Bioorganic & Medicinal Chemistry Letters 28 (2018) 202–206

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry Letters

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

AMP and adenosine are both ligands for adenosine 2B receptor signaling

Jessica K. Holien [a,*], Benjamin Seibt [b], Veena Roberts [b,c], Evelyn Salvaris [b], Michael W. Parker [a,d], Peter J. Cowan [b,c], Karen M. Dwyer [b,c,e]

[a] ACRF Rational Drug Discovery Centre, St. Vincent's Institute of Medical Research, Fitzroy, Victoria 3065, Australia
[b] Immunology Research Centre, St. Vincent's Hospital Melbourne, PO Box 2900, Fitzroy, Victoria 3065, Australia
[c] Department of Medicine, The University of Melbourne, PO Box 2900, Fitzroy, Victoria 3065, Australia
[d] Department of Biochemistry and Molecular Biology, Bio21 Molecular Science and Biotechnology Institute, The University of Melbourne, Fitzroy, Victoria 3010, Australia

ARTICLE INFO

Article history:
Received 4 August 2017
Revised 1 November 2017
Accepted 8 November 2017
Available online 20 November 2017

Keywords:
Adenosine
AMP
$A_{2B}R$
G_q activation
Docking
Homology model

ABSTRACT

Adenosine is considered the canonical ligand for the adenosine 2B receptor ($A_{2B}R$). $A_{2B}R$ is upregulated following kidney ischemia augmenting post ischemic blood flow and limiting tubular injury. In this context the beneficial effect of $A_{2B}R$ signaling has been attributed to an increase in the pericellular concentration of adenosine. However, following renal ischemia both kidney adenosine monophosphate (AMP) and adenosine levels are substantially increased. Using computational modeling and calcium mobilization assays, we investigated whether AMP could also be a ligand for $A_{2B}R$.

The computational modeling suggested that AMP interacts with more favorable energy to $A_{2B}R$ compared with adenosine. Furthermore, AMPαS, a non-hydrolyzable form of AMP, increased calcium uptake by Chinese hamster ovary (CHO) cells expressing the human $A_{2B}R$, indicating preferential signaling via the G_q pathway. Therefore, a putative AMP-$A_{2B}R$ interaction is supported by the computational modeling data and the biological results suggest this interaction involves preferential G_q activation. These data provide further insights into the role of purinergic signaling in the pathophysiology of renal IRI.

Introduction

Adenosine monophosphate (AMP) is generated by the sequential hydrolysis of extracellular adenosine triphosphate (ATP) and adenosine diphosphate (ADP) by the ectonucleotidase CD39 (NTPDase1, ectonucleoside triphosphate diphosphohydrolase-1). AMP is hydrolyzed by CD73 (ecto – 5′ – nucleotidase) to adenosine, which in turn is rapidly metabolized and cleared from the circulation. CD73 and the adenosine receptors (A_1R, $A_{2A}R$, $A_{2B}R$, A_3R) are widely expressed, implicating adenosine signaling in a diverse range of physiological and pathophysiological processes.

The signaling properties of extracellular AMP remain controversial. Over 30 years ago it was first reported that phosphorylated adenosine derivatives were low-affinity adenosine receptor agonists.[1] In 2004 it was proposed that the G-protein-coupled-receptor (GPCR) orphan receptor GPR80/99 was a receptor for extracellular AMP.[2] However, this was quickly discounted because of the endogenous expression of adenosine receptors on HEK293 cells used in the experiments.[3] In 2011 the authors of the update on the classification of adenosine receptors noted "there [was] no good evidence that adenine nucleotides can act on adenosine receptors without being degraded to nucleosides first".[4] However, in 2013 AMP was shown to play a role in thermoregulation mediated by the A_1R.[5] This was supported by *in vitro* studies using HEK293 cells[6] in which AMP activated the human A_1R with equivalent potency to adenosine.

