Cross-talk between Gs and Gq-coupled Pathways in Regulation of IL-4 by A2B Adenosine Receptors in Human Mast Cells.

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a) Running Title: Adenosine A2B Receptors and IL-4

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c) Number of text pages 40
Number of tables 0
Number of figures 10
Number of references 60
Number of words in the Abstract 248
Number of words in the Introduction 578
Number of words in the Discussion 1262

d) Abbreviations:
The abbreviations used are: BAPTA-AM, 1,2-bis(o-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid tetra(acetoxyethyl) ester; CGS21680, 2-p-(2-carboxyethyl)phenethylamino-5'-N-ethylcarboxamidoadenosine; IB-MECA, N6-(3-iodobenzyl)-N-methyl-5'-carbamoyladenosine; IPDX, 3-isobutyl-8-pyrrolidinoxanthine; IP3, inositol 1,4,5-trisphosphate; H89, N-[2-((p-bromocinnamyl)amino)ethyl]-5-isoquinolinesulfonamide, dihydrochloride; NECA, 5'-N-ethylcarboxamidoadenosine; NFAT, nuclear factor of activated T-cells; PBS, phosphate buffered saline; PMA, phorbol myristic acetate; RGS2, regulator of G protein signaling-2; RhoGEF, Rho guanine nucleotide exchange factor; Ro-32-0432, 2-[(Dimethylamino)methyl]-6,7,8,9-tetrahydropyrido[1,2-a]indol-3-yl]-3-(1-methyl-1H-indol-3-yl)maleimide, HCl; Rp-cAMP, Rp-adenosine 2',5'-cyclic monophosphorothioate; U73122, 1-(6-[[1β]-3-methoxyestra-1,3,5-(10)triene-17-yl]amino)hexyl)-1H-pyrrole-2,5-dione; U73343, 1-(6-[[1β]-3-methoxyestra-1,3,5-(10)triene-17-yl]amino)hexyl)-2,5-pyrrolidine-dione.
ABSTRACT

Human mast cells express functional A<sub>2A</sub> and A<sub>2B</sub> adenosine receptors. However, only stimulation of A<sub>2B</sub> but not A<sub>2A</sub> leads to secretion of IL-4, an important step in adenosine receptor-mediated induction of IgE synthesis by B-cells. Here we studied intracellular pathways that link stimulation of A<sub>2B</sub> receptors to IL-4 upregulation in HMC-1 mast cells. Both A<sub>2A</sub> and A<sub>2B</sub> receptors couple to Gs proteins and stimulate adenylate cyclase, but only A<sub>2B</sub> stimulates phospholipase C<sub>β</sub> through coupling to Gq proteins leading to activation of protein kinase C and calcium mobilization. Inhibition of phospholipase C<sub>β</sub> with U73122 completely blocked A<sub>2B</sub> receptor-dependent IL-4 secretion. The protein kinase C inhibitor Ro-32-0432 had no effect on A<sub>2B</sub> receptor-mediated, but inhibited PMA-stimulated IL-4 secretion. In contrast, chelation of intracellular Ca<sup>2+</sup> inhibited both A<sub>2B</sub> receptor- and ionomycin-dependent IL-4 secretion. This Ca<sup>2+</sup>-sensitive pathway most likely includes calcineurin and nuclear factor of activated T-cells (NFAT), because A<sub>2B</sub> receptor-dependent IL-4 secretion was blocked with cyclosporine A or 11R-VIVIT peptide. Gs-linked pathways also play a role in the A<sub>2B</sub> receptor-dependent stimulation of IL-4 secretion; inhibition of adenylate cyclase with 2',5'-dideoxyadenosine, or protein kinase A with Rp-cAMP or H-89, attenuated A<sub>2B</sub> receptor-dependent IL-4 secretion. Although stimulation of adenylate cyclase with forskolin did not increase IL-4 secretion on its own, it potentiated the effect of Pasteurella multocida toxin by 2-fold and ionomycin by 3-fold. Both forskolin and stimulation of A<sub>2B</sub> receptors upregulated NFATc1 protein levels. We conclude that A<sub>2B</sub> receptors upregulate IL-4 through Gq signaling that is potentiated via crosstalk with Gs-coupled pathways.
Adenosine is an intermediate product in the metabolism of ATP. Extracellular adenosine accumulates in inflamed areas due to its release from stressed or damaged cells. Adenosine exerts its action by binding to G protein-coupled adenosine receptors. Four subtypes of adenosine receptors have been cloned and classified as A₁, A₂A, A₂B and A₃ receptors (Fredholm et al., 2001).

There is growing evidence that adenosine plays a role in asthma, a disorder associated with chronic lung inflammation. Elevated concentrations of adenosine are found in bronchoalveolar lavage fluid (Driver et al., 1993) and exhaled breath condensate (Huszar et al., 2002) obtained from asthmatics. Inhaled adenosine (in the form of AMP) provokes bronchoconstriction in asthmatics but not normal subjects, and the magnitude of this response correlates with chronic inflammation (Polosa et al., 2002). Animal models also indicate a pro-inflammatory role of adenosine in the lung. Recent studies in adenosine deaminase-deficient mice, which are characterized by elevated lung tissue levels of adenosine, strongly suggest a causal association between adenosine and an inflammatory phenotype. These mice exhibit a lung phenotype with features of lung inflammation, including bronchial hyperresponsiveness, enhanced mucus secretion, increased IgE synthesis, and elevated levels of pro-inflammatory Th2 cytokines, that is reversed with exogenous adenosine deaminase (Chunn et al., 2001; Zhong et al., 2001; Blackburn et al., 2003).

