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

Ajith A. Welihinda, **Manmeet Kaur**, **Kaviya S. Raveendran**, and **Edward P. Amento**
Molecular Medicine Research Institute, 428 Oakmead Parkway, Sunnyvale, CA 94085

Abstract

Inosine is an endogenous nucleoside that is produced by metabolic deamination of adenosine. Inosine is metabolically more stable (half-life 15h) 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.

Keywords

Inosine; $A_{2A}R$; PAM; cAMP; ERK1/2; cytokines

Corresponding author: Ajith A. Welihinda, Ph.D., awelihinda@mmrx.org.

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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 $G\alpha s$ [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 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, 2mM 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-hA_{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-hA_{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-hA_{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, 10μl 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 8–10 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 AEA061. Cytokine/chemokine levels in the culture supernatants were quantified using ELISA Max kits (BioLegend) and/or bead-based multiplex immunoassays (Eve Technologies).

2.8. Data analysis

Data were analyzed using GraphPad Prism Software. Dose response curves were generated by non-linear regression with a variable slope. Statistical comparisons of the two groups were compared using a paired t-test. Comparisons of multiple groups were performed using one-way ANOVA followed by Tukey's multiple comparisons test.

3. Results

3.1. Inosine dose-dependently induces A_{2A}R-mediated cAMP production

Utilizing a combination of label-free, cell-based, and membrane-based functional assays in conjunction with an equilibrium agonist-binding assay we have demonstrated that inosine is a low-affinity agonist at the A_{2A}R [19]. We have also provided evidence to dismiss the notion that exogenous inosine influences extracellular adenosine levels through the equilibrative nucleoside transporter 1 (ENT1) and ENT2 to activate the A_{2A}R in cellular assays [19]. To further establish that inosine directly activates the A_{2A}R, we evaluated inosine-mediated cAMP production by CHO-K1 cells stably transfected with human A_{2A}R (CHO-hA_{2A}R) in the presence of adenosine deaminase (ADA), an enzyme that eliminates adenosine by catalyzing the hydrolytic deamination of adenosine to inosine. To this end, we utilized a previously described approach that isolates and decreases background signaling and thereby enhances the cAMP signal generated by exogenous inosine [19]. In CHO-hA_{2A}R cells, adenosine induced cAMP production (Fig 1A). The A_{2A}R inverse agonist ZM 241385 blocked the adenosine-mediated cAMP production indicating that the adenosine effects are mediated through the A_{2A}R. Addition of ADA at 3 U/ml (Fig 1A) and 6 U/ml (data not shown) both significantly reduced baseline cAMP levels and completely abolished adenosine-mediated cAMP production by CHO-hA_{2A}R cells suggesting that 3 U/ml of ADA is sufficient to rapidly convert high-affinity agonist adenosine to low-affinity agonist inosine. In the presence of the same concentration of ADA, exogenous inosine increased cAMP production by CHO-hA_{2A}R cells that were blocked by ZM 241385 demonstrating that inosine directly engages the A_{2A}R and is a bonafide agonist at this receptor. Moreover, dose response analysis indicated that ADA increased the EC_{50} without altering E_{max} for inosine-mediated cAMP production in CHO-hA_{2A}R cells (Fig 1B).

3.2. Inosine analog 6-S-[(4-Nitrophenyl)methyl]-6-thioinosine induces A_{2A}R-mediated cAMP production

The inosine analog 6-*S*-[(4-Nitrophenyl)methyl]-6-thioinosine (NBMPR) is a high- and low-affinity inhibitor of ENT1 and ENT2 respectively [22]. ENT1 and ENT2 are major transporters of adenosine and inosine across cell membranes [23]. Therefore, NBMPR has been used in cell-based AR activation assays to reduce the potential impact of cellular uptake, intracellular production and transport of adenosine and inosine. As NBMPR structurally resembles inosine, we examined whether NBMPR induces cAMP production by CHO-hA_{2A}R cells. As shown in Fig 2A, both adenosine as well as NBMPR activated A_{2A}R-mediated cAMP production by CHO-hA_{2A}R cells. To rule out the possibility that NBMPR-dependent activation of the $A_{2A}R$ is not due to an increase in extracellular adenosine upon NBMPR addition via some hitherto unknown mechanism(s), we included ADA in the assay. The addition of ADA at 3 U/ml reduced basal as well as adenosine-induced cAMP production by CHO-$hA_{2A}R$ cells (Fig 2A). In addition, both adenosine- and NBMPR-mediated cAMP production were inhibited by the $A_{2A}R$ inverse agonist ZM 241385 in the presence and in the absence of ADA indicating selectivity of the two agonists. These results suggest that the inosine analog NBMPR stimulates the $A_{2A}R$ resembling the activation seen with inosine.

We demonstrated previously that inosine activates cAMP production by HEK293-$hA_{2A}R$ cytosol-free membrane preparations indicating that inosine directly activates the $A_{2A}R$ [19]. This cell-free assay is completely devoid of potential confounding variables such as influx, intracellular production and efflux of adenosine and inosine. To further establish inosine agonism at the $A_{2A}R$ we evaluated the efficacy of the inosine analog NBMPR on $A_{2A}R$ activation in HEK293-$hA_{2A}R$ membrane preparations. As shown in Fig 2B, NBMPR dose-dependently increased cAMP production and the $A_{2A}R$ inverse agonist ZM 241385 blocked NBMPR-induced cAMP production by HEK293-$hA_{2A}R$ cytosol-free membrane preparations. These results indicate that the inosine analog NBMPR specifically and directly activates the $A_{2A}R$.

3.3. Positive allosteric modulation enhances inosine-mediated $hA_{2A}R$ activation

AEA061, a small molecule that meets the stringent criteria of a positive allosteric modulator (PAM) of the $A_{2A}R$, enhances adenosine-mediated $A_{2A}R$ activation and exerts pharmacological activity in a mouse model of endotoxemia [20]. We sought to examine whether inosine-mediated $A_{2A}R$ activation is also amenable to functional enhancement by AEA061. To this end, we evaluated the effects of AEA061 on inosine-mediated cAMP production by CHO-$hA_{2A}R$ cells, doing so in the presence of ADA to eliminate adenosine. As shown in Figure 3A, inosine induced cAMP production ($p < 0.001$) and the $A_{2A}R$ inverse agonist ZM 241385 blocked this induction ($p < 0.001$). AEA061 by itself did not induce cAMP production. However, it enhanced inosine-mediated cAMP production ($p < 0.05$) that was again blocked by the $A_{2A}R$ inverse agonist ZM 241385 ($p< 0.01$). These results demonstrate that AEA061 has no intrinsic activity towards $hA_{2A}R$-mediated cAMP production, but can allosterically augment inosine-driven $hA_{2A}R$-mediated cAMP production.

ADA is a multifunctional protein with intracellular and extracellular localization. In addition to converting adenosine to inosine, ADA physically interacts with the $A_{2A}R$ and allosterically modulates adenosine binding to the $A_{2A}R$ [24, 25]. To fully understand the effect of AEA061 on inosine-mediated $A_{2A}R$ activation in the context of the natural cellular milieu, we examined the dose response of inosine at the $hA_{2A}R$ in CHO-$hA_{2A}R$ cells under several assay conditions. CHO-$hA_{2A}R$ cells were pretreated with ADA and adenosine 5′-[α,β-methylene] diphosphate, an inhibitor of ecto-5′-nucleotidase to prevent exogenous production of adenosine, then washed and stimulated in the presence or in the absence of ADA with inosine. Regardless of the treatment condition, AEA061 increased the E_{max} (maximal response) for inosine-induced $hA_{2A}R$-mediated cAMP production (Fig 3B).

