₁: [³H]CCPA, final conc. 1.0 nM; A_{2A} agonist: [³H]CGS21680, final conc. 10 nM; A_{2A} antagonist: [³H] ZM 241385, final conc. 2 nM; A₃: [¹²⁵I]AB-MECA, final conc. 0.5 nM), 50 μ l of APEC bistrifluoroacetic acid or Alexa488-APEC in increasing concentrations, and 100 μ l of cell membrane suspension expressing the appropriate receptor diluted with Tris-HCl buffer (50 mM, pH 7.4) containing 10 mM MgCl₂. Non-specific binding was determined using 10 μ M NECA. The tubes were incubated at 25°C for 1 h and then filtered through Whatman GF/B filters under reduced pressure using an MT-24 cell harvester (Brandell, Gaithersburg, MD, USA). Filters were washed rapidly three times with ice-cold buffer. For A₁ and A_{2A} assays, after the filters were washed, they were placed in scintillation vials containing 5 ml of Hydrofluor scintillation buffer and counted using a Perkin Elmer Liquid Scintillation Analyzer. For A₃ assays, the filters were counted using a Packard Cobra II gamma counter. The K_i values were determined using Prism software (GraphPad, San Diego, CA, USA) for all assays.

2.4. Cell culture

CHO and HEK293 cells were grown in DMEM/F12 (1:1), 10% FBS, 2 mM L-glutamine, 50 U/ml penicillin/streptomycin and DMEM, 10% FBS, 2 mM L-glutamine, 50 U/ml penicillin/streptomycin, respectively. Cells were sub-cultured three times a week. Transient transfections were done according to the manufacturer's recommendations with Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). For live-cell imaging or immunocytochemistry, cells were seeded on collagen-coated (0.1% solution, Sigma-Aldrich, St. Louis, MO, USA) glass-bottom (MatTek corporation, Ashland, MA, USA) dishes or coverslips, respectively.

2.5. cAMP and P-CREB measurements

Determinations of cAMP levels and CREB phosphorylation were done as previously described (Schulte et al., 2000, 2003b). Rabbit anti-Ser133-P-CREB was used at a 1:2000 dilution. Graphical and statistical analysis of the results was done with GraphPad Prism5 (nonlinear regression). Statistical significance was analysed with a two-tailed t-test (Fig. 2).

2.6. Live-cell imaging

Cells were seeded into 35 mm diameter glass bottom dishes and transfected the next day. For microscopy, we used cells 20–48 h posttransfection. Cells were washed in Hank's Buffered Salt Solution (HBSS) before addition of fluorescent ligands. After 10 min incubation with the ligand, cells were washed three times in HBSS and investigated under the laser scanning microscope (LSM510 Meta, Zeiss, Jena, Germany). In order to control for and to avoid optical bleed-through between Alexa488 and CFP filter, settings were optimized, and control experiments with untagged receptors and single transfections were performed (not shown).

2.7. Immunocytochemistry

For immunocytochemistry HEK293 cells were transiently transfected with adenosine FLAG-A_{2A} receptors and fixed in 4% paraformaldehyde (10 min at room temperature) 24 h posttransfection. Detection of the FLAG epitope was done as described before (Bryja et al., 2007). Visual evaluation was done on a LSM510 Meta confocal system.

