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Cross-Talk between G_s- and G_q-Coupled Pathways in Regulation of Interleukin-4 by A_{2B} Adenosine Receptors in Human Mast Cells

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ABSTRACT

Human mast cells express functional A_{2A} and A_{2B} adenosine receptors. However, only stimulation of A_{2B}, not A_{2A}, leads to secretion of interleukin (IL)-4, an important step in adenosine receptor-mediated induction of IgE synthesis by B-cells. In this study, we investigate intracellular pathways that link stimulation of A_{2B} receptors to IL-4 up-regulation in HMC-1 mast cells. Both A_{2A} and A_{2B} receptors couple to G_s proteins and stimulate adenylate cyclase, but only A_{2B} stimulates phospholipase Cβ through coupling to G_q proteins leading to activation of protein kinase C and calcium mobilization. Inhibition of phospholipase Cβ completely blocked A_{2B} receptor-dependent IL-4 secretion. The protein kinase C inhibitor 2-{8-[(dimethylamino)-methyl]-6,7,8,9-tetrahydropyrido[1,2-*a*]indol-3-yl}-3-(1-methyl-1*H*-indol-3-yl)maleimide (Ro-32-0432) had no effect on A_{2B} receptor-mediated IL-4 secretion but inhibited phorbol 12-myristate 13-acetate-stimulated IL-4 secretion. In contrast, chelation of intracellular Ca^{2+} inhibited both A_{2B} receptor- and ionomycin-dependent IL-4 secretion. This Ca^{2+}-sensitive pathway probably includes calcineurin and nuclear factor of activated T cells, because A_{2B} receptor-dependent IL-4 secretion was blocked with cyclosporin A or 11R-VIVIT peptide. G_s-linked pathways also play a role in the A_{2B} receptor-dependent stimulation of IL-4 secretion; inhibition of adenylate cyclase or protein kinase A attenuated A_{2B} 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_{2B} receptors up-regulated NFATc1 protein levels. We conclude that A_{2B} receptors up-regulate IL-4 through G_q signaling that is potentiated via cross-talk with G_s-coupled pathways.

Adenosine is an intermediate product in the metabolism of ATP. Extracellular adenosine accumulates in inflamed areas as a result of 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 A_1, A_{2A}, A_{2B}, and A_3 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 patients with asthma. Inhaled adenosine (in the form of AMP) provokes bronchoconstriction in patients with asthma but not in healthy 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

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ABBREVIATIONS: IL, interleukin; NFAT, nuclear factor of activated T cells; NECA, 5′-*N*-ethylcarboxamidoadenosine; IB-MECA, N^6-(3-iodobenzyl)-*N*-methyl-5′-carbamoyladenosine; CGS21680, 2-*p*-(2-carboxyethyl)phenethylamino-NECA; PMA, phorbol 12-myristic 13-acetate; Rp-cAMPs, adenosine 2′,5′-cyclic monophosphorothioate, *R*p-isomer; BAPTA, 1,2-bis(2-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid; AM, tetra(acetoxymethyl) ester; Ro-32-0432, 2-{8-[(dimethylamino)methyl]-6,7,8,9-tetrahydropyrido[1,2-*a*]indol-3-yl}-3-(1-methyl-1*H*-indol-3-yl)maleimide; H89, *N*-[2-((*p*-bromocinnamyl)amino)ethyl]-5-isoquinolinesulfonamide, dihydrochloride; U73122, 1-(6-{[17β-3-methoxyestra-1,3,5-(10)triene-17-yl] amino}hexyl)-1H-pyrrole-2,5-dione; U73343, 1-(6-{[17β-3-methoxyestra-1,3,5-(10)triene-17-yl]amino}hexyl)-2,5-pyrrolidine-dione; PBS, phosphate-buffered saline; RGS, regulator of G protein signaling; 11R-VIVIT, H-Arg-Arg-Arg-Arg-Arg-Arg-Arg-Arg-Arg-Arg-Arg-Gly-Gly-Gly-Met-Ala-Gly-Pro-His-Pro-Val-Ile-Val-Ile-Thr-Gly-Pro-His-Glu-Glu-NH_2; IP_3, inositol 1,4,5-trisphosphate; RhoGEF, Rho guanine nucleotide exchange factor.

mice exhibit a lung phenotype with features of lung inflammation, including bronchial hyper-responsiveness, 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).

