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Enhancement of inosine-mediated $A_{2A}R$ signaling through positive allosteric modulation

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ABSTRACT

Inosine is an endogenous nucleoside that is produced by metabolic deamination of adenosine. Inosine is metabolically more stable (half-life 15 h) than adenosine (half-life < 10 s). Inosine exerts anti-inflammatory and immunomodulatory effects similar to those observed with adenosine. These effects are mediated in part through the adenosine A_{2A} receptor ($A_{2A}R$). Relative to adenosine inosine exhibits a lower affinity towards the $A_{2A}R$. Therefore, it is generally believed that inosine is incapable of activating the $A_{2A}R$ through direct engagement, but indirectly activates the $A_{2A}R$ upon metabolic conversion to higher affinity adenosine. A handful of studies, however, have provided evidence for direct inosine engagement at the $A_{2A}R$ leading to activation of downstream signaling events and inhibition of cytokine production. Here, we demonstrate that under conditions devoid of adenosine, inosine as well as an analog of inosine 6-S-[(4-Nitrophenyl)methyl]-6-thioinosine selectively and dose-dependently activated $A_{2A}R$-mediated cAMP production and ERK1/2 phosphorylation in CHO cells stably expressing the human $A_{2A}R$. Inosine also inhibited LPS-stimulated TNF-α, CCL3 and CCL4 production by splenic monocytes in an $A_{2A}R$-dependent manner. In addition, we demonstrate that a positive allosteric modulator (PAM) of the $A_{2A}R$ enhanced inosine-mediated cAMP production, ERK1/2 phosphorylation and inhibition of pro-inflammatory cytokine and chemokine production. The cumulative effects of allosteric enhancement of adenosine-mediated and inosine-mediated $A_{2A}R$ activation may be the basis for the sustained anti-inflammatory and immunomodulatory effects observed in vivo and thereby provide insights into potential therapeutic interventions for inflammation- and immune-mediated diseases.

1. Introduction

The endogenous purine nucleoside, inosine is formed through the metabolic conversion of adenosine by the enzyme adenosine deaminase (ADA). It is produced both extracellularly as well as intracellularly during normal cell metabolism. Inosine has a longer half-life (15 h; [1]) relative to adenosine (< 10 s; [2]) and consequently, the basal level of inosine in the interstitial fluid can be 2–7 times higher than that of adenosine. In agreement with this observation, in pathological conditions, there is an increase in tissue inosine levels [3–6].

Resembling adenosine, inosine exerts a wide range of anti-inflammatory and immunomodulatory properties. These include inhibition of proinflammatory cytokine and chemokine production [7–9], induction of anti-inflammatory cytokine production [7], improvement of islet transplant survival [10], reduced multiorgan inflammation and prolonged survival in scurfy mice [11] as well as alleviation of clinical signs of myelin oligodendrocyte glycoprotein-induced experimental autoimmune encephalomyelitis [12], allergic lung inflammation [13], streptozotocin-induced non-obese type 1 diabetes [14], TNBS-induced colitis [15] and glycodeoxycholic acid-induced acute pancreatitis [16].

Inosine exerts anti-inflammatory and immunomodulatory effects through specific membrane bound G protein-coupled receptors (GPCRs) termed P_1-purinoceptors, also known as adenosine receptors (AR). There are four AR subtypes termed A_1R, $A_{2A}R$, $A_{2B}R$ and A_3R. Among them, $A_{2A}R$ plays a critical nonredundant role in down-regulating inflammation [17]. $A_{2A}R$ is coupled to the stimulatory G protein Gαs [18]. Adenosine engagement at the $A_{2A}R$ leads to an increase in intracellular cAMP levels as well as phosphorylation of signal-regulated kinase-1 and -2 (ERK1/2). Utilizing a combination of label-free, cell-based, and membrane-based functional assays in conjunction with an equilibrium agonist-binding assay we have recently provided in vitro evidence for direct inosine engagement at the $A_{2A}R$ and subsequent induction of downstream cAMP production and ERK1/2 phosphorylation [19].