3. Results

The newly synthesized compound Alexa488-APEC was assayed for affinity at human adenosine A₁, A_{2A}, and A₃ receptors in competitive radioligand binding experiments (Fig. 1, Table 1). In previous studies we have shown that APEC is inactive at the adenosine A_{2B} receptor (Hide et al., 1992). When using an agonist radioligand, the new compound had K_i values of 149 ± 27 nM and 240 ± 160 nM (mean $\pm$ standard error, S.E.M.) for adenosine A_{2A} and A₃ receptors, respectively, but did not show appreciable binding to the adenosine A₁ receptor even at concentrations up to 10^{-5} M. However, when the antagonist radioligand [³H]-ZM 241385 was used for A_{2A} binding, the K_i value was 2300 ± 410 nM as compared to 215 nM for APEC. Thus, compared to the non-selective parent compound APEC (Jacobson et al., 1989), Alexa488-APEC was moderately selective for the adenosine A_{2A} and A₃ receptors versus the A₁ receptor (Fig. 1, Table 1). In addition, there was a 4.3 fold decrease in adenosine A_{2A} receptor binding affinity and an approximate 12-fold decrease in adenosine A₃ receptor binding affinity upon condensation of APEC with the Alexa Fluor 488 moiety. In order to confirm the low activity of Alexa-488-APEC also at adenosine A_{2B} receptors, which are difficult to study by radioligand binding assays, we determined its capability to induce cAMP production in CHO A_{2B} cells in comparison to NECA-induced cAMP levels (Fig. 1D). At 10^{-5} M Alexa488-APEC induced only 14.8 % of the NECA-induced production of cAMP. Before using Alexa488-APEC extensively for cell stimulation, we determined the stability of

the compound under cell culture conditions. In culture with CHO cells over several days, no degradation products were detectable in the medium by thin layer chromatography (not shown).

In order to investigate the ability of Alexa488-APEC to induce intracellular signaling via the adenosine A_{2A} receptor, we determined elevation of cAMP levels (Fig. 2A) and increases in CREB phosphorylation (P-CREB) at Ser133 (Fig. 2B). CHO cells stably expressing the human adenosine A_{2A} receptor (Klotz et al., 1998) were stimulated with increasing concentrations of Alexa488-APEC for 15 min at 37°C. Subsequent analysis of cAMP production revealed a dose-dependent increase in cAMP (EC₅₀=12.8 nM (8.7 to 18.9 nM) mean (95% CI)) in CHO cells expressing the human adenosine A_{2A} receptor but not in untransfected control cells (Fig. 2A). The Alexa488-APEC-induced increase in cAMP levels was completely abolished by preincubation with the adenosine A₂ receptor-selective reversible, competitive antagonist ZM-241, 385 (10 µM) (Poucher et al., 1995) confirming the specificity of the ligand-receptor interaction (Fig. 1A). Further downstream in the signaling pathway, we also detected dose-dependent induction of P-CREB with an EC₅₀= 512.4 (155.8 to 1685) nM (mean, 95% CI) for Alexa488-APEC stimulation in adenosine A_{2A} receptor-transfected CHO cells but not in untransfected control cells (Fig. 2B). The adenosine A_{2A} receptor-induced response on P-CREB was sensitive to pretreatment with the cAMP-dependent protein kinase (PKA) inhibitor H89, arguing for a direct regulation of CREB through cAMP and PKA. For cAMP and P-CREB measurements, 10 µM of forskolin, a direct activator of adenylyl cyclase, served as a positive control.

With the aim to use Alexa488-APEC to trace adenosine A_{2A} receptor trafficking in living cells, we used a cyan fluorescent protein (CFP)-tagged adenosine A_{2A} receptor (A_{2A}-CFP, (Hoffmann et al., 2005)) transiently transfected into CHO cells. In unstimulated cells, A_{2A}-CFP was localized primarily to membranes suggesting that the mature receptor is properly folded and inserted into the plasma membrane (own results not shown, but see (Hoffmann et al., 2005)). Due to high background fluorescence, it was not possible to visualize adenosine A_{2A} receptors with Alexa488-APEC from time point zero of the stimulation without removing unbound agonist. Thus, we started evaluation of living cells 10 min after addition of Alexa488-APEC and three short washes in HBSS (Fig. 3). In transiently transfected CHO cells Alexa488-APEC and A_{2A}-CFP redistributed in parallel, with an almost complete overlap, from a localization predominantly in membranes to one in the cytosol, indicating that the Alexa488-APEC-bound A_{2A}-CFP underwent agonist-mediated endocytosis (Fig. 3A). To quantify the agonist-induced internalization of the human adenosine A_{2A} receptor, we used a FLAG-tagged version of the receptor and overexpressed it in HEK293 cells. For quantification of receptor internalization, we switched to HEK293 cells, which have a larger cytoplasmic volume than CHO cells, making it easier to distinguish between membranous and cytosolic labeling. We used the nonselective adenosine receptor agonist NECA (2 µM, 1 h) to study receptor redistribution in fixed cells after anti-FLAG immunocytochemistry by manual counting of three different categories: predominantly membranous, membranous and cytosolic, and predominant cytosolic localization of the FLAG-A_{2A} receptor (Fig. 3B). Indeed, a net internalization was detected, indicating that agonist stimulation promotes internalization of a proportion of the exogenously expressed adenosine A_{2A} receptors. In order to confirm the specificity of the interaction of Alexa488-APEC with adenosine A_{2A} receptors and to exclude a parallel constitutive internalization of Alexa488-APEC in endosomes, we used the competitive antagonist ZM 241,385. This compound was chosen for its selectivity to adenosine A_{2A} receptors and due to the fact that there is no detectable nonspecific binding in untransfected cells (Palmer et al., 1995). Figure 3C shows that Alexa488-APEC (10 µM) binding was almost completely abolished in the presence of 10 µM ZM 241,385. It is also important to note that Alexa488-APEC was neither associated with the membrane of untransfected cells, nor internalized in these cells, indicating that unspecific binding and uptake were negligible (not shown).