Up-regulation of IL-4 plays a major role in the development of asthma. This cytokine induces polarization of T cells toward 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 naive 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 shown that adenosine acting on A_{2B} 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 A_{2A} and A_{2B} receptors (Feoktistov and Biaggioni, 1995, 1998; Feoktistov et al., 2003a). Both A_2 subtypes of adenosine receptors activate adenylate cyclase via G_s-protein. However, only the A_{2B} receptor has been shown to be coupled also to phospholipase Cβ via a GTP-binding protein of the G_q 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 dependence 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 up-regulation 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 of between 3 and 6 × 10^5 cells/ml by dilution with Iscove's medium supplemented with 10% (v/v) fetal bovine serum, 2 mM glutamine, 1.2 mM α-thioglycerol, and antibiotic-antimycotic mixture (Invitrogen, Carlsbad, CA). Cells were kept under humidified atmosphere of air/CO_2 (19:1) at 37°C.

Chemicals. 5′-*N*-Ethylcarboxamidoadenosine (NECA), N^6-(3-iodobenzyl)-*N*-methyl-5′-carbamoyladenosine (IB-MECA), and CGS21680 were purchased from Sigma/RBI (Natick, MA). Phorbol 12-myristic 13-acetate (PMA), ionomycin, forskolin, adenosine 2′,5′-cyclic monophosphorothioate, *R*p-isomer (Rp-cAMPs), and dimethyl sulfoxide were 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. Adenylate cyclase inhibitor 2′,5′-dideoxyadenosine, cell-permeable calcium chelator BAPTA-AM, protein kinase C inhibitor Ro-32-0432, protein kinase A inhibitor H89, cyclosporin A, cell-permeable NFAT inhibitor 11R-VIVIT peptide, phospholipase C inhibitor U73122 and its inactive analog U73343 were purchased from Calbiochem (San Diego, CA). *Pasteurella multocida* toxin was obtained from List Biological Laboratories (Campbell, CA).

Measurement of cAMP Accumulation. HMC-1 (2 × 10^6 cells/ml) were preincubated in 200 μl of 150 mM NaCl, 2.7 mM KCl, 0.37 mM NaH_2PO_4, 1 mM $MgSO_4$, 1 mM $CaCl_2$, 5 g/l D-glucose, and 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 (TRK432; GE Healthcare, Little Chalfont, Buckinghamshire, UK).

Measurement of [^{3}H]Inositol Phosphate Formation. Formation of inositol phosphates was determined using a modification of the procedure described by Seuwen et al. (1988). HMC-1 cells (5 × 10^6 cells/ml) were labeled to equilibrium with [*myo*-^{3}H]inositol (2 μCi/ml; PerkinElmer Life and Analytical Sciences, Boston, MA) for 18 h in inositol-free DMEM. In some experiments, 1 μg/ml *P. 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 × 10^6 cells/ml in 150 mM NaCl, 2.7 mM KCl, 0.37 mM NaH_2PO_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 *P. multocida* toxin, U73122, or U73343. After preincubation for 15 min at 37°C, NECA or its vehicle was 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 of 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, Hercules, CA). Free inositol and glycerophosphoinositol were eluted with 1.25 ml of H_2O 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 pretreated with 1 μg/ml *P. multocida* toxin for 18 h. Before experiments, cells were washed twice with PBS and resuspended at a concentration of 2 × 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 (Eagle's minimum essential medium, Joklik modification; Sigma) was used instead of Iscove's medium. After 15 min of preincubation, reactions were started by addition of stimulants and continued for 6 h under humidified atmosphere of air/CO_2 (19:1) at 37°C. At the end of this incubation period, the culture media were collected by centrifugation at 12,500*g* for 1 min at 4°C. IL-4 and IL-8 concentrations were measured using enzyme-linked immunosorbent assay 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 of serum-free Iscove's medium containing 1.5 μl of Fugene 6. After 15-min

incubation at room temperature, the transfection mixture was added to 5×10^5 cells suspended in 500 μl of growth medium.

Cells were cotransfected with cDNA described under *Results* section and luciferase reporters at a ratio of 5:1. The ratio 20:1 was used for the IL-4 firefly luciferase reporter/control *Renilla reniformis* 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) was obtained from UMR cDNA Resource Center (Rolla, MO), 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 *R. reniformis* luciferase plasmid pRL-SV40 was purchased from Promega (Madison, WI). Twenty-four hours after transfections, cells were incubated in the presence of reagents indicated under *Results* for an additional 6 h. Reporter activity was measured using a Dual-Luciferase Reporter Assay System (Promega). Firefly luciferase reporter activities were normalized against *R. reniformis* luciferase activities from the coexpressed pRL-SV40 and expressed as relative luciferase activities over basal (set as 1).