Relative to the control (without the pretreatment), both ADA pretreatment and ADA pretreatment plus subsequent ADA addition increased the EC_{50} of inosine-mediated cAMP production (decreased affinity of the $hA_{2A}R$; Fig 3C). AEA061 reduced the EC_{50} of inosine (increased $hA_{2A}R$ affinity to inosine) in ADA pre-treated CHO-$hA_{2A}R$ cells ($p < 0.01$) but not in cells without the ADA pretreatment alone or with ADA pretreatment and subsequent ADA addition. These results suggest that the positive allosteric modulator AEA061 exhibits differential effects on the affinity and efficacy of the $A_{2A}R$ to inosine. AEA061 enhances efficacy of the $A_{2A}R$ towards inosine both in the presence and absence of membrane-bound adenosine and ADA. However, AEA061 enhances $A_{2A}R$ affinity to inosine only in the absence of membrane-bound adenosine and ADA.

3.4. Positive allosteric modulation enhances inosine-mediated $hA_{2A}R$-dependant ERK1/2 activation

We demonstrated previously that $A_{2A}R$ activation by both adenosine and inosine leads to ERK1/2 phosphorylation [19]. To determine whether the PAM of the $A_{2A}R$, AEA061, potentiates inosine-inducible, $A_{2A}R$-mediated ERK1/2 activation, we evaluated the effects of AEA061 on ERK1/2 phosphorylation in CHO-$hA_{2A}R$ cells. Consistent with our previous observations, inosine increased ERK1/2 phosphorylation (Fig 4A) indicating that inosine effects are mediated through the $A_{2A}R$. Moreover, AEA061 increased both basal and inosine-induced ERK1/2 phosphorylation (Fig 4A). The $A_{2A}R$ inverse agonist ZM 241385 reversed inosine-, AEA061- and inosine plus AEA061-mediated ERK1/2 phosphorylation. Dose response analyses indicated that AEA061 alone enhanced the E_{max} (maximal response; Fig 4B) regardless of the assay conditions. AEA061 reduced EC_{50} (increased affinity) to inosine with and without the ADA pre-treatment (Fig 4C). Although not statistically significant, AEA061 lowered the EC_{50} to inosine in CHO-$hA_{2A}R$ cells with ADA pre-treatment and subsequent ADA addition. These results collectively suggest that both AEA061- and inosine-induced ERK1/2 phosphorylation are mediated through the $A_{2A}R$ and that AEA061 functions both as an agonist as well as a PAM with respect to ERK1/2 activation, a sharp contrast to its effect on cAMP activation where AEA061 functions solely as a PAM.

3.5. Inosine suppresses the production of pro-inflammatory cytokines through the $A_{2A}R$

Inosine exerts anti-inflammatory effects through inhibition of the production of pro-inflammatory cytokines and chemokines [7, 26–28]. Using pharmacological tools, Haskó et al. [7] demonstrated that inosine-mediated suppression of TNF-α production is mediated through both A_1R and $A_{2A}R$ in vitro. To further assess the role of $A_{2A}R$ in inosine-mediated suppression of cytokine production, we investigated inosine's effects on cytokine production by monocytes isolated from $A_{2A}R$ intact and deficient mice. To distinguish adenosine effects from those of inosine, we utilized ADA which converts adenosine to inosine. Consistent with the role of the $A_{2A}R$, activation of the receptor with the selective agonist CGS 21680 in the absence and in the presence of ADA inhibited TNF-α production by $A_{2A}R$-intact but not $A_{2A}R$-deficient monocytes (Fig 5A and 5B). In the absence of ADA, inosine at 30 μM and 100 μM inhibited LPS-stimulated TNF-α production by 18.6 ± 3.7% ($p < 0.01$) and 30.1 ± 1.3% ($p < 0.001$) by $A_{2A}R$ intact monocytes respectively (Fig 5A). The same concentrations of inosine inhibited LPS-stimulated TNF-α production by 5.2 ± 1.3% and

16.3 ± 0.7% ($p < 0.001$) in $A_{2A}R$ deficient monocytes. These results indicate that inosine-mediated inhibition of TNF-α production is reduced by 46% ($p < 0.01$) in $A_{2A}R$-deficient monocytes compared with receptor-intact monocytes suggesting that inosine-mediated inhibition of TNF-α at least in part mediated through the $A_{2A}R$. The inclusion of ADA in the assay prevents $A_{2A}R$ activation by adenosine as it converts extracellular adenosine produced by monocytes to inosine. When the confounding effects of adenosine were eliminated, inosine-mediated inhibition of LPS-stimulated TNF-α production continued to be reduced in both $A_{2A}R$ intact and $A_{2A}R$ deficient monocytes (Fig 5B). In the presence of ADA, inosine at 30 μM and 100 μM inhibited LPS-stimulated TNF-α production by 13.2 ± 1.9% ($p < 0.01$) and 16.8 ± 1.6% ($p < 0.01$) in $A_{2A}R$ intact monocytes respectively (Fig 5B). The same concentrations of inosine inhibited LPS-stimulated TNF-α production by 5.2 ± 2.2% and 12.4 ± 1.2% ($P < 0.01$) in $A_{2A}R$ deficient monocytes, indicating a 2.5- and a 1.4-fold reduction in the inhibition of TNF-α production in $A_{2A}R$ deficient monocytes in comparison with $A_{2A}R$-intact monocytes at 30 μM and 100 μM inosine respectively. These results indicate that inosine-mediated $A_{2A}R$ activation leads to inhibition of TNF-α production as is the case for adenosine-mediated $A_{2A}R$ activation. Inhibition of TNF-α production in $A_{2A}R$ deficient monocytes by inosine and not by $A_{2A}R$ selective agonist CGS 21680 suggests that inhibitory effects of inosine are also mediated through other adenosine receptor subtype(s).

3.6. PAM of the $A_{2A}R$ enhances inosine-mediated suppression of pro-inflammatory cytokines production

To examine whether allosteric enhancement of inosine-mediated $A_{2A}R$ stimulation leads to increased suppression of cytokine production, we evaluated the effects of inosine in the presence and in the absence of AEA061 on TNF-α production by LPS-stimulated monocytes. To assess the potential for $A_{2A}R$ activation by adenosine produced via the salvage pathway, we performed the assays with and without ADA. Consistent with the anti-inflammatory role of the $A_{2A}R$, selective activation of this receptor with CGS 21680 in the absence of ADA inhibited TNF-α production by LPS-stimulated $A_{2A}R$-intact monocytes by 39% and the PAM of the $A_{2A}R$ enhanced this CGS 21680-mediated inhibition to 49% (Fig 6A; $p < 0.001$). Under the same conditions, CGS 21680 alone and in combination with AEA061 reduced LPS-stimulated production in $A_{2A}R$ deficient monocytes by only 6.7% and 14% respectively ($p < 0.001$; Fig 6A) demonstrating the inhibitory effects of these agents are mediated through the $A_{2A}R$. Similarly, both inosine and AEA061 as single agents dose-dependently inhibited TNF-α production by LPS-stimulated $A_{2A}R$ intact monocytes in the absence of ADA. In combination, they produced even greater inhibition than either agent alone indicating allosteric enhancement (Fig 6A). Although the higher concentration of AEA061 (0.3 μM) both alone and in combination produced a greater percent inhibition of TNF-α production by $A_{2A}R$ intact monocytes than AEA061 at 0.1 μM, the difference in percent inhibition of TNF-α production between $A_{2A}R$ intact and deficient monocytes had narrowed. This suggests loss of selectivity of AEA061 towards the $A_{2A}R$ at higher concentrations. In the presence of ADA (Fig 6B), inosine and AEA061 produced the same general pattern of inhibition of TNF-α production by $A_{2A}R$ intact and deficient monocytes. However, the overall percent inhibition of TNF-α production was somewhat lower in the presence of ADA relative to the absence of ADA. Inhibition of production of the pro-inflammatory chemokine CCL3 by inosine and AEA061 in the absence of ADA (Fig 6C) closely resembles that of TNF-α (Fig 6A) in $A_{2A}R$ intact and deficient monocytes under the same conditions. However, in the presence of ADA, the difference in CCL3 inhibition (Fig 6D) between $A_{2A}R$ intact and deficient monocytes was more prominent. Inosine and AEA061 also individually inhibited CCL4 production by monocytes (Fig 6E) and the combination of these two agents produced greater inhibition than each agent alone suggesting allosteric potentiation of the $A_{2A}R$. The addition of ADA reduced the inhibition of CCL4 production under the same conditions, but increased the difference in inhibition between $A_{2A}R$ intact and deficient monocytes (Fig 6F). Collectively, these results indicate that positive allosteric potentiation of inosine-mediated $A_{2A}R$ activation inhibits pro-inflammatory cytokine/chemokine production by monocytes.