To further delineate the internalization mechanisms for adenosine A_{2A} receptors, we treated the cells with hyperosmolar sucrose, which is known to inhibit clathrin-mediated endocytosis (Heuser et al., 1989). As shown in Fig. 3D, hyperosmolar sucrose (0.45 M) prevented agonist-induced internalization of A_{2A}-CFP, pointing to adenosine A_{2A} receptor internalization through clathrin-mediated endocytosis. Indeed, co-localization immunocytochemistry experiments in unstimulated and NECA-stimulated cells confirmed this assumption since clathrin as a marker for clathrin-coated vesicles co-localized with FLAG-A_{2A} in agonist-treated cells (Fig. 4). Furthermore, NECA-stimulated A_{2A}-CFP receptors could also be found in Rab5-positive early endosomes as investigated in living cells overexpressing Rab5-GFP and A_{2A}-CFP (Fig. 4).

4. Discussion

Our results show that the moderately selective, novel compound Alexa488-APEC promotes cAMP production and CREB phosphorylation via the human adenosine A_{2A} receptor. Further, Alexa488-APEC can be used as a tool for the investigation of kinetics of adenosine A_{2A} receptor trafficking in living cells. We provide evidence that human adenosine A_{2A} receptors ectopically expressed in both CHO and HEK293 cells internalize upon agonist stimulation via clathrin-coated pit endocytosis promoting a localization of the receptor in early endosomes. Fluorescently-tagged receptors and ligands have been used for the investigation of the G_i-coupled adenosine A₁ and A₃ receptor trafficking (Matharu et al., 2001; Ferguson et al., 2002; Briddon et al., 2004; Milojevic et al., 2006; Middleton et al., 2007). However, regarding the G_s-coupled adenosine A_{2A} and A_{2B} receptors, little information on the kinetics of internalization and subcellular localization of internalized receptors is available.

The analysis of receptor dynamics using fluorescently labeled agonists is challenging since visualization is compromised by the ratio of excess fluorescence in the medium and bound fluorescence on the cell surface/receptor. To deal with this problem, we incubated with Alexa488-APEC for 10 min followed by three rapid rinses with HBSS, thus removing unbound ligand and enhancing the signal-to-noise ratio. However, this made it impossible to assess receptor redistribution during the early phase of stimulation dominated by receptor-ligand recognition. Another technical issue is related to relative receptor expression levels and detection levels of bound Alexa488-APEC. In our hands, it was not possible to detect Alexa488-APEC binding to adenosine A_{2A} receptors stably expressed in CHO cells. The receptor number on these cells, however, was calculated to be lower than that of GABAergic striatopallidal neurons (Kull et al., 1999). This suggests that Alexa488-APEC cannot be used for cells expressing low levels of adenosine A_{2A} receptors but could be useful for cells endogenously expressing high levels of this receptor, such as GABAergic striatopallidal neurons or leukocytes. The sensitivity of this assay could be enhanced by increasing the fluorescence yield or by the development of fluorescent ligands with higher selectivity and affinity. With regard to its limited selectivity, it is also important to address the usefulness of Alexa488-APEC for tracing adenosine A_{2A} receptors in cells expressing multiple adenosine receptor subtypes, especially those that express adenosine A_{2A} and A₃ receptors. These issues will be subjects of future investigations.

