Biochemical Pharmacology 147 (2018) 55–66

Contents lists available at ScienceDirect

Biochemical Pharmacology

journal homepage: www.elsevier.com/locate/biochempharm

Characterisation of endogenous A_{2A} and A_{2B} receptor-mediated cyclic AMP responses in HEK 293 cells using the GloSensor™ biosensor: Evidence for an allosteric mechanism of action for the A_{2B}-selective antagonist PSB 603

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ARTICLE INFO

Article history:
Received 21 August 2017
Accepted 23 October 2017
Available online 26 October 2017

Chemical Compounds studied in this article:
Adenosine (PubChem CID: 60961)
BAY 60-6583 (PubChem CID: 11717831)
CGS 21680 (PubChem CID: 10256643)
Forskolin (PubChem CID: 47936)
NECA (PubChem CID: 448222)
PSB 603 (PubChem CID: 44185871)
SCH 58261 (PubChem CID: 176408)
XAC (PubChem CID: 5697)
ZM 241385 (PubChem CID: 176407)

Keywords:
Adenosine receptor
Cyclic AMP
Kinetics
Allosterism
PSB 603
A_{2B} receptor

ABSTRACT

Endogenous adenosine A_{2B} receptors (A_{2B}AR) mediate cAMP accumulation in HEK 293 cells. Here we have used a biosensor to investigate the mechanism of action of the A_{2B}AR antagonist PSB 603 in HEK 293 cells. The A_{2A} agonist CGS 21680 elicited a small response in these cells (*circa* 20% of that obtained with NECA), suggesting that they also contain a small population of A_{2A} receptors. The responses to NECA and adenosine were antagonised by PSB 603, but not by the selective A_{2A}AR antagonist SCH 58261. In contrast, CGS 21680 responses were not antagonised by high concentrations of PSB 603, but were sensitive to inhibition by SCH 58261. Analysis of the effect of increasing concentrations of PSB 603 on the response to NECA indicated a non-competitive mode of action yielding a marked reduction in the NECA E_{MAX} with no significant effect on EC_{50} values. Kinetics analysis of the effect of PSB 603 on the A_{2B}AR-mediated NECA responses confirmed a saturable effect that was consistent with an allosteric mode of antagonism. The possibility that PSB 603 acts as a negative allosteric modulator of A_{2B}AR suggests new approaches to the development of therapeutic agents to treat conditions where adenosine levels are high.

1. Introduction

Adenosine acts via four (A_1, A_{2A}, A_{2B} and A_3) specific G protein-coupled receptors (GPCRs) [1]. The A_1 and A_3 receptors couple to Gi/o proteins and inhibit adenylyl cyclase activity whilst the A_{2A} and A_{2B} receptors preferentially couple to Gs proteins and stimulate the formation of cyclic AMP (cAMP) [1–3]. The crystal structure of the A_{2A} receptor (A_{2A}AR) in both antagonist [4] and agonist [5] bound conformations has been determined in recent years. The adenosine A_{2B} receptor (A_{2B}AR), which is closely related to the A_{2A}AR, is the least well defined of the four adenosine receptors and has low affinity for the endogenous agonist, adenosine [3,6]. A_{2B}ARs have been reported to have important roles in inflammation, fibrosis, angiogenesis and tumour progression [3,7–11] making them an important therapeutic target for drug discovery.

Abbreviations: A_{2A}AR, A2A adenosine receptor; A_{2B}AR, A2B adenosine receptor; BAY 60-6583, (2-[[6-Amino-3,5-dicyano-4-[4-(cyclopropylmethoxy)phenyl]-2-pyridinyl]thio]-acetamide; HEK293G, Human Embryonic Kidney 293 cell line stably expressing the GloSensor™ biosensor; DMEM, Dulbecco modified eagles medium; FCS, fetal calf serum; HBSS, HEPES buffered saline solution; NECA, 5′-(N-Ethylcarboxamido)adenosine; CGS 21680, 4-[2-[[6-Amino-9-(N-ethyl-β-D-ribofuranuronamidosyl)-9H-purin-2-yl]amino]ethyl]benzene propanoic acid hydrochloride; XAC, Xanthine amine congener; PSB 603, 8-[4-[4-(4-chlorophenzyl)piperazide-1-sulfonyl)phenyl]]-1-propylxanthine; ZM 241385, 4-(2-[7-Amino-2-(2-furyl)[1,2,4]triazolo[2,3-a][1,3,5]triazin-5-ylamino]ethyl)phenol; SCH 58261, 2-(2-Furanyl)-7-(2-phenylethyl)-7H-pyrazolo [4,3-e] [1,2,4]triazolo[1,5-c]pyrimidin-5-amine; cAMP, cyclic AMP; ADA, adenosine deaminase.