Upregulation of IL-4 plays a major role in the development of asthma. This cytokine induces polarization of T-cells towards a Th2 phenotype that ultimately leads to a Th2 inflammatory response associated with both systemic and local production of allergen-specific IgE (Steinke and Borish, 2001). Mast cells have been proposed to
provide the earliest source of IL-4 to naïve T-cells, which is necessary to initiate and amplify their differentiation to a Th2 phenotype (Wang et al., 1999). Mast cell-derived IL-4 has also been proposed to induce IgE synthesis in B-cells (Gauchat et al., 1993). Elevated levels of IL-4 and IgE can act synergistically to increase mast cell FcεRI receptor expression and mediator release (Yamaguchi et al., 1999). Activation of mast cells by IgE, in turn, can stimulate production of IL-4 in mast cells (Plaut et al., 1989; Okayama et al., 1995), thus further amplifying an inflammatory cycle.

We have recently shown that adenosine acting on A2B receptors stimulates generation of IL-4 in human mast cells HMC-1 and induces IgE synthesis in B-cells (Ryzhov et al., 2004). HMC-1 cells express functional A2A and A2B receptors (Feoktistov and Biaggioni, 1995; Feoktistov and Biaggioni, 1998; Feoktistov et al., 2003a). Both A2 subtypes of adenosine receptors activate adenylate cyclase via Gs-protein. However, only A2B receptor has been shown to be coupled also to phospholipase Cβ via a GTP-binding protein of the Gq family leading to stimulation of protein kinase C, and the release of intracellular calcium (Feoktistov and Biaggioni, 1995; Linden et al., 1999).

Several studies have focused on the signaling requirements that lead to the release of IL-4 mediated by cross-linking of FcεRI receptors in mast cells. IL-4 has been shown to be regulated at the transcriptional level by calcium-dependent activation of nuclear factor of activated T-cells (NFAT) (Weiss et al., 1996). Calcium dependency of this process is supported by the finding that receptor-mediated signal transduction leading to IL-4 expression can be bypassed using the calcium ionophore ionomycin (Plaut et al., 1989). In contrast, the signaling transduction involved in regulation of IL-4 by adenosine
remains unknown. In the present study we examined intracellular pathways that link stimulation of $A_2B$ adenosine receptors to IL-4 upregulation in HMC-1 mast cells.
MATERIALS AND METHODS

Cell culture

Human mast HMC-1 cells, a generous gift from Dr. J.H. Butterfield (Mayo Clinic, Rochester, MN), were maintained in suspension culture at a density between 3 and 6 x 10^5 cells/ml by dilution with Iscove’s medium supplemented with 10 % (vol/vol) fetal bovine serum, 2 mM glutamine, 1.2 mM α-thioglycerol, and antibiotic-antimycotic mixture (Gibco BRL, Gaithersburg, MD). Cells were kept under humidified atmosphere of air/CO₂ (19:1) at 37° C.

Chemicals

5'-N-ethylcarboxamidoadenosine (NECA), N⁶-(3-iodobenzyl)-N-methyl-5'-carbamoyladenosine (IB-MECA), and 2-p-(2-carboxyethyl)phenethylamino-NECA (CGS21680) were purchased from Research Biochemicals (Natick, MA). Phorbol myristic acetate (PMA), ionomycin, forskolin, Rp-adenosine 2',5'-cyclic monophosphorothioate (Rp-cAMP) were from Sigma (St. Louis, MO). Adenylate cyclase inhibitor 2',5'-dideoxyadenosine, 1,2-bis(o-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid tetra(acetoxymethyl) ester (BAPTA-AM), cell-permeable protein kinase C inhibitor 2-8-[(Dimethylamino)methyl]-6,7,8,9-tetrahydropyrido[1,2-a]indol-3-yl]-3-(1-methyl-1H-indol-3-yl)maleimide, HCl (Ro-32-0432), N-[2-((p-bromocinnamyl) amino)ethyl]-5-isouquinolinesulfonamide, dihydrochloride (H89), cyclosporine A, cell-permeable NFAT inhibitor 11R-VIVIT peptide, phospholipase C inhibitor 1-(6-[[17β-3-methoxyestra-1,3,5-(10)triene-17-yl] amino]hexyl)-1H-pyrrole-2,5-dione (U73122) and its inactive analog 1-(6-[[17β-3-methoxyestra-1,3,5-(10)triene-17-yl] amino]hexyl)-2,5-
pyrrolidine-dione (U73343) were purchased from Calbiochem (San Diego, CA). Pasteurella multocida toxin was obtained from List Biological Laboratories (Campbell, CA). Dimethyl sulfoxide was purchased from Sigma (St. Louis, MO). When used as a solvent, final dimethyl sulfoxide concentrations in all assays did not exceed 1% and the same dimethyl sulfoxide concentrations were used as vehicle controls.

**Measurement of cAMP accumulation**

HMC-1 (2 x 10^6 cells/ml) were preincubated in 200 µl of 150 mM NaCl, 2.7 mM KCl, 0.37 mM NaH₂PO₄, 1 mM MgSO₄, 1 mM CaCl₂, 5 g/l D-glucose, 10 mM HEPES-NaOH, pH 7.4, 1 U/ml adenosine deaminase and 1 mM papaverine in the absence or presence of 2',5'-dideoxyadenosine for 15 min at 37°C. Forskolin (1 µM) or NECA (10 µM) were added to cells, and the incubation was allowed to proceed for 3 min at 37°C. The reaction was stopped by the addition of 50 µl of 25% trichloroacetic acid. The extracts were washed five times with 10 volumes of water-saturated ether. Cyclic AMP concentrations were determined using cAMP assay kit (TRK.432; Amersham Corp., Arlington Heights, IL).