We show that agonist-stimulated adenosine A_{2A} receptors co-localize with clathrin and Rab5, indicating that endocytosis is mediated through clathrin-coated vesicles, which upon internalization mature to Rab5-positive early endosomes. This is confirmed by the fact that hyperosmolar sucrose, which blocks clathrin-coated vesicle formation (Heuser et al., 1989), also blocked adenosine A_{2A}-CFP receptor uptake. Co-localization with the Rab5-positive early endosomes suggests, further, that internalized adenosine A_{2A} receptors are being recycled (Drake et al., 2006) resulting in transient desensitization and resensitization as previously shown (Mundell et al., 1998a). Agonist-induced G protein-coupled receptor endocytosis

through clathrin-dependent mechanisms is known to be mediated by the multipurpose scaffold protein β -arrestin (Lin et al., 1997; Dewire et al., 2007). Indeed previous results show that adenosine A_{2A} receptors can internalize alone or in the presence of dopamine D_2 receptors (Hillion et al., 2002; Burgueño et al., 2003) and interact with β -arrestin in a G protein-coupled receptor kinase 2 (GRK2)-dependent manner (Khoa et al., 2006). Further it was shown that adenosine A_{2A} receptor desensitization, an event that is closely linked to receptor phosphorylation and endocytosis, is dependent on a conserved Thr298 (Palmer et al., 1997), important for agonist (CGS21680)-induced association with β -arrestin1 (Khoa et al., 2006). In this context it is worthwhile mentioning that the endosomal ligand/receptor/ β -arrestin complex was previously shown to represent an alternative high affinity ternary complex implicating that internalized receptors are ligand-bound (Gurevich et al., 1997).

Receptor internalization is – according to current knowledge – not only a negative feedback response to agonist stimulation resulting in desensitization, but also a means to promote the formation of signaling endosomes (Dewire et al., 2007). One of the best studied pathways activated by G protein-coupled receptor signaling endosomes is the mitogen-activated protein kinase pathway (Pierce et al., 2000). It was shown that two routes of extracellular signal-regulated kinase1/2 signaling are activated by G protein-coupled receptors: a rapid, transient pathway depending on the activation of heterotrimeric G proteins, and a slow and prolonged pathway based on β -arrestin scaffolding of the ERK1/2 cascade (Ahn et al., 2004). In the case of the adenosine A_{2A} receptor, however, a fast and rather transient activation of ERK1/2 was reported, which does not support β -arrestin-mediated endosomal signaling to ERK1/2 (Schulte et al., 2000). Thus, agonist-induced internalization of adenosine A_{2A} receptors might mainly serve desensitization purposes, even though other pathways possibly associated with endosomal signaling such as c-jun-N-terminal kinases or Src kinases have not been studied.

In summary, this basic characterization of the novel, fluorescent adenosine A_{2A} receptor agonist Alexa488-APEC shows that we have synthesized an appropriate tool for the study of receptor dynamics. Further, we report that adenosine A_{2A} receptors internalize in an agonist-dependent manner through clathrin coated vesicles leading to redistribution to Rab5-positive endosomes.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Fig. 1.

Inhibition of radioligand binding by Alexa488-APEC and its precursor APEC at three subtypes of adenosine receptors. Competition radioligand binding studies were done at the adenosine $\text{A}_{2\text{A}}$ (A. using agonist ^3H CGS21680; C. using antagonist ^3H ZM241,385), A_1 (B. using agonist ^3H CCPA), and A_3 (B. using agonist ^{125}I AB-MECA) receptors. For a summary of K_i values obtained refer to Table 1. Specific binding was approximately (cpm): A_1 , 500; $\text{A}_{2\text{A}}$, 1300 (agonist), 700 (antagonist); A_3 , 1600. Non-specific binding in each case was in the range of 20 – 30% of total counts. In D, cAMP production in CHO $\text{A}_{2\text{B}}$ cells in response to increasing concentrations of NECA and 10^{-5} M Alexa-488-APEC are shown (mean $\pm$ SD).