Western Blot Analysis of NFATc1 and NFATc2 Protein Levels. HMC-1 cells (10^7) were washed in ice-cold PBS and then lysed in 0.5 ml of radioimmunoprecipitation assay buffer (PBS, 1% Nonidet P-40, 0.5% sodium deoxycholate, and 0.1% SDS) that contained 0.1 M Na_2CO_3 and a 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*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), preincubated in sample buffer (Invitrogen) at 70°C for 5 min, were resolved on NuPAGE Bis-Tris gradient 4 to 12% gel (Invitrogen), and transferred to PVDF membranes (Millipore Corporation, Billerica, MA) by electroblotting. Membranes were blocked with 3% (w/v) dry fat-free milk in Tris-buffered saline with 0.05% Tween 20 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) at a dilution of 1:300 by incubating at room temperature for 1 h. After washing with Tris-buffered saline with 0.05% Tween 20, 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).

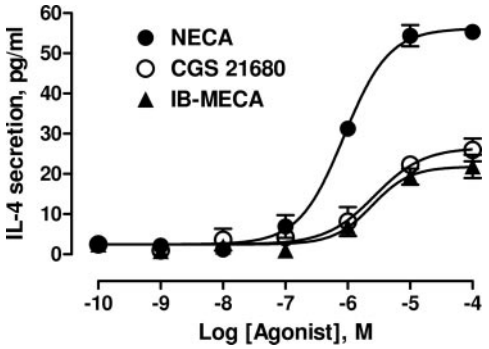

Fig. 1. Stimulation of IL-4 production by adenosine receptor agonists in HMC-1. Concentration-response curves for IL-4 secretion induced by the nonselective agonist NECA (●), by the selective A_{2A} agonist CGS21680 (○) or by the selective A_3 agonist IB-MECA (▲). Values are expressed as mean ± S.E.M. (n = 3).

Results

Role of Adenosine Receptor Subtypes in IL-4 Secretion. HMC-1 cells express mRNA for the A_{2A}, A_{2B}, and A_3 but not A_1 subtypes 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 EC_{50} of 0.9 μM, close to the reported affinity of this agonist at A_{2B} adenosine receptors (Feoktistov and Biaggioni, 1997). In contrast, the A_{2A} receptor agonist CGS21680 and the A_3 agonist IB-MECA had no effect when used at selective concentrations (<1 μM). These results are consistent with a pharmacological profile of A_{2B} adenosine receptors and agree with previously reported inhibition of NECA-induced IL-4 secretion in mast cells by the selective A_{2B} antagonist 3-isobutyl-8-pyrrolidinoxanthine (Ryzhov et al., 2004). From the data presented in Fig. 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 G_q-Linked Signaling Pathways in A_{2B} Receptor-Dependent Up-Regulation of IL-4. We have shown previously that A_{2B} adenosine receptors stimulate phosphoinositide turnover and calcium mobilization in HMC-1 cells via a pertussis toxin-insensitive mechanism, indicating involvement of $G_{q/11}$ proteins and phospholipase Cβ. (Feoktistov and Biaggioni, 1995). To determine whether this signaling pathway is involved in A_{2B} receptor-dependent up-regulation of IL-4, we initially evaluated the role of $G_{q/11}$ proteins in IL-4 up-regulation using a reporter assay. As seen in Fig. 2, NECA (10 μM) increased IL-4 reporter activity in HMC-1 cells by 5.0 ± 0.1-fold. Overexpression of the preferential $G_{q/11}$ inhibitor RGS2 (Heximer et al., 1997, 1999; Tang et al., 2003) resulted in 75% inhibition of NECA-induced IL-4 reporter activity, indicating the involvement of $G_{q/11}$ proteins in the regulation of IL-4 transcription. In contrast, overex-