Discussion

Inosine, a metabolite of adenosine, exerts anti-inflammatory and immunomodulatory effects in vivo [28]. These effects are at least in part mediated through the $A_{2A}R$, a member of a class of purinergic G protein-coupled receptors. Inosine is less potent than adenosine in activating the $A_{2A}R$ [29]. Hence, the prevailing notion is that the inosine effects observed in vivo are not generated by direct inosine engagement at the $A_{2A}R$ but rather mediated indirectly through receptor activation by adenosine produced from the metabolic conversion of inosine. However, a handful of studies have provided initial circumstantial evidence for direct activation of $A_{2A}R$ by inosine [7, 13, 27]. Recently, utilizing a combination of label-free, cell-based, and membrane-based functional assays in conjunction with an equilibrium agonist-binding assay we demonstrated that inosine directly engages the $A_{2A}R$ and activates signaling events downstream of the receptor leading to cAMP production and ERK1/2 phosphorylation [19]. In the present study, we sought to provide additional proof for $A_{2A}R$ activation through direct engagement of inosine at the receptor as well as determine if inosine-mediated engagement of the receptor was amenable to allosteric modulation.

We employed several approaches to rule out indirect and confounding effects of adenosine on $A_{2A}R$ activation. We pretreated cells with adenosine deaminase (ADA) to rid the cells of endogenously produced adenosine and adenosine 5′-[α,β-methylene] diphosphate, an inhibitor of ecto-5′-nucleotidase, to halt endogenous/exogenous production of adenosine and inosine. Moreover, we utilized ADA during the treatment phase of the assay to eliminate adenosine produced during the short duration of the $A_{2A}R$ activation assay. Under these conditions, exogenous inosine as well as the inosine analog NBMPR but not adenosine stimulated activation of the $A_{2A}R$. This inosine- and NBMPR-mediated $A_{2A}R$ activation was dose-dependent and was inhibitable by the $A_{2A}R$ inverse agonist ZM 241385 providing strong evidence for inosine agonism at this receptor.

Our data indicate that inosine inhibits pro-inflammatory cytokine and chemokine production in an $A_{2A}R$-dependent manner in vitro. The level of inhibition of pro-inflammatory cytokine production achieved with inosine in mouse monocytes is consistent with that published by Haskó et al. [7] and is also in agreement with the EC_{50} values for inosine-mediated $A_{2A}R$ activation in CHO-h$A_{2A}R$ cells reported here. These results strongly support the contention that the activation of the $A_{2A}R$ by inosine leads to a reduction in the production of pro-inflammatory cytokines and chemokines. Our results also indicate that ADA in general reduced the inhibition of pro-inflammatory cytokine and chemokine production in both $A_{2A}R$ intact and deficient monocytes. This is consistent with the fact that extracellular adenosine produced by monocytes/macrophages during the assay is rapidly deaminated by exogenous ADA to inosine that has lower affinity than adenosine at the $A_{2A}R$, hence lower inhibition of TNF-α production by $A_{2A}R$ intact monocytes. The reduced inhibition of TNF-α production in $A_{2A}R$-deficient monocytes in the presence of ADA suggests that inosine is also less potent than adenosine in activating $A_{2A}R$-independent pathways [30, 31] leading to inhibition of TNF-α production.

On the basis of crystal structure predictions and site-directed mutagenesis studies, the critical residues that form the adenosine binding pocket in the $A_{2A}R$ have been identified [32, 33]. The nature of the interactions of inosine with these residues may explain the observed low potency of the receptor to inosine [19, 34]. To better understand the molecular mechanism of $A_{2A}R$ recognition by adenosine and inosine, Deganutti et al. [35] utilized supervised molecular dynamics simulation, a computational method that allows identification and characterization of multiple stable receptor conformations such as orthosteric, allosteric and meta-binding states. They reported an overlap of meta-stable states predicted for binding of inosine and adenosine to the $A_{2A}R$ suggesting that both agonists share a common molecular mechanism for receptor activation [35]. Their findings are in agreement with our previous [19] as well as present experimental evidence for functional agonism of inosine at the $A_{2A}R$ and explain the molecular mechanism of inosine-mediated receptor activation.

Inosine and the inverse agonist ZM 241385 both bind to the same site at the $A_{2A}R$ with different affinities (inosine has a lower affinity). Consistent with these observations, ZM 241385 at 100 nM completely reversed the effects of 100 μM inosine in CHO-hA2AR cells. However, the same concentration of ZM 241385 (100 nM) was not able to fully reverse the effects mediated by inosine at a higher concentration (1000 μM). This incomplete inhibition is most likely due to mass action as the parental CHO-K1 cells do not express any adenosine receptor subytpes and in addition, the ZM 241385 concentration used was 10,000–fold less than that of inosine. Partial inhibition of NBMPR-mediated $A_{2A}R$ activation by ZM 241385 may be explained also by the law of mass action.

The molecular mechanism of $A_{2A}R$ activation is consistent with conformational selection where binding of agonists and inverse agonists/antagonists stabilizes the receptor in active and inactive conformations respectively [36]. The active conformation(s) engage Gs and adenylate cyclase to produce cAMP whereas the inactive conformation(s) do not. Binding of a PAM such as AEA061 to the $A_{2A}R$ facilitates this process as indicated by enhanced efficacy and potency of the agonist at the receptor [20]. Since AEA061 does not activate $A_{2A}R$-mediated cAMP production in the absence of an agonist [20], AEA061 by itself does not stabilize $A_{2A}R$ conformation(s) that engage Gs. Depending on the cell type, agonist engagement at the $A_{2A}R$ leads to Gs-mediated, cAMP-dependent and small G protein $p21^{ras}$-mediated, cAMP-independent ERK1/2 phosphorylation [37, 38]. Therefore, it is conceivable that AEA061 by itself, in the absence of an agonist, stabilizes an $A_{2A}R$

conformation(s) that is capable of engaging p21^{ras} to activate ERK1/2, thereby exhibiting an agonist-like behavior.

Previously we demonstrated that the synthetic small molecule AEA061 does not activate the $A_{2A}R$ by itself but augments adenosine-mediated $A_{2A}R$ activation, and thus bears the hallmarks of positive allosteric modulation [20]. Our data indicate that AEA061 also enhances inosine-mediated $A_{2A}R$ responsiveness in the base-line state as well as under conditions that isolate and eliminate the confounding effects of adenosine. Although the PAM of the $A_{2A}R$, AEA061, enhances the maximal response of inosine-mediated cAMP production and ERK1/2 phosphorylation under all three assay conditions, it exhibits differential effects with respect to EC_{50} for cAMP production and ERK1/2 activation. AEA061 reduces the EC_{50} for inosine-mediated ERK1/2 phosphorylation regardless of the assay condition. In contrast, AEA061 reduces the EC_{50} for inosine-mediated cAMP production with ADA pretreatment and subsequent ADA addition. It is possible that ADA, in itself an allosteric modulator of the $A_{2A}R$ [24, 25], differentially influences AEA061 effects on cAMP and ERK1/2 pathways.