Fig. 2.

Alexa488-APEC is a functional agonist at the adenosine A_{2A} receptor. (A) Stimulation of CHO cells stably transfected with the human adenosine A_{2A} receptor induced cAMP production in a dose-dependent manner (EC_{50} =12.8 nM (8.7–18.9) mean (95% CI)). Agonist-induced cAMP production was completely abolished by preincubation with the competitive adenosine A₂ receptor antagonist ZM 241,385 (10 µM). 10 µM forskolin was used as a positive control. Data are normalized to maximal cAMP levels induced by 10 µM Alexa488-APEC. ‡ indicates a significant difference between Alexa488-APEC alone and in combination with ZM 241,385. n=3. (B) Stimulation of the human adenosine A_{2A} receptor stably expressed in CHO cells (filled squares) with increasing concentrations of Alexa488-APEC evoked a dose-dependent

increase in CREB phosphorylation (P-CREB), which was sensitive to PKA inhibition by H89. ‡ indicates a significant difference between Alexa488-APEC alone and in combination with H89. Forskolin (10 μ M) was used as a positive control. Results are normalized to maximal cAMP levels induced by 12.5 μ M Alexa488-APEC. The lower panel shows representative immunoblots of P-CREB in CHO A_{2A} as well as untransfected CHO cells (n=3). Untransfected CHO cells (open circles) did not respond to Alexa488-APEC. n=3 (mean $\pm$ SD).

Fig. 3. Specific interaction of Alexa488-APEC with adenosine A_{2A} receptors induced receptor internalization. (A) Living CHO cells transiently transfected with adenosine A_{2A} -CFP receptors were observed with a laser scanning microscope 10 – 60 min post stimulation with Alexa488-APEC. The bar graph in B shows a quantification of HEK293 cells expressing a FLAG- A_{2A} receptor showing predominantly cytosolic, membranous and cytosolic, or predominantly membranous receptor labeling. Three hundred cells per condition were manually counted. (C) Alexa488-binding is abolished in the presence of 10 μ M ZM 241,385. (D) Agonist (2 μ M NECA)-induced A_{2A} -CFP receptor internalization is blocked in the presence of hyperosmolaric sucrose.

Fig. 4.

Agonist-stimulated adenosine A_{2A} receptors internalize through clathrin-dependent endocytosis and localize to early endosomes. FLAG-A_{2A} receptors were expressed in HEK293 cells for immunocytochemistry using an anti-FLAG (Cy3, red) and anti-clathrin (Cy2, green) antibody. The upper panels show co-localization of FLAG-A_{2A} and clathrin in fixed, unstimulated cells and after 1 h stimulation with NECA (1 μM). The lower panels show co-localization of A_{2A}-CFP with Rab5-GFP. Living HEK293 cells co-transfected with A_{2A}-CFP (red) and Rab5-GFP (green) are shown before and after 1 h stimulation with 1 μM NECA. n=3. Arrows indicate some of the Rab5-positive and adenosine A_{2A} receptor-containing vesicles,

which were identified by live cell imaging as newly formed vesicles. Yellow color indicates co-localization.

Table 1
Comparison of the dissociation constant K_i of the parent APEC and its fluorescent derivative Alexa488-APEC with regard to agonist binding to adenosine A_1 , A_{2A} and A_3 receptor (NA, <10% displacement at 10^{-5} M) and antagonist binding at the A_{2A} receptor. Values are given as mean $\pm$ S.E.M.

Compound	A_{2A} agonist, K_i		A_3 K_i		A_1 K_i		A_{2A} antagonist, K_i	
	Mean (nM)	S.E.M. (nM)	Mean (nM)	S.E.M. (nM)	Mean (nM)	S.E.M. (nM)	Mean (nM)	S.E.M. (nM)
Alexa488-APEC	149	27	240	160	No binding	37	2300	410
APEC	35	5	20	2	450		215	80