The canonical ligand for all four receptors is adenosine, the potency of which varies according to receptor density and the type of response measured. The receptors are localized on the cell surface and belong to the GPCR family.[4] Structural information of the GPCR family as a whole has expanded rapidly.[7] Within the adenosine family of receptors the $A_{2A}R$ has been most thoroughly studied and the crystalline structure determined,[8] which has enhanced the understanding of both orthosteric and allosteric binding sites. In addition a number of studies have demonstrated that adenosine receptors can exist as homodimers and heterodimers, which may impact GPCR function (reviewed in 7).

The $A_{2B}R$ has been implicated in the pathophysiology of renal ischemic-reperfusion injury (IRI) by increasing post-ischemic blood flow[9] and limiting TNF-α release from neutrophils.[10] The $A_{2B}R$ is a G_s-coupled protein receptor and its activation increases

* Corresponding author.
E-mail address: jholien@svi.edu.au (J.K. Holien).
[e] Permanent address: School of Medicine, Faculty of Health, Deakin University. Locked Bag 20000, Geelong, Victoria 3220, Australia.

https://doi.org/10.1016/j.bmcl.2017.11.019
0960-894X/

intracellular adenylyl cyclase activity and cAMP levels. $A_{2B}R$ is also coupled to G_q proteins, which upon $A_{2B}R$ engagement activate phosphatidylinositol-phospholipase C, triggering a series of steps resulting in the opening of calcium channels. In many cells G_s coupling appears preferred.[4] The $A_{2B}R$ is widely expressed throughout the body. However, because of the relatively low potency of adenosine for this receptor it may not be fully activated under physiological conditions but rather activated in pathological states, such as hypoxia, when the pericellular adenosine concentration is significantly elevated. Indeed the $A_{2B}R$ is upregulated on the renal vasculature following ischemia,[10] mediated by the transcription factor HIF-1α,[11] and this heightened expression is maintained for at least 4 weeks.[12] The coordinated increase in the expression of $A_{2B}R$ and local concentration of adenosine in response to kidney ischemia implicates purinergic signaling in the pathophysiology of renal IRI. Indeed augmenting adenosine signaling, particularly via the $A_{2B}R$, prior to acute kidney ischemia disease mitigates injury (reviewed in 13).

Although the effects of adenosine receptor signaling in acute renal IRI have been attributed to the generation of high concentrations of adenosine, we have recently reported an increase in kidney levels of both AMP and adenosine, with a concomitant fall in ATP and ADP.[14] We have also previously demonstrated that both serum AMP and adenosine concentrations were increased following the intravenous injection of pro-inflammatory collagen.[15] This observation together with the evidence of increased $A_{2B}R$ expression in acute renal IRI, led to the hypothesis that AMP, in addition to adenosine, may be a ligand for the $A_{2B}R$. This potential interaction is investigated here using computational modeling and calcium mobilization assays.

Materials and methods

Homology modeling and docking

A homology model of $A_{2B}R$ was created based on the model of $A_{2A}R$ in complex with adenosine[17] and the first published $A_{2A}R$ crystal structure[16] using Modeller.[24] The stereochemical quality of the model was checked using ProCheck V 3.5.[18] This model was then used to dock adenosine, AMP and AMPαS using the Dock-Geom module within Sybyl-X 2.1.1 (Certara L.P.). A protomol was created based upon the position of adenosine as seen in the $A_{2A}R$ crystal structure. The bloat was increased to 2 Å (default 0) and threshold reduced to 0.35 (default 0.5). Full flexibility of the ligands, the protein hydrogens and protein heavy atoms was allowed. The top 20 solutions were retained for each ligand and were visually analyzed in Pymol (The PyMOL Molecular Graphics System, Version 1.8 Schrödinger, LLC).

Molecular dynamics

The molecular dynamics program NAMD[26] was used to minimize the docked complexes of $A_{2B}R$ with adenosine or AMP. These complexes were initially solvated with TIP3P water using the Solvate plugin within VMD version 1.9. The rotate to minimize volume was selected, the boundary reduced to 1.8 and the box padding increased to 20 Å in all directions; all other parameters were kept at default. Charges were then neutralized with NaCl using the autoionize plugin within VMD version 1.9 using default settings. Each structure was minimized and equalized for 1 ns (2 fentosecond time step) under the CHARMM27 all-atom force field at 298 K. Langevin dynamics were used with group pressure and Langevin piston were turned on. Trajectory snap shots were collected every picosecond.