**Measurement of [³H] inositol phosphates formation**

Formation of inositol phosphates was determined using a modification of the procedure described by K. Seuwen et al (Seuwen et al., 1988). HMC-1 (5 x 10^6 cells/ml) were labeled to equilibrium with myo-[³H] inositol (2 µCi/ml, DuPont-NEN, Boston, MA) for 18 h in inositol-free DMEM medium. In some experiments 1µg/ml Pasteurella multocida toxin was also included in this incubation medium. The HMC-1 cells were
then washed twice with phosphate buffered saline (PBS) and resuspended at a concentration of $3 \times 10^6$ cells/ml in 150 mM NaCl, 2.7 mM KCl, 0.37 mM NaH$_2$PO$_4$, 1 mM MgSO$_4$, 1 mM CaCl$_2$, 5 g/l D-glucose, 10 mM HEPES-NaOH, pH 7.4 and 1 U/ml adenosine deaminase containing 20 mM LiCl$_2$ in the absence or presence of Pasteurella multocida toxin, U73122 or U73343. After preincubation for 15 min at 37° C, NECA or its vehicle were added to cells, and the incubation was allowed to proceed for 30 min at 37° C. Reaction was terminated by replacing the incubation buffer with 200 μl ice-cold 10 mM formic acid (pH 3). After 30 min, this solution containing the extracted inositol phosphates and inositol was collected and diluted with 800 μl of 5 mM NH$_3$ solution (final pH 8 - 9). The resulting mixture was then applied to a column containing 0.2 ml anion exchange resin (AG 1-X8, formate form, 200 - 400 mesh; Bio-Rad Laboratories, Richmond, CA). Free inositol and glycerophosphoinositol were eluted with 1.25 ml of H$_2$O and 1 ml of 40 mM ammonium formate/formic acid, pH 5. Total inositol phosphates were eluted in the single step with 1 ml of 2 M ammonium formate/formic acid, pH 5, and radioactivity was measured by liquid scintillation counting.

**Measurement of IL-4 and IL-8 secretion**

In some studies HMC-1 cells were pre-treated with 1 μg/ml Pasteurella multocida toxin for 18 h. Prior to experiments, cells were washed twice with PBS and resuspended at a concentration of $2 \times 10^6$ cells/ml in serum-free Iscove's media containing 2 mM glutamine, 1.2 mM α-thioglycerol and 1 U/ml adenosine deaminase in the absence or presence of inhibitors. In experiments using BAPTA-AM to chelate intracellular Ca$^{2+}$, a calcium free medium (Joklik MEM, catalog # M0518, Sigma, St Louis, MO) was used.
instead of Iscove’s medium. After 15 min preincubation, reactions were started by addition of stimulants and continued for 6 h under humidified atmosphere of air/CO₂ (19:1) at 37° C. At the end of this incubation period, the culture media were collected by centrifugation at 12,500 x g for 1 min at 4° C. IL-4 and IL-8 concentrations were measured using ELISA kits (R&D Systems, Minneapolis, MN).

Transfections and luciferase reporter assay.

HMC-1 cells were transfected using Fugene 6 transfection reagent (Roche, Indianapolis, IN). Plasmid DNA (0.5 µg) was mixed with 25 µl serum-free Iscove’s medium containing 1.5 µl Fugene 6. After 15 min incubation at room temperature, the transfection mixture was added to 5 x 10⁵ cells suspended in 500 µl of growth medium.

Cells were co-transfected with cDNA described in the Results section and luciferase reporters at a ratio of 5 : 1. The ratio 20 : 1 was used for IL-4 firefly luciferase reporter : control Renilla luciferase reporter combination. IL-4 promoter-driven luciferase reporter, a firefly luciferase reporter plasmid, comprising 5' flanking -269 to +11 base pairs of the human IL-4 gene (Li-Weber et al., 1998) was kindly provided by Dr. Min Li-Weber (German Cancer Research Center, Heidelberg, Germany). Luciferase reporter of NFAT-mediated transcriptional activation pNFAT-luc was purchased from Stratagene (La Jolla, CA). cDNA encoding RGS2 in pcDNA3.1 expression vector (Invitrogen, San Diego, CA) was obtained from UMR cDNA Resource Center (www.cdna.org) and cDNA encoding the RGS box of p115 RhoGEF in pcDNA3.1 was kindly provided by Dr. Tatyana Voyno-Yasenetskaya (University of Illinois, Chicago, IL). A control constitutively active Renilla luciferase plasmid pRL-SV40 was purchased
from Promega (Madison, WI). Twenty-four hours after transfections, cells were incubated in the presence of reagents indicated in the Results section for additional 6 hours. Reporter activity was measured using a Dual-Luciferase Reporter Assay System (Promega). Firefly luciferase reporter activities were normalized against Renilla luciferase activities from the co-expressed pRL-SV40, and expressed as relative luciferase activities over basal (set as 1).

**Western Blot analysis of NFATc1 and NFATc2 protein levels.**

HMC-1 (10^7 cells) were washed in ice-cold PBS and then lysed in 0.5 ml of RIPA buffer (PBS, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS) that contained 0.1 M Na₂CO₃ and 1:10 dilution of a protease inhibitor cocktail (Roche, Indianapolis, IN) for 60 min on ice. Cellular debris was centrifuged for 15 min at 12,500 x g, and supernatants containing total cellular proteins were stored at -80°C. To ensure even gel loading, cell protein concentrations were determined by Coomassie Plus - The Better Bradford™ Assay Kit (Pierce Chemical, Rockford, IL) following manufacturer’s instructions. Samples (20 µg of protein), pre-incubated in sample buffer (Invitrogen, San Diego, CA) at 70°C for 5 min, were resolved on NuPAGE Bis-Tris gradient 4 to 12% gel (Invitrogen, San Diego, CA), and transferred to PVDF membranes (Millipore Corporation, Billerica, MA) by electroblotting. Membranes were blocked with 3% (wt/vol) dry fat-free milk in Tris-buffered saline with 0.05% Tween (TBST) for 60 min at room temperature.