Our data indicate that positive allosteric modulation of the $A_{2A}R$ by AEA061 enhances inosine-mediated inhibition of pro-inflammatory cytokine and chemokine production by splenic monocytes/macrophages. Inhibition of cytokine and chemokine production by AEA061 alone in the absence of an exogenously added agonist is consistent with the fact that cells produce extracellular adenosine and inosine during normal cell metabolism in amounts sufficient to engage the $A_{2A}R$ allowing allosteric enhancement with AEA061. As AEA061 exhibits no allosteric effects towards A_1R and A_3R and weak activity towards $A_{2B}R$ (unpublished data), the $A_{2A}R$-independent inhibition of cytokine production by AEA061 at higher concentrations may be mediated through the low-affinity $A_{2B}R$. Although inosine does not activate $A_{2B}R$ in stable cell lines overexpressing the receptor in vitro [19, 29], $A_{2B}R$ is the principal mediator of inosine activity in the bladder [39]. Therefore, it is conceivable that the inhibition of cytokine and chemokine production observed in $A_{2A}R$ deficient monocytes with AEA061 in the presence of exogenously added ADA is due to enhancement of extracellularly produced inosine mediating $A_{2B}R$ activation. Further investigation will be necessary to rigorously examine this possibility.

In summary, the major outcomes of the present investigation are the demonstration of inosine agonism at the $A_{2A}R$, the amenability of inosine-mediated $A_{2A}R$ activation to allosteric modulation and the effect of inosine- and PAM- mediated $A_{2A}R$ activation on pro-inflammatory cytokine and chemokine production. Our data indicate that, as is the case for adenosine, positive allosteric modulation of the $A_{2A}R$ with AEA061 potentiates inosine-mediated cAMP production, ERK1/2 phosphorylation and inhibition of pro-inflammatory cytokine and chemokine production. We previously proposed that both adenosine and inosine are natural agonists of the $A_{2A}R$ and are important to mount a robust and effective immunomodulatory response. Adenosine with a shorter half-life (<10 sec) initiates $A_{2A}R$ activation and upon conversion to metabolically more stable inosine (half-life 15 h) sustains $A_{2A}R$ signaling to prolong anti-inflammatory and immunomodulatory responses. The PAM of the $A_{2A}R$ AEA061 potentiates and preserves the natural temporal and physical pattern of both adenosine-mediated and inosine-mediated $A_{2A}R$ activation to harness the full potential of the endogenous A_{2A}R-dependent mechanisms tasked to effectively downmodulate inflammation. Given the intricacies of GPCR signaling, it is conceivable that potentiation of adenosine- and inosine-mediated A_{2A}R activation through a PAM may have qualitatively and quantitatively different immunomodulatory responses as opposed to A_{2A}R activation through a high-affinity agonist. If proven, positive allosteric modulation of the A_{2A}R may provide new insights into potential therapeutic interventions for immune- and inflammation-mediated diseases.

Acknowledgments

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Abbreviations

ADA	adenosine deaminase
Ado	adenosine
AR	adenosine receptor
A_1R	adenosine A_1R
A_{2A}R	adenosine A_{2A} receptor
A_{2B}R	adenosine A_{2B} receptor
A_3R	adenosine A_3 receptor
cAMP	cyclic adenosine monophosphate
CGS 21680	4-[2-[[6-Amino-9-(N-ethyl-β-D-ribofuranuronamidosyl)-9H-purin-2-yl]amino]ethyl] benzenepropanoic acid hydrochloride
ENT1	equilibrative nucleoside transporter 1
ENT2	equilibrative nucleoside transporter 2
ERK1/2	extracellular signal-regulated kinase-1 and -2
GPCR	G protein-coupled receptor
HBSS	Hanks’ balanced salt solution
HEPES	4-(2-Hydroxyethyl) piperazine-1-ethanesulfonic acid, N-(2-Hydroxyethyl)piperazine-N′-(2-ethanesulfonic acid
HTRF	homogeneous time resolved fluorescence
Ino	inosine
LPS	lipopolysaccharide
NBMPR	6-*S*-[(4-Nitrophenyl)methyl]-6-thioinosine

PAM	positive allosteric modulator
PBS	phosphate buffered saline
SD	standard deviation
SEM	standard error of the mean
TNBS	2,4,6-trinitrobenzenesulfonic acid
ZM 241385	4-(2-[7-Amino-2-(2-furyl)[1,2,4]triazolo[2,3-a][1,3,5]triazin-5-ylamino]ethyl)phenol

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Highlights

- Under conditions devoid of adenosine, inosine dose-dependently activates the $A_{2A}R$.
- Inosine-mediated $A_{2A}R$ activation increases cAMP and phospho-ERK1/2 levels.
- Inosine inhibits TNF-α, CCL3 and CCL4 production by monocytes via $A_{2A}R$ activation.
- PAM of the $A_{2A}R$ potentiates inosine-mediated receptor signaling.
- PAM of the $A_{2A}R$ inhibits TNF-α, CCL3 and CCL4 production.

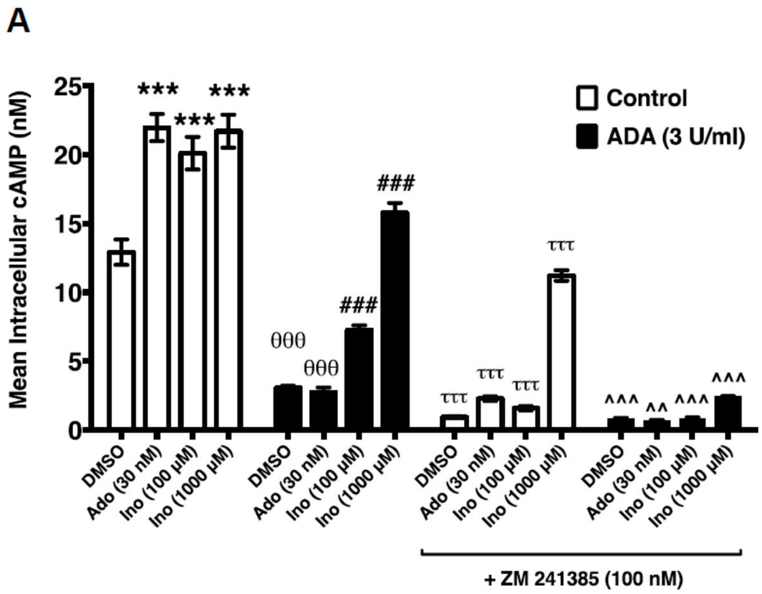


Fig. 1a

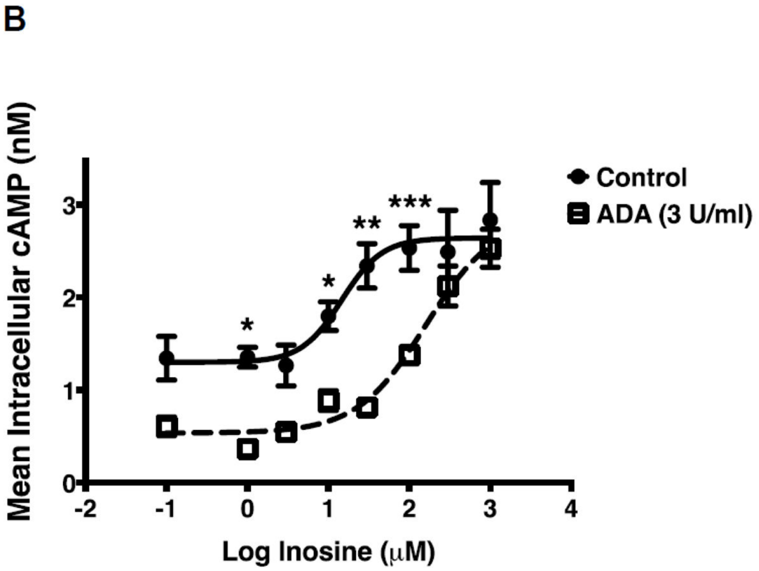


Fig. 1b

Fig. 1.

Inosine directly and dose-dependently stimulates $hA_{2A}R$-mediated cAMP production. CHO-$hA_{2A}R$ cells were incubated with adenosine (control) or inosine with and without ADA (3 U/ml) and in the presence/absence of the $hA_{2A}R$-selective inverse agonist ZM 241385 for 10 min (A). Mean intracellular cAMP levels ± SEM of a representative experiment are shown (n=3; ***, < 0.001 vs DMSO; θθθ, p < 0.001 vs without ADA; ###, p < 0.001 vs DMSO with ADA; τττ, < 0.001 vs without ADA and ZM 241385; ^^, p < 0.001 vs with ADA but without ZM 241385). Dose response of $hA_{2A}R$-mediated cAMP production to inosine (B). Mean intracellular cAMP levels ± SEM of a representative experiment are shown (n=3; ***, < 0.001 vs ADA).