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https://doi.org/10.1016/j.bcp.2017.10.013
0006-2952/

Whilst there are a number of selective ligands available for the A_1, A_3 and A_{2A} receptors, these are more limited for A_{2B}AR [3,12–15]. However, a selective A_{2B}AR antagonist has been recently developed (PSB 603) which has also been used as a radioligand (^{3}H-PSB-603) [16]. This compound has been used to investigate the amino acids involved in the interaction of agonists and antagonists with A_{2B}AR [6]. This study showed that whilst Trp247, Val250 (both in transmembrane 6;TM6), and Ser279 (TM7) were important for the binding of nucleoside-based agonists, Leu81 (TM3), Asn186 (TM5) and Val250 (TM6) were crucial for binding of the xanthine-derived antagonist PSB 603 [6]. These data suggest that PSB 603 may bind to a different set of amino acids to those used by the endogenous ligand adenosine, and this raises the possibility of an allosteric mechanism of action.

Allosteric ligands bind to a topographically distinct site (allosteric) from that occupied by the endogenous agonist (orthosteric site) and elicit a conformational change that can lead to alterations in the affinity or efficacy of the ligand occupying the orthosteric binding site [17–20]. Key features of an allosteric mechanism of action are that the effect is saturable (i.e. reaches a limiting maximal effect), can depend on the specific ligand occupying the orthosteric site (probe dependence) and provides scope for both positive and negative effects on ligand binding and/or function [17–20]. An allosteric mechanism of action can provide a drug with a number of potential advantages such as introducing greater selectivity for the target, producing an effect that may depend on concurrent binding of the natural ligand and, in the case of negative allosteric regulators, a non-competitive effect that is resistant to high concentrations of the endogenous orthosteric agonist. This may have advantage for A_{2B}AR directed therapeutics that are designed to address conditions such as ischemia and inflammation where levels of adenosine may be very high.

In the present study we have characterised the cAMP responses elicited by endogenous A_{2B}AR expressed in HEK 293 cells [21] with particular reference to the potential for allosteric interactions. We also provide evidence for a minor population of endogenous A_{2A}AR in this cell line. To help with this characterisation, we have used the following adenosine receptor antagonists (their respective Ki values for A_{2A}AR and A_{2B}AR given in parentheses): XAC (1 nM [22], 73 nM [23]); ZM 241385 (1.4 nM, 32 nM [23]); SCH 58261 (0.6 nM, 5011 nM [24]) and PSB 603 (Ki > 10,000 nM, 0.5 nM [16]). We have studied real-time kinetic changes in cAMP levels using the GloSensor™ biosensor (Promega) in intact living cells [25,26]. The GloSensor™ technology is based on an engineered form of firefly luciferase encompassing a cAMP-binding domain from protein kinase A (RIIβB; [26]). Upon binding of cAMP, in the presence of the GloSensor™ substrate [26], the resultant conformational change in the GloSensor™ biosensor leads to light emission that can be detected by an automated plate-reader. This assay lends itself nicely to the study of GPCR mediated cAMP modulation in both endogenous and over expressed systems. For example, it has been used to study the $G\alpha_s$-coupled β2-adrenergic receptor found endogenously in HEK293 cells [27] or over-expressed in HEK293 cells, and used to dissect intracellular signalling [28]. Furthermore, $G\alpha_{i/o}$-coupled responses can be determined from their ability to inhibit forskolin-stimulated cAMP responses (e.g. for the metabotropic glutamate receptor expressed in CHO K1 cells [29]) or reduce basal levels of cAMP (e.g. the succinate receptor 1 in HEK293 cells [30]). Here we have used the GloSensor™ biosensor to study the pharmacological profile, and mechanism of action of PSB 603 as an antagonist, of G_s-coupled A_{2B}ARs endogenously expressed in HEK293 cells.