NFATc1 and NFATc2 were detected with commercially available mouse monoclonal antibodies (Santa Cruz Biotechnology, Santa Cruz, CA) at a dilution of 1:200.
by incubating at 4°C overnight. β-actin was determined using rabbit polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA) at a dilution of 1:300 by incubating at room temperature for 1 h. After washing with TBST, the membranes were incubated with a peroxidase-conjugated secondary antibody for 60 min at room temperature. The membranes were washed again and the bands were visualized with an enhanced chemiluminescence method (Nesbitt and Horton, 1992). The immunoreactivity of protein bands was quantified by a densitometer using NIH image software (http://rsb.info.nih.gov/nih-image).
RESULTS

Role of adenosine receptor subtypes in IL-4 secretion

HMC-1 cells express mRNA for A2A, A2B, A3 but not A1 subtype of adenosine receptors (Meade et al., 2002; Feoktistov et al., 2003b). Figure 1 shows that only the nonselective adenosine receptor agonist NECA stimulated IL-4 secretion in HMC-1 with an EC50 of 0.9 µM, close to the reported affinity of this agonist at A2B adenosine receptors (Feoktistov and Biaggioni, 1997). In contrast, the A2A receptor agonist CGS 21680 and the A3 agonist IB-MECA had no effect when used at selective concentrations (<1 µM). These results are consistent with a pharmacological profile of A2B adenosine receptors, and agree with previously reported inhibition of NECA-induced IL-4 secretion in mast cells by the selective A2B antagonist IPDX (Ryzhov et al., 2004). From the data presented in Figure 1, we selected a concentration of NECA (10 µM) producing maximal response to perform inhibitory analysis of intracellular signaling pathways leading to stimulation of IL-4 secretion.

Role of Gq-linked signaling pathways in A2B receptor-dependent upregulation of IL-4

We have previously shown that A2B adenosine receptors stimulate phosphoinositide turnover and calcium mobilization in HMC-1 cells via a pertussis toxin-insensitive mechanism, indicating involvement of Gq/11 proteins and phospholipase Cβ. (Feoktistov and Biaggioni, 1995). To determine whether this signaling pathway is involved in A2B receptor-dependent upregulation of IL-4, we initially evaluated the role of Gq/11 proteins in IL-4 upregulation using a reporter assay. As seen in Figure 2, NECA (10 µM) increased IL-4 reporter activity in HMC-1 cells by 5.0±0.1 fold. Overexpression
of the preferential Gq/11 inhibitor RGS2 (Heximer et al., 1997; Heximer et al., 1999; Tang et al., 2003) resulted in 75% inhibition of NECA-induced IL-4 reporter activity, indicating the involvement of Gq/11 proteins in the regulation of IL-4 transcription. In contrast, overexpression of p115 RhoGEF RGS, a selective inhibitor of pertussis toxin-insensitive G12/13 proteins (Hains et al., 2004) not coupled to phospholipase Cγ (Neves et al., 2002), had no significant effect on stimulation of reporter activity by NECA. To further ascertain the role of phospholipase Cβ activation in stimulation of IL-4 secretion, HMC-1 cells were incubated in the presence of 10 µM NECA and increasing concentrations of cell-permeable phospholipase C inhibitor U73122 or its inactive structural analog U73343. Only U73122, but not U73343 inhibited NECA-induced phosphoinositide turnover (Figure 3A) and IL-4 secretion (Figure 3B) at concentrations in the low micromolar range. These results demonstrate functional engagement of phospholipase Cβ in NECA-induced IL-4 upregulation in mast cells.

We then analyzed intracellular pathways downstream from phospholipase Cβ. Hydrolysis of the membrane lipid phosphatidylinositol 4,5-bisphosphate by phospholipase Cβ produces diacylglycerol leading to stimulation of protein kinase C, and inositol 1,4,5-trisphosphate (IP₃) that mediates the release of intracellular calcium. We found that stimulation of protein kinase C by 10 nM phorbol 12-myristate 13-acetate (PMA) or increase in intracellular Ca²⁺ by 1 µM ionomycin induced IL-4 secretion in HMC-1 cells by 3.6±0.2-fold and 2.2±0.2-fold over basal levels, respectively (data not shown). However, the cell-permeable protein kinase C inhibitor Ro-32-0432 decreased only PMA-induced but not NECA-induced IL-4 secretion (Figure 4A). In contrast, chelation of intracellular Ca²⁺ by BAPTA-AM inhibited IL-4 secretion, induced by both...
ionomycin and NECA, to a similar extent (Figure 4B) indicating an important role of Ca\textsuperscript{2+}-dependent processes in A\textsubscript{2B} receptor-dependent regulation of IL-4.

Among many calcium targets, calcineurin is known as the most important activator of NFAT (Im and Rao, 2004). To assess the role of this signaling pathway in A\textsubscript{2B} receptor-dependent IL-4 production, we initially used cyclosporine A, which, in complex with an endogenous protein cyclophilin, binds to calcineurin and inhibits its catalytic activity (Liu \textit{et al.}, 1991). As seen in Figure 5 (left panel), inhibition of calcineurin with cyclosporine A effectively blocked the NECA-induced IL-4 secretion. Inhibition of catalytic activity of calcineurin by cyclosporine A prevents activation of NFAT, but it can also affect many other intracellular substrates of calcineurin. Therefore, we used the cell-permeable inhibitor of calcineurin-NFAT interaction, 11R-VIVIT peptide (Aramburu \textit{et al.}, 1999) to specifically block NFAT activation. As seen in Figure 5 (right panel), 11R-VIVIT peptide inhibited NECA-induced IL-4 secretion, confirming an important role of NFAT activation by calcineurin in A\textsubscript{2B} receptor-dependent stimulation of IL-4 secretion.

Taken together, our results indicate that coupling of A\textsubscript{2B} receptors to Gq/11 proteins ultimately leads to upregulation of IL-4 in HMC-1 that involves stimulation of phospholipase C\textbeta, synthesis of IP\textsubscript{3}, release of Ca\textsuperscript{2+} from intracellular stores, stimulation of calcium-dependent phosphatase calcineurin and activation of NFAT.

**Role of Gs-linked signaling pathways in A\textsubscript{2B} receptor-dependent upregulation of IL-4**

In addition to stimulation of phospholipase C\textbeta via G proteins of the Gq/11 family, A\textsubscript{2B} adenosine receptors also stimulate adenylate cyclase via Gs proteins (Feoktistov and
Biaggioni, 1995). To elucidate a potential role of adenylate cyclase activation in A2B receptor-mediated regulation of IL-4 production, we studied the effects of 2',5'-dideoxyadenosine, a known inhibitor of the adenylate cyclase catalytic activity (Johnson et al., 1997). We initially demonstrated that 2',5'-dideoxyadenosine inhibited NECA-stimulated adenylate cyclase in HMC-1 in a concentration-dependent manner; cAMP accumulation was almost completely blocked by 100 µM 2',5'-dideoxyadenosine (Figure 6A). 2',5'-dideoxyadenosine also inhibited NECA-stimulated IL-4 secretion (Figure 6B). However, inhibition was only partial, reaching 49±3% in the presence of 100 µM 2',5'-dideoxyadenosine. In contrast, 2',5'-dideoxyadenosine did not inhibited NECA-stimulated IL-8 secretion, a previously described cAMP-independent process (Feoktistov and Biaggioni, 1995) and used in this study as a negative control.