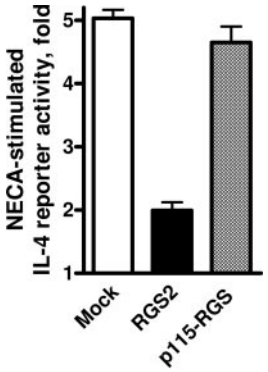

Fig. 2. Effect of expression of RGS2 and p115 RhoGEF-RGS on NECA-dependent IL-4 promoter activity. Activation of IL-4 gene promoter was studied by cotransfection of IL-4 luciferase reporters together with vectors encoding RGS2 and the RGS box of p115 RhoGEF (indicated on graph as p115-RGS) or with an 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 h. The results from three experiments are expressed as mean ± S.E.M. of NECA-induced stimulation of luciferase activity.

pression of p115 RhoGEF RGS, a selective inhibitor of pertussis toxin-insensitive $G_{12/13}$ proteins (Hains et al., 2004) not coupled to phospholipase Cβ (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 (Fig. 3A) and IL-4 secretion (Fig. 3B) at concentrations in the low micromolar range. These results demonstrate functional engagement of phospholipase Cβ in NECA-induced IL-4 up-regulation 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 (IP_3) that mediates the release of intracellular calcium. We found that stimulation of protein kinase C by 10 nM PMA or increase in intracellular Ca^{2+} by 1 μM ionomycin induced IL-4 secretion in HMC-1 cells by 3.6 $\pm$ 0.2- and 2.2 $\pm$ 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 (Fig. 4A). In contrast, chelation of intracellular Ca^{2+} by BAPTA-AM inhibited IL-4 secretion, induced by both ionomycin and NECA, to a similar extent (Fig. 4B) indicating an important role of Ca^{2+}-dependent processes in A_{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_{2B} receptor-dependent IL-4 production, we initially used cyclosporin A, which, in complex with an endogenous protein cyclophilin, binds to calcineurin and inhibits its catalytic activity (Liu et al., 1991). As seen in Fig. 5, left, inhibition of calcineurin with cyclosporin A effectively blocked the NECA-induced IL-4 secretion. Inhibition of catalytic activity of calcineurin by cyclosporin 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 et al., 1999) to specifically block NFAT activation. As seen in Fig. 5, right, 11R-VIVIT peptide inhibited NECA-induced IL-4 secretion, confirming an important role of NFAT activation by calcineurin in A_{2B} receptor-dependent stimulation of IL-4 secretion.

Taken together, our results indicate that coupling of A_{2B} receptors to $G_{q/11}$ proteins ultimately leads to up-regulation of IL-4 in HMC-1 that involves stimulation of phospholipase Cβ, synthesis of IP_3, release of Ca^{2+} from intracellular stores, stimulation of calcium-dependent phosphatase calcineurin, and activation of NFAT.

Role of G_s-Linked Signaling Pathways in A_{2B} Receptor-Dependent Up-Regulation of IL-4. In addition to stimulation of phospholipase Cβ via G proteins of the $G_{q/11}$