Calcium immobilization assay

One confluent 175 cm^2 flask of GeneBLAzer® CHO-hA_{2B} cells (Invitrogen) were washed with phosphate buffered saline and cells removed with TrypLE. The reaction was stopped with DMEM containing dialyzed fetal bovine serum. 40,000 cells per well were seeded on a black 96-well plate with clear bottom and incubated overnight at 37 °C, 5% CO_2. The calcium assay was performed using the Fluo-4 Direct™ Calcium Assay Kit (Invitrogen) according to the manufacturer's instructions. Briefly, test compounds (obtained from Tocris Bioscience) were injected by FLUOstar Galaxy and fluorescence was immediately measured for 140 s each sample (excitation 485 nm and emission 520 nm). Calcium assays were performed as triplicates and the end concentration of DMSO was 1%. The average of measured fluorescence over a time interval was subtracted from the blank which also contained 1% DMSO.

cAMP assay

One confluent 175 cm^2 flask of GeneBLAzer® CHO-hA_{2B} cells (Invitrogen) was washed with phosphate buffered saline and cells removed with TrypLE. The reaction was stopped with DMEM containing dialyzed fetal bovine serum. 5000 cells per well were seeded on a black 384-well plate with clear bottom and incubated overnight at 37 °C, 5% CO_2. The cAMP assay was performed using the GeneBLAzer® ADORA2B-CRE-*bla* CHO-K1 Assay Protocol, Cat. No. K1780. Briefly, test compounds (obtained from Tocris Bioscience) were added to the cells and incubated for 5 h in 37 °C, 5% CO_2. This was followed by addition of Live-BLAzer™-FRET substrate Mixture and incubated in the dark for 2 h at room temperature. The fluorescent cAMP/FRET signal was then detected using the FLUOstar® Galaxy (excitation 410 nm and emission 530 nm). 5-(*N*-Ethylcarboxamido) adenosine (NECA) was used to generate the standard curve as it is a non-hydrolysable compound. All experiments were performed in triplicate.

Results and discussion

To explore the hypothesis that AMP was a ligand for $A_{2B}R$, we analyzed the docking potential of adenosine, AMP and AMPαS using computational modeling. Adenosine was chosen as the canonical ligand; AMP as the putative ligand and AMPαS as it is a non-hydrolysable analogue of AMP and was used for *in vitro* studies.

A homology model of $A_{2B}R$ was created using the combined $A_{2A}R$ crystal structures as a template.[16,17] The quality of the model was assessed, with 98.9% of the amino acids residing in the allowed regions of the Ramachandran plot. Furthermore, Procheck[18] states that the amino acid properties, including main-chain and side-chain parameters, bond lengths and bond angles were within or better than an average 2.5 Å resolution crystal structure, thus this model was deemed suitable for docking studies.

All three ligands (adenosine, AMP and AMPαS) were docked convincingly into the $A_{2B}R$ model. AMP and AMPαS docked in an almost identical manner (Fig. 1A), suggesting that AMPαS is a good substitute for AMP. Because of this, only AMP will be discussed. Interestingly, AMP scored significantly higher than adenosine, with docking scores of 8.4 and 6.8 respectively.

The adenosine rings of all ligands shared similar hydrophobic interactions with residues Leu 86, Phe 173, Glu 174, Met 182, Val 250, Met 272, Ile 276 and Asn 254. The adenosine rings also all contained hydrogen bonds between the side-chains of Asn 254 (with N1 of the adenine ring), Glu 174 (with amino group of adenine ring) and Ser 279 (with ribose oxygen) (Fig. 1B,C). Since the adenosine portion of all three ligands was orientated in the $A_{2B}R$ model