Inhibition of protein kinase A, further downstream from adenylate cyclase, with the inhibitory cAMP analog Rp-cAMP also resulted in partial inhibition of NECA-induced IL-4 secretion, but had no effect on NECA-induced IL-8 secretion (Figure 6C). Furthermore, blocking the ATP binding site of protein kinase A with 1µM H-89 inhibited NECA-stimulated IL-4 secretion by 46±3%, but had no effect on NECA-stimulated IL-8 secretion (data not shown).

**Interaction of signaling pathways linked to A2B receptors in stimulation of IL-4 secretion**

Inhibitory analysis of signaling cascades activated by A2B receptors in HMC-1 indicated involvement of intracellular pathways linked to activation of both phospholipase Cβ and adenylate cyclase in regulation of IL-4 secretion. To understand
how these pathways may interact, we evaluated the effects of stimulation of each of these pathways alone, or together, on IL-4 secretion. We used forskolin to simulate the effect of A2B receptors on Gs-adenylate cyclase pathways without activation of Gq-phospholipase Cβ. To ensure that we do not overstimulate these pathways, we conducted ancillary studies and determined that 1µM forskolin and 10 µM NECA produced similar levels of cAMP accumulation in HMC-1 (Figure 7A). Gq-phospholipase Cβ pathway was stimulated by Pasteurella multocida toxin (Wilson and Ho, 2004). Incubation of HMC-1 cells with 1 µg/ml Pasteurella multocida toxin resulted in stimulation of phosphoinositide turnover comprising approximately 60% of that induced by 10 µM NECA (Figure 7B).

As seen in Figure 7C, Pasteurella multocida toxin stimulated IL-4 secretion by 1.9±0.2 fold. Forskolin had no effect on its own, but potentiated the effect of Pasteurella multocida toxin on IL-4 secretion, resulting in 2.8±0.2-fold stimulation (p<0.05, unpaired, two-tailed t-test, compared to stimulation with Pasteurella multocida toxin alone). Forskolin potentiated also the effect of the calcium ionophore ionomycin, increasing stimulation of IL-4 secretion from 1.8±0.2 to 3.4±0.2-fold (p<0.01, t-test, Figure 7C). These data indicate that cross-talk between Gs- and Gq-linked pathways occurs downstream from IP3-dependent mobilization of intracellular calcium.

We then used a luciferase reporter assay to determine whether these signaling pathways interact to regulate IL-4 transcription. As seen in figure 8A, forskolin had no significant effect on IL-4 reporter activity, but enhanced stimulation of IL-4 promoter by ionomycin from 2.1±0.5-fold to 5.8±0.5-fold (p<0.01, t-test). Furthermore, forskolin per se had no effect on pNFAT-luc reporter activity driven by a minimal promoter containing
four consecutive NFAT binding sites, but potentiated stimulation of the reporter by ionomycin from 12±1-fold to 32±6-fold (p<0.05, t-test, Figure 8B). These data indicate that interaction between Gs- and Gq-linked pathways occurs upstream from stimulation of IL-4 promoter, and that enhancement of Ca^{2+} signal by cAMP-dependent pathway takes place at the NFAT-binding site.

**Effects of NECA and forskolin on NFAT protein levels in HMC-1**

Proteins belonging to the NFAT family of transcription factors play a central role in regulation of IL-4 synthesis in mast cells (Weiss and Brown, 2001; Weiss et al., 1996). Calcium-dependent dephosphorylation of NFAT proteins by calcineurin results in their translocation to the nucleus and binding to DNA. Because forskolin alone had no effect on IL-4 secretion, direct stimulation of this pathway by Gs-adenylate cyclase-dependent processes appears unlikely. We hypothesized then that stimulation of Gs-adenylate cyclase by A_{2B} adenosine receptors might lead to increase of NFAT levels, thus increasing the pool of nuclear factors available for stimulation by calcineurin via Gq-phospholipase Cβ pathway. To test this hypothesis, we measured the protein levels of NFATc1 and NFATc2, factors shown to bind to the NFAT site in the IL-4 promoter and activate IL-4 transcription (Boise et al., 1993; Timmerman et al., 1997). As seen in Figure 9, incubation of HMC-1 with NECA for 1 h increased NFATc1 immunoreactivity on Western blots of cell lysates by 64%. Forskolin also increased levels of NFATc1 proteins by 46%. In contrast, both NECA and forskolin had virtually no effect on NFATc2 protein levels in HMC-1.
DISCUSSION

There is growing evidence that A2B adenosine receptors play an important role in respiratory disorders associated with lung inflammation such as asthma and chronic obstructive pulmonary disease (Polosa et al., 2002; Fozard, 2003; Holgate, 2005). Research in this field has provided a basis for developing A2B receptor antagonists as a new therapeutic approach to asthma (Feoktistov et al., 1998; Kim et al., 2000; Feoktistov et al., 2001; Hayallah et al., 2002; Varani et al., 2005; Zablocki et al., 2005; Holgate, 2005; Cacciari et al., 2005). Recently we presented evidence that adenosine triggers IL-4 production in mast cells, and that this, in turn, induces IgE synthesis by B-lymphocytes, thus providing a regulatory loop for amplification of allergic reactions (Ryzhov et al., 2004). In the present study we investigated intracellular pathways that link activation of adenosine receptors to IL-4 production in HMC-1, a mastocytoma cell line that shares phenotypic characteristics with human lung mast cells (Nilsson et al., 1994).