2. Materials and methods

2.1. Cultured cells

The cAMP GloSensor™ (20F) biosensor [26] expressed in HEK293 (HEK293G) cells was obtained from Promega (Madison, WI). HEK293G cells were maintained in Dulbecco modified eagles medium (DMEM) supplemented with 2 mM L-glutamine, 10% FCS (fetal calf serum) and 200 μg/ml hygromycin B at 37 °C 5% CO_2. Once confluent, cells were dislodged from the flask surface by gentle shaking after incubation in 0.25% trypsin and cell pellet formed following 5 min 1000 g centrifugation. For the GloSensor™ assay, cells were re-suspended in DMEM supplemented with 2 mM L-glutamine and 10% FCS and seeded at a density of 35000 cells/well on poly-l-lysine treated clear bottomed white walled 96 well plates. Cells were incubated at 37°C 5% CO_2 overnight prior to assay.

2.2. GloSensor™ assay

The GloSensor™ assay was carried out as per manufacturer's instructions (Promega, Madison, WI, USA). Briefly, this was as follows; Media was aspirated and cells were incubated in 100 μl HBSS (HEPES buffered saline solution pH 7.45; Sodium pyruvate 2 mM, NaCl 145 mM, D-Glucose 10 mM, KCL 5 mM, $MgSO_4{\cdot}7H_2O$ 1 mM, HEPES 10 mM, $CaCl_2$ 1.7 mM, $NaHCO_3$ 1.5 mM) containing 4–6% GloSensor™ cAMP reagent and incubated for 2 h at final experimental temperature of 35°C. Luminescence was measured on an EnVision® Multilabel Plate Reader (Perkin Elmer, Massachusetts, USA) continuously over 60 min, averaging 1 read per well every 1.5 min, following the addition of 100 μl HBSS in the presence or absence of Forskolin (10 nM–10 μM), NECA (5′-(N-Ethylcarboxamido) adenosine, 10 nM-30 μM), Adenosine (100 nM-100 μM), BAY 60-6583 (2-[[6-Amino-3,5-dicyano-4-[4-(cyclopropylmethoxy)phenyl]-2-pyridinyl]thio]-acetamide, 1 pM-30 μM) or CGS 21680 (4-[2-[[6-Amino-9-(N-ethyl-β-D-ribofuranuronamidosyl)-9H-purin-2-yl]amino] ethyl]benzene propanoic acid hydrochloride, 30 nM-30 μM). Antagonist action was monitored following 30 min pre-incubation with HBSS in the presence or absence of XAC (xanthine amine congener), PSB 603 (8-[4-[4-(4-Chlorophenzyl)piperazide-1-sulfonyl)phenyl]]-1-propylxanthine), ZM 241385 (4-(2-[7-Amino-2-(2-furyl)[1,2,4] triazolo[2,3-a][1,3,5]triazin-5-ylamino]ethyl)phenol or SCH 58261 (2-(2-Furanyl)-7-(2-phenylethyl)-7H-pyrazolo [4,3-e][1,2,4] triazolo [1,5-c]pyrimidin-5-amine).

2.3. Data analysis

Determinations of agonist potency, antagonist affinity and equilibrium dissociation constants were made by fitting data within GraphPad Prism version 5.03 for Windows (GraphPad Software, San Diego California USA, www.graphpad.com).

To obtain the antagonist equilibrium dissociation constants (K_B) a modified form of the Gaddum equation was used as described by Lazareno and Birdsall [31]:

$$K_B = \frac{IC_{50}}{[A]/EC_F - 1}$$

where IC_{50} is the molar concentration of antagonist (B) required to decrease by 50% the response mediated by the fixed molar concentration of agonist (A) in the absence of antagonist; and EC_F the molar concentration of agonist that, in the absence of antagonist, mediated the same response as that obtained in the presence of an IC_{50} concentration of antagonist. Agonist concentration response curves were simultaneously obtained (in the absence of antagonist).