GPCRs initiate signaling upon binding of cognate ligands at evolutionarily conserved sites termed orthosteric sites. In addition, GPCRs also contain allosteric sites that are topologically distinct from the orthosteric sites. Therefore, structural determinants of ligand binding at

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the orthosteric and allosteric sites are inherently different. Unlike orthosteric ligands, allosteric ligands have little or no intrinsic ability to activate GPCRs upon engagement at the allosteric site. They only modulate orthosteric ligand-mediated receptor function through conformational changes that manifest as altered affinity and/or efficacy of the receptors towards orthosteric ligands, hence they preserve the endogenous orthosteric ligand-mediated physiological responses. Allosteric ligands that enhance the orthosteric ligand-mediated responses are termed positive allosteric modulators (PAMs).

To examine the potential of allosteric enhancement of the $A_{2A}R$ function to alter inflammatory immune responses in vitro and in vivo, we developed a series of compounds with PAM activity. One of these compounds, AEA061, is a small molecule that meets the stringent criteria of a PAM of the $A_{2A}R$ [20]. AEA061 has no intrinsic activity towards either rat or human $A_{2A}Rs$ but enhances the affinity and maximal response of the receptors to adenosine. AEA061 is selective towards $A_{2A}R$. It does not affect potency or efficacy of A_1R or A_3R but increases the potency of $A_{2B}R$ by two-fold without altering efficacy (unpublished data). Positive allosteric modulation of the $A_{2A}R$ with AEA061 inhibits inflammatory cytokine and chemokine production in vitro and reduces circulating plasma TNF-α and MCP-1 levels and increases plasma IL-10 in endotoxemic $A_{2A}R$ intact, but not in $A_{2A}R$ deficient, mice [20].

We sought to further establish that inosine directly, and not through its conversion to adenosine via salvage pathways, activates the $A_{2A}R$. To this end, we examined the ability of inosine as well as the inosine analog 6-*S*-[(4-Nitrophenyl)methyl]-6-thioinosine (NBMPR) to activate the $A_{2A}R$ in cell-based and cell-free assays in the presence of ADA and AEA061. We demonstrated that inosine as well as NBMPR activates the $A_{2A}R$ in the presence and in the absence of ADA. Moreover, both inosine and NBMPR inhibit pro-inflammatory cytokine and chemokine production by $A_{2A}R$ intact, but not by $A_{2A}R$ deficient mouse splenic monocytes. We next sought to determine if inosine-mediated $A_{2A}R$ activation is amenable to allosteric modulation. We now present data supporting the hypothesis that positive allosteric modulation of the $A_{2A}R$ enhances inosine-mediated $A_{2A}R$ activation as demonstrated by increased cAMP production, ERK1/2 phosphorylation and inhibition of pro-inflammatory cytokine and chemokine production.

2. Materials and methods

2.1. Mice

Male BALB/cJ mice and $A_{2A}R$ null mice (C;129S-Adora2atm1Jfc/J; Jackson Laboratories) were housed at 68–72 °F with a 12 h light/dark cycle, fed normal rodent chow and water ad libitum and were kept in a pathogen-free environment. A protocol approved by the Animal Care and Use Committee of the Molecular Medicine Research Institute was used in this study.

2.2. Materials

CGS 21680, NBMPR, and ZM 241385 were purchased from Tocris Biosciences. Growth media and adenosine deaminase were obtained from Lonza and Worthington Biochemical Corporation respectively. Rolipram, adenosine, inosine, adenosine 5′-[α,β-methylene] diphosphate and LPS (*E. coli* O111:B4) and all the reagents (unless otherwise stated) were purchased from Sigma-Aldrich.

2.3. Cell culture

CHO-K1 cells stably expressing human $A_{2A}R$ (CHO-h$A_{2A}R$; [21]) were grown in DMEM/F-12 (1:1) supplemented with 10% FBS, 2 mM glutamine and G418 (0.2 mg/ml). All cells were maintained at 37 °C in a 5% CO_2 incubator.