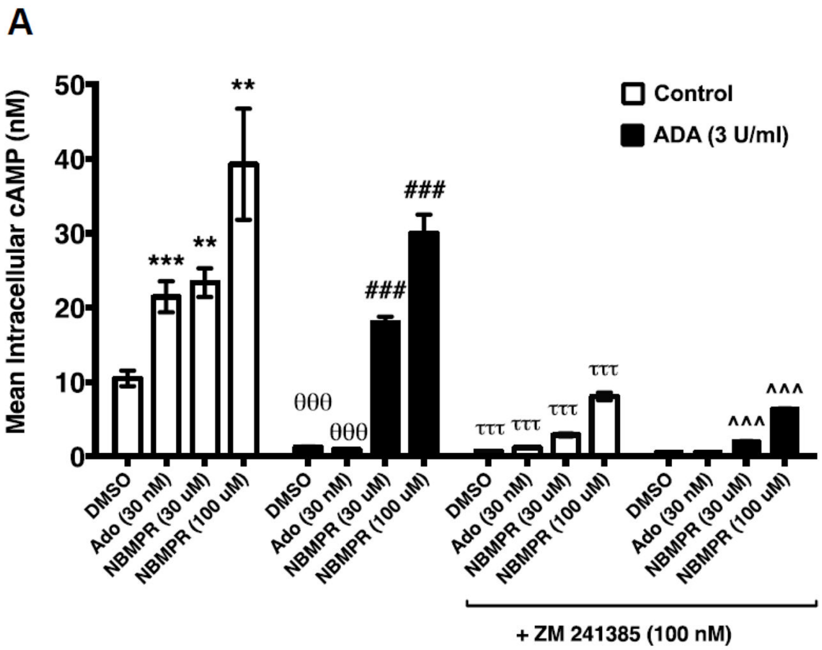


Fig. 2a

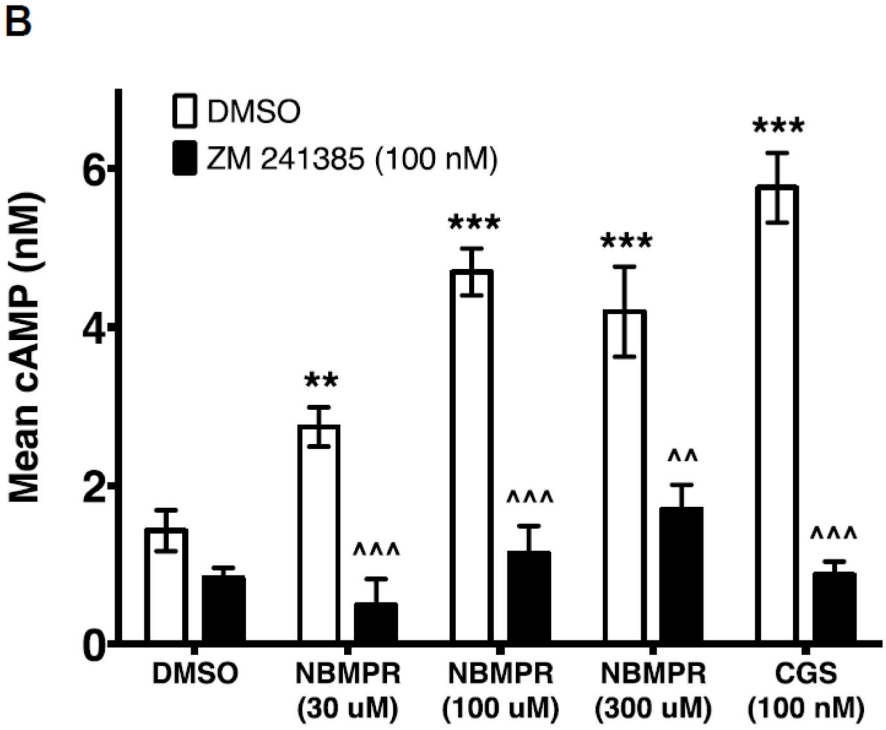


Fig. 2b

Fig. 2.

Inosine analog NBMPR dose-dependently induces $hA_{2A}R$-mediated cAMP production in cellular and cell-free membrane assays.

CHO-hA$_{2A}$R cells were incubated with adenosine (control) or NBMPR with and without ADA (3 U/ml) and in the presence/absence of the hA$_{2A}$R-selective inverse agonist ZM 241385 for 10 min (A). Mean intracellular cAMP levels ± SEM of a representative experiment are shown (n=3; **, < 0.01 vs DMSO; ***, < 0.001 vs DMSO; θθθ, $P < 0.001$ vs without ADA; ###, $p < 0.001$ vs DMSO with ADA; τττ, < 0.001 vs without ADA and ZM 241385; ^^^, $p < 0.001$ vs with ADA but without ZM 241385). CHO-hA$_{2A}$R cell membranes were incubated with indicated concentrations of NBMPR and CGS 21680 (CGS) in the presence and in the absence of the A$_{2A}$R-selective inverse agonist ZM 241385 for 30 min (B). Mean cAMP production ± SEM of representative experiments are shown (n=6; ** and ***, $p < 0.05$ and $p < 0.001$ vs DMSO respectively; ^^ and ^^^, $p < 0.05$ and $p < 0.001$ vs without ZM 241385 respectively).

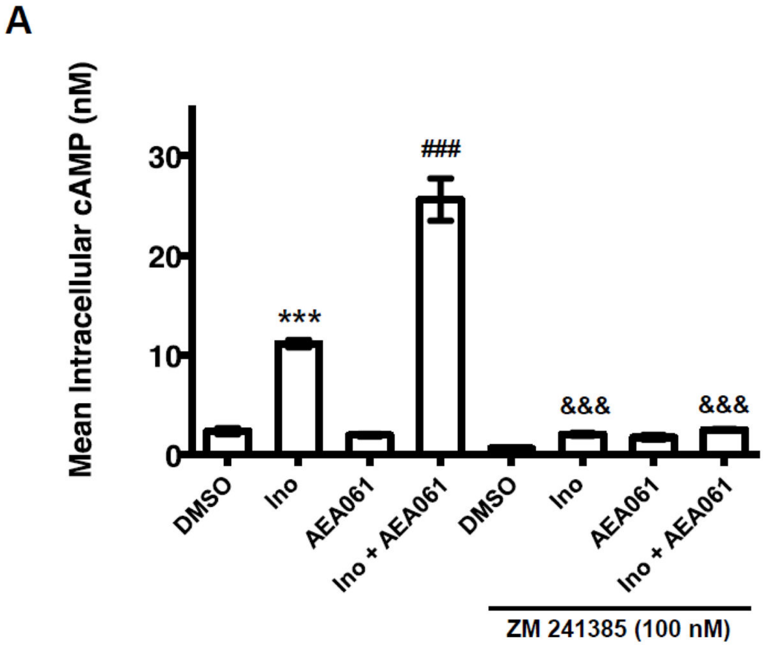

A
Mean Intracellular cAMP (nM)
30
20
10
0
###
***
&&&
&&&
DMSO
Ino
AEA061
Ino + AEA061
DMSO
Ino
AEA061
Ino + AEA061
ZM 241385 (100 nM)