Expression of adenosine receptors in HMC-1 has been previously characterized. These cells express mRNA for A2A, A2B and A3 adenosine receptors (Meade et al., 2002; Feoktistov et al., 2003b). There is, however, no evidence of functional coupling of A3 receptors to adenylate cyclase or phospholipase Cβ in HMC-1 (Feoktistov et al., 2003b), whereas both A2A and A2B are linked to stimulation of adenylate cyclase (Feoktistov and Biaggioni, 1995). In addition, A2B receptors are also linked to stimulation of phospholipase Cβ through coupling to pertussis toxin-insensitive Gq/11 proteins (Feoktistov and Biaggioni, 1995). In agreement with previous results (Ryzhov et al., 2004), only stimulation of A2B, but not A2A or A3 receptors, induced IL-4 secretion,
implying an important role of phospholipase Cβ-linked pathways in regulation of IL-4 production.

Indeed, our studies employing inhibitors and activators of phospholipase Cβ-linked pathways (Figure 10) revealed their essential role in A2B receptor-dependent IL-4 generation. Activation of Gq with Pasteurella multocida toxin stimulated phospholipase Cβ and IL-4 secretion. Overexpression of the preferential Gq inhibitor RGS2 significantly reduced A2B receptor-dependent stimulation of IL-4 reporter. Stimulation of IL-4 secretion by NECA, mediated via A2B receptors, was completely blocked by U73122, a phospholipase C inhibitor, but was insensitive to its inactive structural analog U73343. The products of phospholipase Cβ enzymatic activity, IP3 and diacylglycerol, stimulate Ca2+ mobilization and protein kinase C, respectively. Our results indicate that Ca2+ mobilization, but not protein kinase C stimulation, contribute to IL-4 upregulation by A2B receptors; IL-4 secretion was stimulated by increasing intracellular calcium with ionomycin and, conversely, chelation of intracellular Ca2+ with BAPTA-AM attenuated both ionomycin- and NECA-induced IL-4 secretion. In contrast, inhibition of protein kinase C with Ro-32-0432 had no effect on A2B receptor-dependent IL-4 secretion. Inhibition of calcineurin downstream from calcium mobilization with cyclosporine A blocked A2B receptor-dependent IL-4 secretion. Furthermore, 11R-VIVIT peptide, a selective blocker of calcineurin-NFAT interaction, also inhibited this process.

The results of our study delineated a signal transduction pathway from A2B receptors, via Gq, phospholipase Cβ, IP3, mobilization of intracellular calcium, calcineurin and NFAT, to IL-4 production. This is in agreement with the reported property of other Gq-coupled receptors to stimulate NFAT in PC12 and Jurkat cells (Boss
et al., 1996). These data also explain why stimulation of A2B receptors coupled to Gs and Gq proteins, but not A2A receptors coupled only to Gs, induced IL-4 in HMC-1.

Our study also revealed the existence of cross-talk between Gs- and Gq-dependent pathways stimulated by A2B adenosine receptors. We demonstrated for the first time that Gs-adenylate cyclase-linked pathways positively modulate IL-4 secretion in human mast cells. The role of cAMP in the regulation of inflammatory responses remains controversial. Molecules elevating intracellular cAMP levels have been reported to inhibit cytokine GM-CSF, IL-5 and MIP-1α production in cord blood-derived mast cells (Shichijo et al., 1999). We have previously reported that A2B adenosine receptors stimulated generation of IL-8 in HMC-1 independently from cAMP (Feoktistov and Biaggioni, 1995). In the current study, we found that A2B receptor-mediated stimulation of IL-4, but not that of IL-8, was attenuated by the adenylate cyclase inhibitor 2',5'-dideoxyadenosine, or by the protein kinase A inhibitors Rp-cAMP and H-89. The inhibition produced by these compounds was partial, suggesting that the Gs-adenylate cyclase-protein kinase A pathway is not obligatory for IL-4 secretion, but it is rather important for modulation of signal transduction via Gq-phospholipase Cβ pathway. Indeed, stimulation of Gq-phospholipase Cβ pathways with Pasteurella multocida toxin was associated with increased IL-4 secretion, and stimulation of Gs-adenylate cyclase-linked pathways with forskolin potentiated this response, whereas forskolin alone had no effect. The observation that forskolin potentiates ionomycin-induced IL-4 promoter activity and secretion implies that cross-talk between these pathways occurs downstream from calcium mobilization.
Of interest, stimulation of the cAMP-protein kinase A pathway in CD4+ T-cells results in up-regulation of IL-4 production (Lacour et al., 1994; Tokoyoda et al., 2004). It has been proposed that this mechanism involves protein kinase A-dependent stimulation of NFAT (Tokoyoda et al., 2004). In HMC-1, stimulation of Gs-adenylate cyclase-protein kinase A pathway has no effect on IL-4 secretion in the absence of Gq-phospholipase Cβ-dependent stimulation of NFAT. Therefore, it is unlikely that Gs-adenylate cyclase-protein kinase A pathway stimulates NFAT directly, but rather facilitates stimulation mediated via Gq-phospholipase Cβ-dependent pathways. One possible explanation could involve upregulation of NFAT by Gs-adenylate cyclase-protein kinase A-dependent mechanisms, thus increasing the pool of NFAT available for stimulation via Gq-phospholipase Cβ-dependent pathways. Our results support this possibility; both stimulation of A2B receptors with NECA and stimulation of Gs-adenylate cyclase pathway with forskolin upregulated NFATc1 protein levels. Our results do not exclude, however, that there could be other protein kinase A-dependent pathways involved in potentiation of IL-4 secretion stimulated via Gq-phospholipase Cβ-dependent pathways. For example, protein kinase A can promote accumulation of NFAT in the nucleus by inhibiting glycogen synthase kinase 3 (Fang et al., 2000), the enzyme that regulates the nuclear export of NFAT (Beals et al., 1997). Therefore, it is possible to infer that activated protein kinase A might inhibit the nuclear export of NFAT by inactivating glycogen synthase kinase 3 in HMC-1, and that a longer presence of NFAT in the nucleus might augment the transcription of IL-4. It is also possible that cAMP will induce or activate other transcription factors that are involved in the transcription of IL-4 stimulated by NFAT. All of these potential mechanisms can contribute to the observed
protein kinase A-dependent potentiation of IL-4 secretion stimulated by Gq-phospholipase Cβ-dependent pathways. We do not imply, however, that the positive regulation of NFAT and IL-4 by cAMP observed in our study is a universal phenomenon. Indeed, there is evidence for cell-specific differences in the regulation of NFAT/IL-4 by cAMP, with both positive and negative interactions being reported (Pouw-Kraan et al., 1992; Lacour et al., 1994; Tsuruta et al., 1995; Wirth et al., 1996; Borger et al., 1996; Sheridan et al., 2002; Tokoyoda et al., 2004).