2.4. Cell-based cAMP assay

CHO-h$A_{2A}R$ [21] cells were seeded in 96-well half-area white plates (Greiner bio-one; 5×10^3 cells/well) in the absence of G418 20 h prior to assay. Cells were either pretreated or left untreated (control) prior to stimulation. For the pretreatment, CHO-h$A_{2A}R$ cells were washed twice with Hanks' balanced salt solution (HBSS) and incubated in HBSS containing adenosine deaminase (ADA; 3 U/ml) and adenosine 5′[α,β-methylene] diphosphate (50 μM) for 15 min at 37 °C. Both control and pretreated cells were washed twice with HBSS and incubated with rolipram (50 μM), adenosine 5′-[α,β-methylene] diphosphate (50 μM), adenosine, inosine and NBMPR at indicated concentration(s) in the presence or in the absence of ZM 241385 (100 nM) for 10 min at 37 °C. Pretreated cells were also incubated with the same assay components in the presence of ADA (3 U/ml) for 10 min at 37 °C. Intracellular cAMP levels were quantified using an HTRF assay kit (Cisbio).

2.5. Cell-free membrane-based cAMP assay

HEK293-h$A_{2A}R$ cell membranes (PerkinElmer) were incubated in HBSS, containing adenosine 5′-[α,β-methylene] diphosphate (50 μM) and ADA (3 U/ml) at 37 °C for 20 min. Membranes were washed twice with 33 mM HEPES containing 0.1% Tween 20 and stimulated with the same buffer containing 100 μM ATP, 2 μM GTP, 10 μM GDP, 2 μM $MgCl_2$, 150 mM NaCl, 50 μM adenosine 5′-[α,β-methylene] diphosphate, 50 μM rolipram, ADA (3 U/ml) and NBMPR (0–300 μM) or CGS 21680 (100 nM) in the presence and in the absence of ZM 241385 (100 nM) in half-area white plates (Greiner bio-one; 4.5 μg protein/well) for 30 min at 37 °C. cAMP levels were quantified using an HTRF assay kit (Cisbio).

2.6. ERK1/2 phosphorylation assay

CHO-h$A_{2A}R$ cells [21] were seeded in 96-well plates (Greiner bio-one; 2.5×10^4 cells/well) in the absence of G418 20 h prior to assay. The medium was replaced with medium lacking serum and incubated for an additional 3 h. Cells were either pretreated or left untreated (control) prior to stimulation. For the pretreatment, CHO-h$A_{2A}R$ cells were washed twice with HBSS and incubated in HBSS containing ADA (3 U/ml) and adenosine 5′-[α,β-methylene] diphosphate (50 μM) for 15 min at 37 °C. Control as well as pretreated cells were washed with warm HBSS to remove ADA and incubated with adenosine 5′-[α,β-methylene] diphosphate (50 μM), inosine and AEA061 at indicated concentration(s) in the presence or in the absence of ZM 241385 (100 nM) for 10 min at 37 °C. Pretreated cells were also incubated with the same assay components in the presence of ADA (3 U/ml) for 10 min at 37 °C. The assay was terminated by aspirating the assay buffer and incubating cells with lysis buffer (50 μl/well) at room temperature with shaking for 10 min. Phospho ERK1/2 levels were detected using an Alphascreen Surefire kit (PerkinElmer) according to the manufacturer's suggested protocol. Briefly, 10 μl of the lysate was transferred to a ProxiPlate-384 (PerkinElmer) and incubated with 10 μl of assay detection mixture at room temperature in the dark for 2 h. Fluorescent emissions were quantified using an EnSpire multimode plate reader (PerkinElmer).

2.7. Cytokine assays

Splenic monocytes/macrophages were isolated from 8 to 10 weeks old male BALB/cJ mice and $A_{2A}R$ null mice by plastic adherence after incubation for 2 h at 37 °C and 5% CO_2, followed by washing with warm media to remove nonadherent cells. Monocytes/macrophages were seeded in 96-well plates (2×10^5 cells per well) and stimulated with lipopolysaccharide (LPS; 50 ng/ml) in RPMI 1640 containing 1% heat inactivated FBS for 4 h in the presence and in the absence of ADA (3 U/ml) and indicated concentration(s) of inosine, CGS 21680 and