Fig. 3a

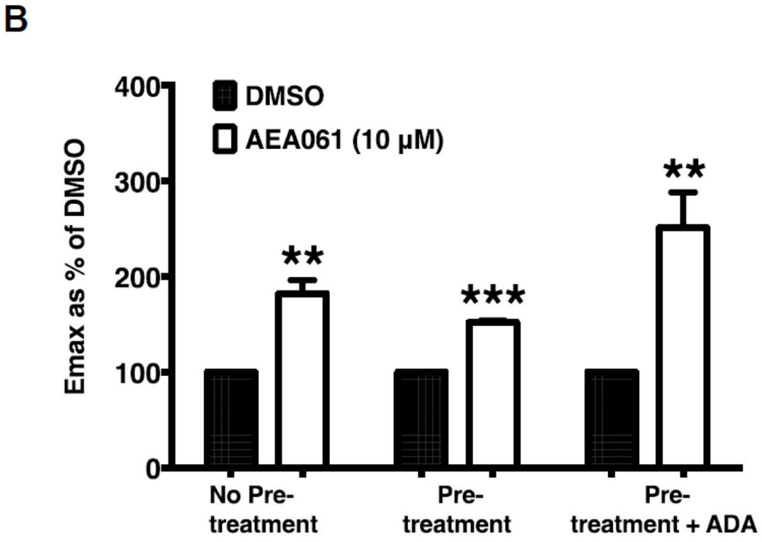

B
DMSO
AEA061 (10 μM)
Emax as % of DMSO
400
300
200
100
0
**
***
**
No Pre-treatment
Pre-treatment
Pre-treatment + ADA

Fig. 3b

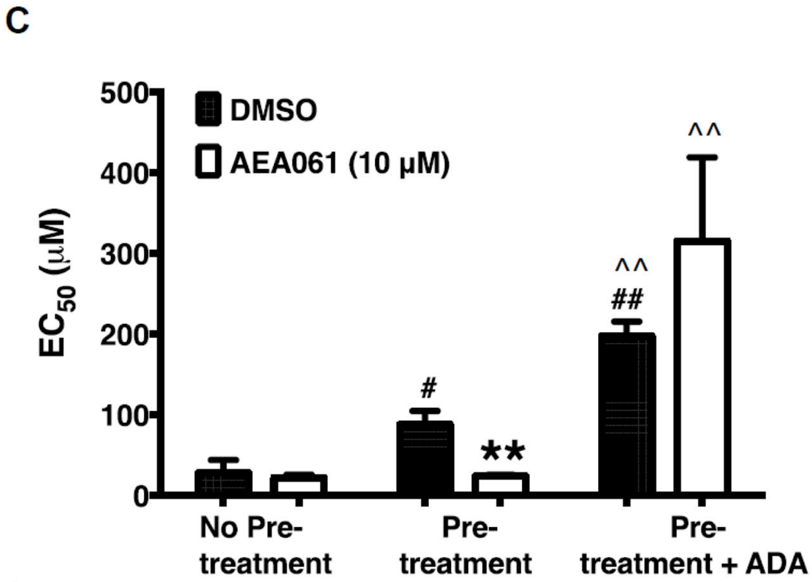


Fig. 3c

Fig. 3.

PAM of the $A_{2A}R$ AEA061 enhances inosine-inducible, $A_{2A}R$-mediated cAMP production. CHO-$hA_{2a}R$ cells were incubated with inosine (Ino; 1000 µM) and AEA061 (10 µM) in the presence and in the absence of ZM 241385 (100 nM) for 10 min (A). Mean cAMP levels ± SEM of a representative experiment is shown (n=3; ***, $p < 0.001$ vs DMSO; ###, < p < 0.001 vs inosine; &&&, $p < 0.001$ vs without ZM 241385). AEA061 increases E_{max} (B) and alters EC_{50} (C) of inosine-mediated $A_{2A}R$ activation. CHO-$A_{2A}R$ cells were incubated with AEA061 and varying concentrations of inosine for 10 min. Mean E_{max} (B) and EC_{50} (C) values of four experiments with SEM are shown (**, < 0.01 vs DMSO; # and ##, $p < 0.05$ and 0.01 vs without ADA pretreatment; ^^, $p < 0.01$ vs ADA pretreatment without subsequent ADA addition respectively).

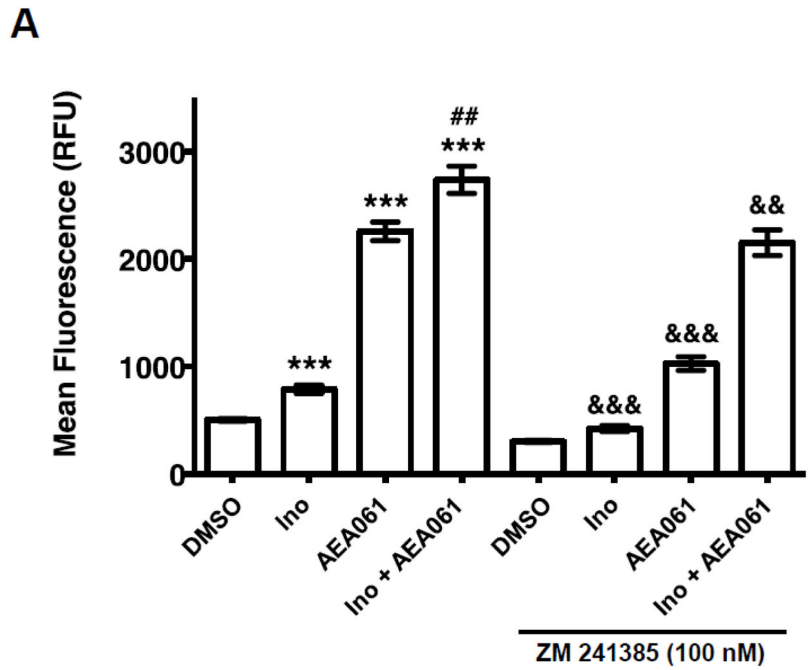

A
Mean Fluorescence (RFU)
0
1000
2000
3000
***
***
##
***
&&&
&&&
&&
DMSO
Ino
AEA061
Ino + AEA061
DMSO
Ino
AEA061
Ino + AEA061
ZM 241385 (100 nM)

Fig. 4a

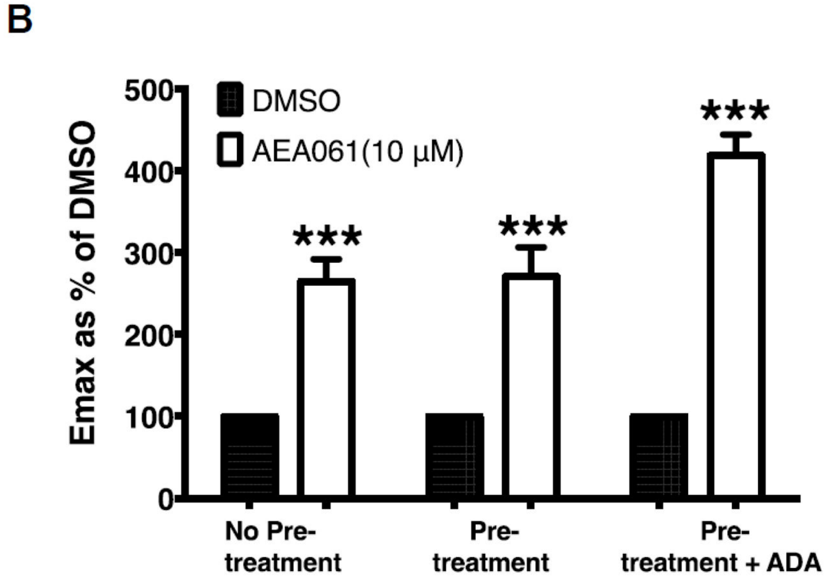

B
Emax as % of DMSO
0
100
200
300
400
500
■ DMSO
□ AEA061(10 µM)
***
***
***
No Pre-treatment
Pre-treatment
Pre-treatment + ADA

Fig. 4b

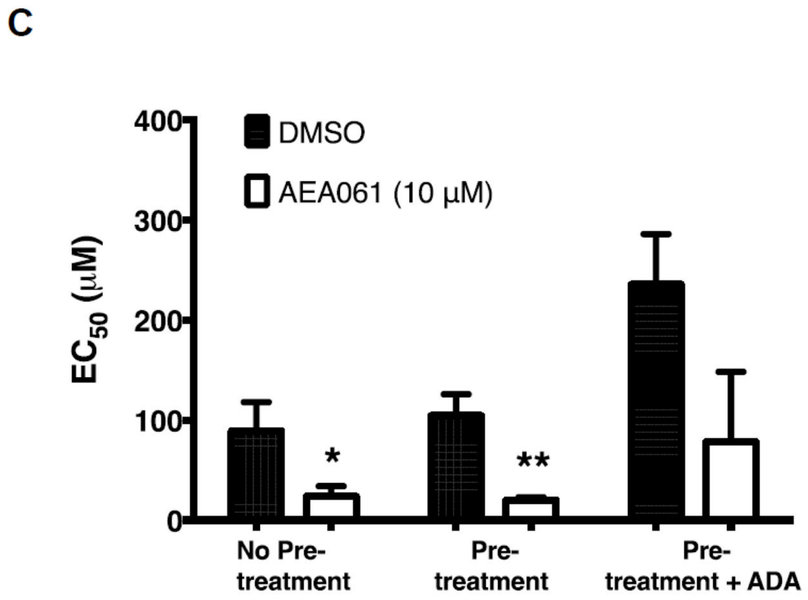


Fig. 4c

Fig. 4.