In summary, our data explain the necessity and underscore the importance of dual coupling of A2B receptors to Gs/Gq proteins with concurrent stimulation of diverse intracellular pathways for adenosine-dependent regulation of IL-4 production in human mast cells (Figure 10). A2B adenosine receptors induce IL-4 generation via Gq-mediated stimulation of phospholipase Cβ, IP3-mediated mobilization of intracellular Ca2+ and activation of NFAT by calcineurin. This process is potentiated via Gs-mediated stimulation of adenylate cyclase and activation of protein kinase A, and may involve the increase in protein levels of NFATc1. The existence of cross-talk between Gq-phospholipase Cβ and Gs-adenylate cyclase signaling pathways in regulation of IL-4 secretion enables A2B receptors, coupled to both Gq and Gs, to effectively stimulate IL-4 production in mast cells and contribute to the allergic inflammatory response associated with asthma.
REFERENCES


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FOOTNOTES

a) This work was supported by NIH grants R01 HL70073 and R01 HL76306 (to IF), and P01 HL56693 and HL67232 (to IB).

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FIGURE LEGENDS

Figure 1. Stimulation of IL-4 production by adenosine receptor agonists in HMC-1

Concentration-response curves for IL-4 secretion induced by the nonselective agonist NECA (closed circles), by the selective A2A agonist CGS21680 (open circles) or by the selective A3 agonist IB-MECA (closed triangles). Values are expressed as mean±SEM (n=3).

Figure 2. Effect of expression of RGS2 and p115 RhoGEF-RGS on NECA-dependent IL-4 promoter activity.

Activation of IL-4 gene promoter was studied by cotransfection of IL-4 luciferase reporters together with vectors encoding RGS2 and the RGS box of p115 RhoGEF (indicated on graph as p115-RGS) or with an empty pcDNA3.1 vector (mock) in HMC-1. Twenty-four hours after transfections, cells were incubated in the presence or absence of 10 µM NECA for additional 6 hours. The results from three experiments are expressed as mean±standard error of NECA-induced stimulation of luciferase activity.

Figure 3. Effect of phospholipase C inhibition on phosphoinositide turnover and IL-4 production in HMC-1

Effects of phospholipase C inhibitor U73122 (closed circles) and inactive control analog U73343 (open circles) on accumulation of inositol phosphates (A) and IL-4 secretion (B) from cells stimulated with 10 µM NECA. In the absence of inhibitors, 10 µM NECA increased accumulation of [3H] inositol phosphates from 2993±63 to 4233±75.
dpm and concentrations of IL-4 in the medium from 3.3±0.4 to 62±6 pg/ml. Values are presented as mean±SEM (n=4).

**Figure 4. Effects of protein kinase C inhibition and calcium chelation on IL-4 production in HMC-1**

A. Effects of the protein kinase C inhibitor Ro-32-0432 on IL-4 secretion from cells stimulated with 10 µM NECA (closed circles) or 10 nM PMA (open circles) for 6 hrs. In the absence of the inhibitor, NECA increased concentrations of IL-4 in the medium from 3.7±0.3 to 63.2±3.6 pg/ml and 10 nM PMA increased IL-4 from 3.7±0.3 to 13.5±2.1 pg/ml. Values are presented as mean±SEM (n=3).

B. Effect of the calcium chelator BAPTA-AM on IL-4 secretion from cells stimulated with 10 µM NECA (closed circles) or 1 µM ionomycin (open circles) for 6 hrs. In the absence of BAPTA-AM, 10 µM NECA increased concentrations of IL-4 in the medium from 3.6±0.4 to 25.4±1.5 pg/ml and 1 µM ionomycin increased IL-4 from 3.6±0.4 to 7.9±1.1 pg/ml. Please note that these experiments were conducted using a Ca²⁺-free medium. Values are presented as mean±SEM (n=3).

**Figure 5. Effect of calcineurin inhibition on IL-4 production in HMC-1**

Effect of cyclosporine A (left panel) and 11R-VIVIT peptide (right panel) on IL-4 secretion from cells stimulated with 10 µM NECA. In the absence of inhibitors, 10 µM NECA increased concentrations of IL-4 in the medium from 5.3±0.1 to 78.4±6.2 pg/ml. Values are presented as mean±SEM (n=3).
Figure 6. **Effects of adenylate cyclase and protein kinase A inhibition on cytokine production in HMC-1**

A. Effect of adenylate cyclase inhibitor 2′,5′-dideoxyadenosine on cAMP accumulation in cells stimulated with 10 µM NECA. In the absence of 2′,5′-dideoxyadenosine, 10 µM NECA increased cAMP levels from 3.2±0.6 to 19.4±2.9 pmol/10^6 cells. Values are presented as mean±SEM (n=3) of NECA-stimulated response.

B. Effect of adenylate cyclase inhibitor 2′,5′-dideoxyadenosine on IL-4 (closed circles) or IL-8 (open circles) secretion from cells stimulated with 10 µM NECA. In the absence of 2′,5′-dideoxyadenosine, 10 µM NECA increased concentrations of IL-8 in the medium from 210±9.5 to 1,209±27 pg/ml. Values are presented as mean±SEM (n=3) of NECA-stimulated response.