AEA061 potentiates inosine-inducible, A_{2A}R-mediated ERK1/2 phosphorylation. CHO-hA_{2A}R cells were incubated with inosine (Ino; 1000 µM) and AEA061 (10 µM) in the presence and in the absence of ZM 241385 for 10 min (A). Phospho ERK1/2 levels were quantified using a FRET-based detection kit. Mean FRET ratios ± SEM of a representative experiment are shown (n=3; ***, $p < 0.001$ vs DMSO; ##, < 0.01 vs AEA061; && and &&&, $p < 0.01$ and 0.001 vs without ZM 241385 respectively). AEA061 enhances E_{max} (B) and reduces EC_{50} (C) of inosine-mediated ERK1/2 phosphorylation. CHO-A_{2a}R cells were pre-treated and incubated with AEA061 and varying concentrations of inosine for 10 min. Mean E_{max} (B) and EC_{50} (C) values of three independent experiments with SEM are shown (* and ***, < 0.05 and 0.001 vs DMSO respectively).

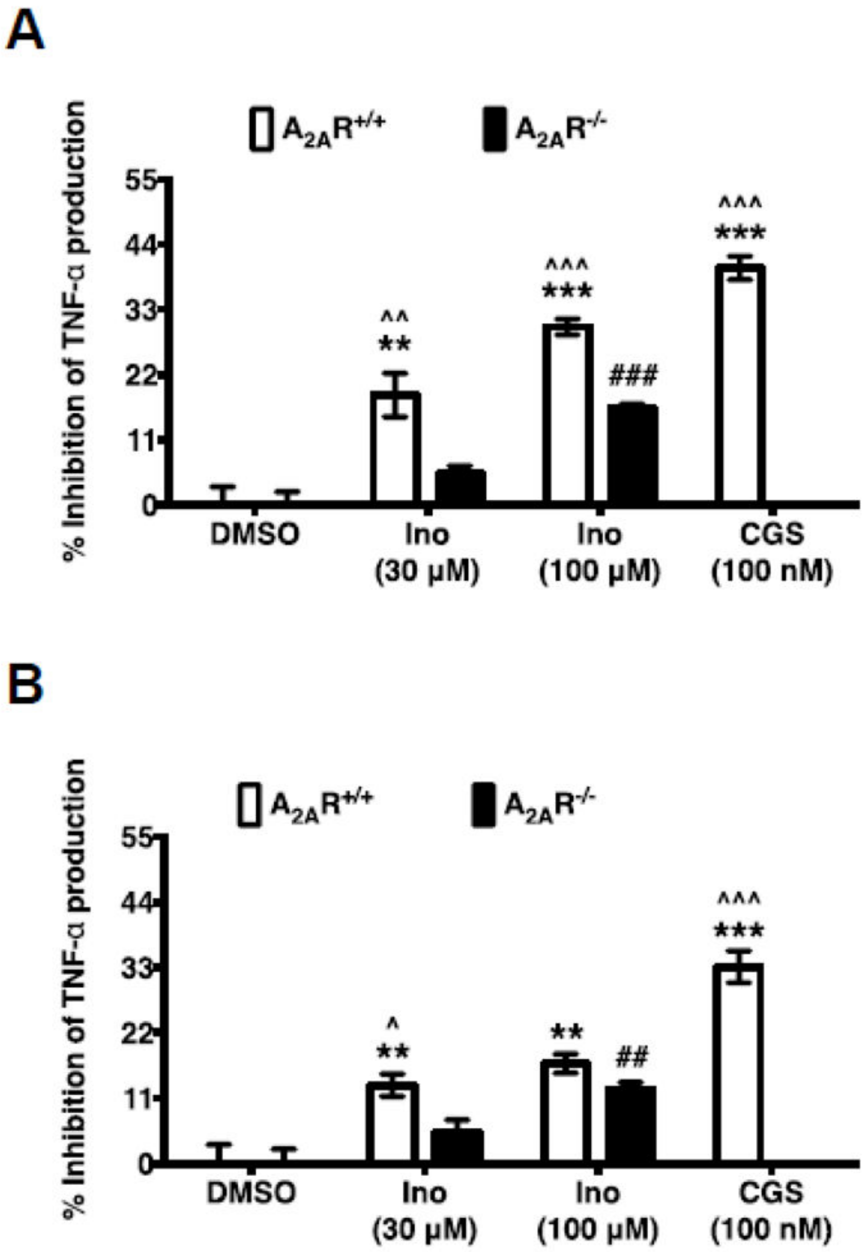
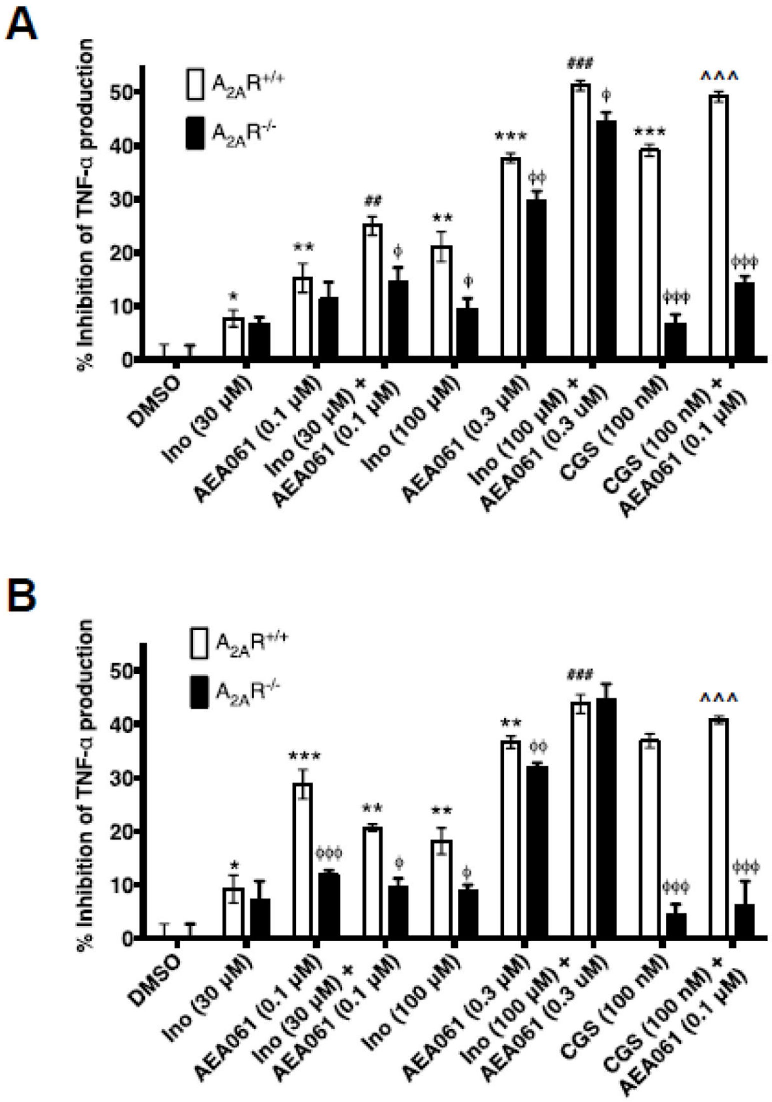
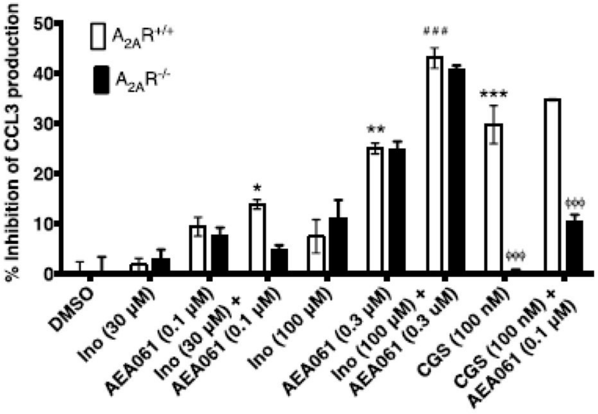


Fig. 5.

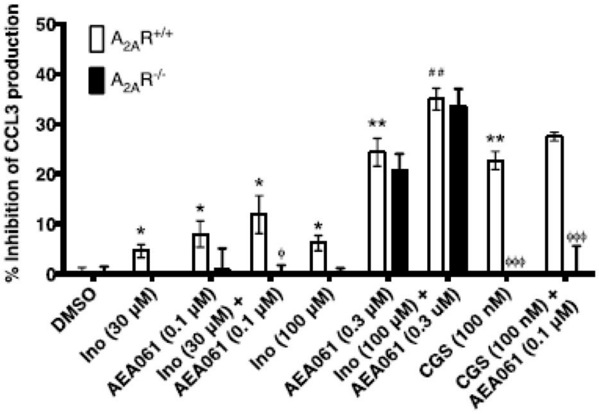
Inosine-mediated $A_{2A}R$ activation inhibits TNF-α production by mouse splenic monocytes. $A_{2A}R$ intact and deficient mouse splenic monocytes were stimulated with LPS (50 ng/ml), inosine (Ino) and CGS 21680 (CGS) in the absence (A) and in presence (B) of ADA (3 U/ml) for 4 h. Mean % inhibition of TNF-α production relative to DMSO (control) of a representative experiment performed in triplicate with SEM are shown (n=3; ** and ***, $p <$ 0.01 and 0.001 vs $A_{2A}R^{+/+}$ DMSO; ## and ###, $p < 0.01$ and 0.001 vs $A_{2A}R^{-/-}$ DMSO; ^, ^^ and ^^^, $p < 0.05$, 0.01 and 0.001 vs $A_{2A}R^{-/-}$ respectively).


A
% Inhibition of TNF-α production
0
10
20
30
40
50
$A_{2A}R^{+/+}$
$A_{2A}R^{-/-}$
*
**
##
**
***
ϕϕ
###
ϕ
***
ϕϕϕ
^^^
ϕϕϕ
ϕ
ϕ
DMSO
Ino (30 μM)
AEA061 (0.1 μM)
Ino (30 μM) + AEA061 (0.1 μM)
Ino (100 μM)
AEA061 (0.3 μM)
Ino (100 μM) + AEA061 (0.3 uM)
CGS (100 nM)
CGS (100 nM) + AEA061 (0.1 μM)
B
% Inhibition of TNF-α production
0
10
20
30
40
50
$A_{2A}R^{+/+}$
$A_{2A}R^{-/-}$
*
***
ϕϕϕ
**
ϕ
**
ϕ
**
ϕϕ
###
ϕϕϕ
^^^
ϕϕϕ
DMSO
Ino (30 μM)
AEA061 (0.1 μM)
Ino (30 μM) + AEA061 (0.1 μM)
Ino (100 μM)
AEA061 (0.3 μM)
Ino (100 μM) + AEA061 (0.3 uM)
CGS (100 nM)
CGS (100 nM) + AEA061 (0.1 μM)

C


% Inhibition of CCL3 production
50
40
30
20
10
0
A2AR+/+
A2AR-/-
*
**
###
***
ϕϕϕ
ϕϕϕ
DMSO
Ino (30 μM)
AEA061 (0.1 μM)
Ino (30 μM) + AEA061 (0.1 μM)
Ino (100 μM)
AEA061 (0.3 μM)
Ino (100 μM) + AEA061 (0.3 uM)
CGS (100 nM)
CGS (100 nM) + AEA061 (0.1 μM)

D


% Inhibition of CCL3 production
50
40
30
20
10
0
A2AR+/+
A2AR-/-
*
*
*
ϕ
*
**
##
**
ϕϕϕ
ϕϕϕ
DMSO
Ino (30 μM)
AEA061 (0.1 μM)
Ino (30 μM) + AEA061 (0.1 μM)
Ino (100 μM)
AEA061 (0.3 μM)
Ino (100 μM) + AEA061 (0.3 uM)
CGS (100 nM)
CGS (100 nM) + AEA061 (0.1 μM)

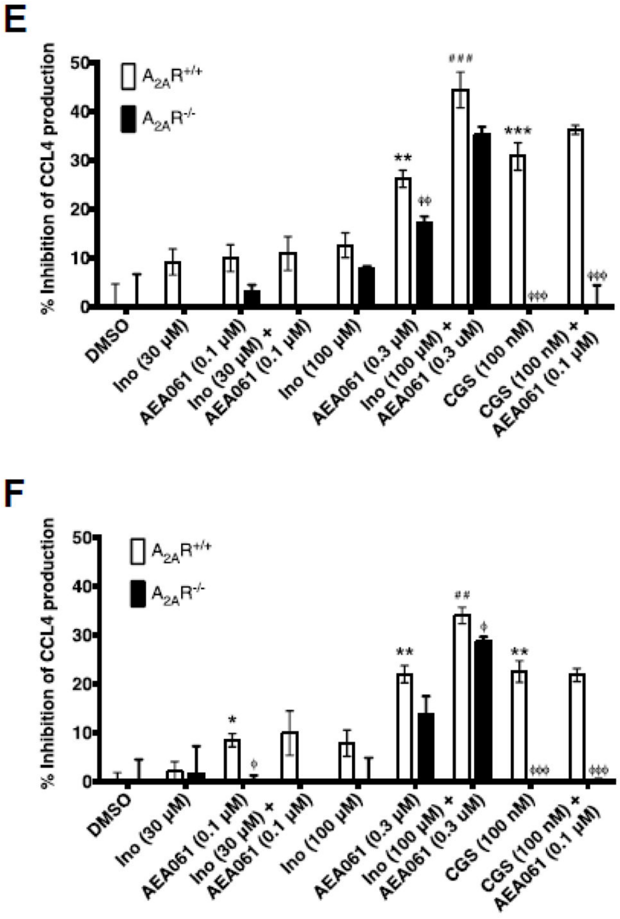


Fig. 6.

PAM of the $A_{2A}R$ potentiates inosine-mediated inhibition of TNF-α in an $A_{2A}R$ dependent manner. Splenic monocytes isolated from $A_{2A}R$ intact and deficient mice were stimulated with LPS (50 ng/ml), inosine (Ino), CGS 21680 (CGS) in the absence (A, C, E) and in presence (B, D, F) of ADA (3 U/ml) for 4 h. Mean percent inhibition of TNF-α (A & B), CCL3 (C & D) and CCL4 (E & F) production relative to DMSO (control) of a representative experiment performed in replicates of four with SEM are shown (n=4; *, ** and ***, $p <$ 0.05, 0.01 and 0.001 vs DMSO; ## and ###, $p < 0.01$ and 0.001 vs $A_{2A}R^{+/+}$ AEA061; ^^^, $p < 0.001$ vs CGS 21680; φ, φφ and φφφ, < 0.05, 0.01 and 0.001 vs $A_{2A}R^{+/+}$ respectively).