C. Effect of protein kinase A inhibitor Rp-cAMP on IL-4 (closed circles) or IL-8 (open circles) secretion from cells stimulated with 10 µM NECA. Values are presented as mean±SEM (n=3) of NECA-stimulated response.

Figure 7. **Potentiation of Pasteurella multocida toxin- and ionomycin-induced stimulation of IL-4 by forskolin**

A. Effects of 10 µM NECA, 1µM forskolin or their vehicle on cAMP accumulation in HMC-1. Values are presented as mean±SEM (n=3).
B. Effects of 10 µM NECA, 1µg/ml Pasteurella multocida toxin (PMT) or their vehicle on accumulation of inositol phosphates in HMC-1. Values are presented as mean±SEM (n=4).

C. Effects of 1µM forskolin, 1µg/ml Pasteurella multocida toxin (PMT), combination of 1µM forskolin and 1µg/ml Pasteurella multocida toxin, 1µM ionomycin, combination of 1µM forskolin and 1µM ionomycin, or their vehicle on IL-4 secretion from HMC-1 cells. Values are presented as mean±SEM (n=3).

Figure 8. Interaction of cAMP and Ca²⁺-dependent pathways at the NFAT-binding site and IL-4 promoter.

Effects of 10µM NECA, 1µM forskolin, 1µM ionomycin, combination of 1µM forskolin and 1µM ionomycin or their vehicle, on IL-4 reporter activity (A), or activity of pNFAT-luc reporter driven by a minimal promoter under control of 4X NFAT binding sequences (B), in HMC-1 cells. Values are presented as mean±SEM (n=3).

Figure 9. Effects of NECA and forskolin on NFAT protein levels

A. Western blot analysis of NFATc1 and NFATc2 protein levels in resting HMC-1 (control) and cells stimulated with 10 µM NECA or 1µM forskolin. Arrows indicate positions of NFATc1 splice variants (A, B, and C) commonly present in various cells (Chuvpilo et al., 1999; Monticelli et al., 2004). A representative blot of two experiments is shown.
B. Levels of NFAT proteins quantified from Western blot data by densitometry and expressed as a percentage of corresponding levels in resting cells normalized to β-actin protein levels used as internal control.

**Figure 10. Schematic representation of A2B receptor-stimulated intracellular pathways involved in regulation of IL-4 production in HMC-1**

HMC-1 cells express functional A2B receptors (A2BAR) coupled to adenylate cyclase (AC) via Gs-protein. Activation of this pathway results in accumulation of cAMP and stimulation of protein kinase A (PKA). A2BAR are coupled also to phospholipase Cβ (PLCβ) via a GTP-binding protein of the Gq family. Activation of this pathway results in increase in diacylglycerol (DAG) and inositol trisphosphate (IP3). DAG stimulates protein kinase C (PKC). IP3 activates mobilization of calcium from intracellular stores. (Feoktistov and Biaggioni, 1995).

In this study, we present evidence that A2BAR stimulate IL-4 production via Gq-mediated stimulation of phospholipase Cβ, IP3-mediated mobilization of intracellular Ca2+ and activation of NFAT by calcineurin. This process was blocked by the Gq inhibitor RGS2, phospholipase C inhibitor U73122, the calcium chelator BAPTA-AM, the calcineurin inhibitor cyclosporine A, the calcineurin-NFAT interaction inhibitor 11R-VIVIT peptide, but not by the PKC inhibitor Ro-32-0432. A2BAR also modulate IL-4 production via Gs-mediated stimulation of adenylate cyclase and activation of protein kinase A. A2BAR-stimulated IL-4 production was attenuated by the adenylate cyclase inhibitor 2',5'-dideoxyadenosine (ddADO), and the protein kinase A inhibitors Rp-cAMP and H-89. Stimulation of Gs-adenylate cyclase pathways with forskolin did not have an
effect on its own, but potentiated IL-4 production associated with stimulation of Gq-phospholipase Cβ with *Pasteurella multocida* toxin (PMT) or mobilization of intracellular Ca^{2+} with ionomycin. Broken arrow in the diagram signifies the potentiating effect of Gs-adenylate cyclase-protein kinase A stimulation on IL-4 production.
Figure 1
Figure 2

NECA-stimulated IL-4 reporter activity, fold

Mock
RGS2
p115-RGS
Figure 3

Graph A: NECA-stimulated PI turnover, %
- Y-axis: NECA-stimulated PI turnover, %
- X-axis: Log [Inhibitor], M
- Symbols: U73343 (open circle), U73122 (filled circle)

Graph B: NECA-stimulated IL-4 secretion, %
- Y-axis: NECA-stimulated IL-4 secretion, %
- X-axis: Log [Inhibitor], M
- Symbols: U73343 (open circle), U73122 (filled circle)
Figure 4

A

Stimulation of IL-4 secretion, %

NECA

PMA

Log [Ro-32-0432], M

B

Stimulation of IL-4 secretion, %

NECA

Ionomycin

Log [BAPTA-AM], M
Figure 5

NECA-stimulated IL-4 secretion, %

Cyclosporin A, nM

11R-VIVIT, μM

0 0.1 1 10
Figure 6
Figure 7
Figure 8
Figure 9

A

NFATc1
C
B
A

NFATc2

β-actin

Control  NECA  Forskolin

B

Δ NFAT, %

<table>
<thead>
<tr>
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Figure 10

A_2BAR → Gs → AC → cAMP

Forskolin, ddADO

Gs → Gq → PLCβ → IP_3 → DAG → PKC → Ca^{2+} → Calcineurin → NFAT → IL-4

RGS2 → Gq → PMT

U73122

Ro-32-0432 → PKC

DAG

Ro-32-0432

Calcineurin → 11R-VIVIT

RGS2

IP_3

BAPTA-AM

Cyclosporine A

Calcineurin

NFAT

IL-4

Rp-cAMP, H-89

PMT

Ionomycin

Ca^{2+}

BAPTA-AM

Cyclosporine A

11R-VIVIT

RGS2

IP_3

BAPTA-AM

Cyclosporine A

11R-VIVIT