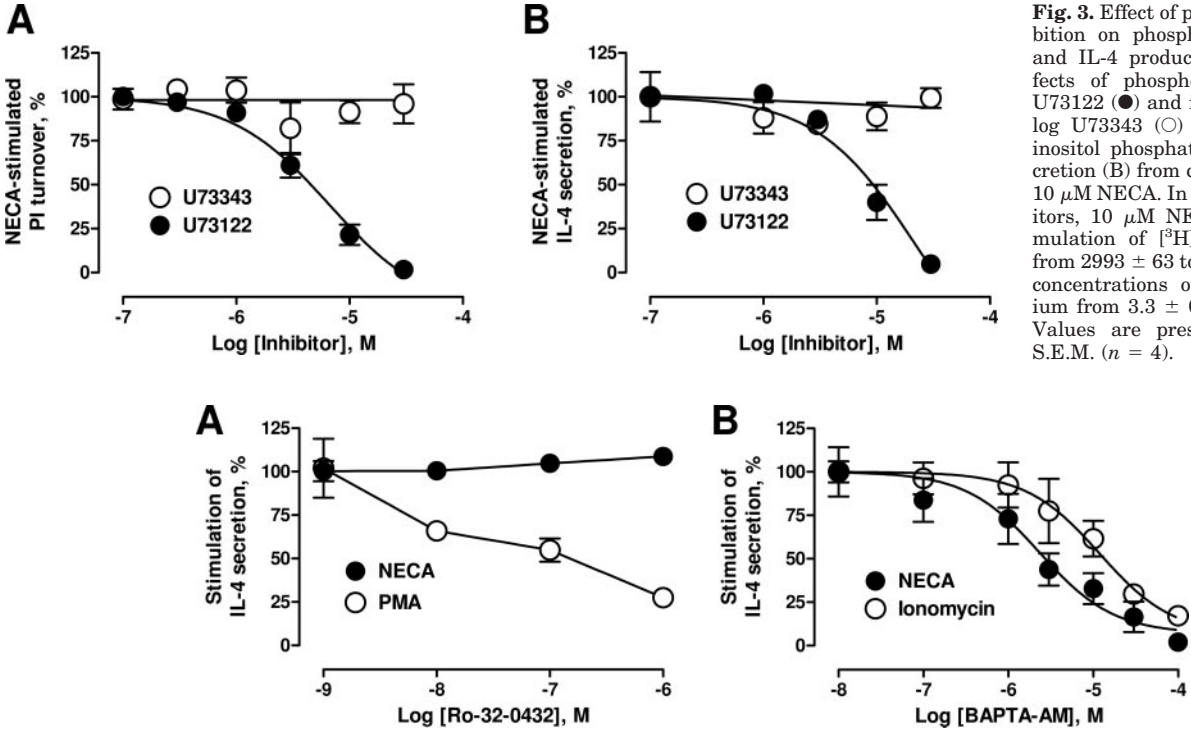

Fig. 3. Effect of phospholipase C inhibition on phosphoinositide turnover and IL-4 production in HMC-1. Effects of phospholipase C inhibitor U73122 (●) and inactive control analog U73343 (○) on accumulation of inositol phosphates (A) and IL-4 secretion (B) from cells stimulated with 10 μM NECA. In the absence of inhibitors, 10 μM NECA increased accumulation of [^{3}H]inositol phosphates from 2993 $\pm$ 63 to 4233 $\pm$ 75 dpm and concentrations of IL-4 in the medium from 3.3 $\pm$ 0.4 to 62 $\pm$ 6 pg/ml. Values are presented as mean $\pm$ S.E.M. (n = 4).

Fig. 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 (●) or 10 nM PMA (○) for 6 h. In the absence of the inhibitor, NECA increased concentrations of IL-4 in the medium from 3.7 $\pm$ 0.3 to 63.2 $\pm$ 3.6 pg/ml and 10 nM PMA increased IL-4 from 3.7 $\pm$ 0.3 to 13.5 $\pm$ 2.1 pg/ml. Values are presented as mean $\pm$ S.E.M. (n = 3). B, effect of the calcium chelator BAPTA-AM on IL-4 secretion from cells stimulated with 10 μM NECA (●) or 1 μM ionomycin (○) for 6 h. In the absence of BAPTA-AM, 10 μM NECA increased concentrations of IL-4 in the medium from 3.6 $\pm$ 0.4 to 25.4 $\pm$ 1.5 pg/ml and 1 μM ionomycin increased IL-4 from 3.6 $\pm$ 0.4 to 7.9 $\pm$ 1.1 pg/ml. Please note that these experiments were conducted using a Ca^{2+}-free medium. Values are presented as mean $\pm$ S.E.M. (n = 3).

family, A_{2B} adenosine receptors also stimulate adenylate cyclase via G_s proteins (Feoktistov and Biaggioni, 1995). To elucidate a potential role of adenylate cyclase activation in A_{2B} 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 (Fig. 6A). 2′,5′-Dideoxyadenosine also inhibited NECA-stimulated IL-4 secretion (Fig. 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 inhibit NECA-stimulated IL-8 secretion, a cAMP-independent process described previously (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-cAMPs, also resulted in partial inhibition of NECA-induced IL-4 secretion but had no effect on NECA-induced IL-8 secretion (Fig. 6C). Furthermore, blocking the ATP binding site of protein kinase A with 1 μM 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 A_{2B} Receptors in Stimulation of IL-4 Secretion. Inhibitory analysis of signaling cascades activated by A_{2B} 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 A_{2B} receptors on G_s-adenylate cyclase pathways without activation of G_q-phospholipase Cβ. To ensure that we did not overstimulate these pathways, we conducted ancillary studies and determined that 1 μM forskolin and 10 μM NECA produced similar levels of cAMP accumulation in HMC-1 (Fig. 7A). G_q-phospholipase Cβ pathway was stimulated by *P. multocida* toxin (Wilson and Ho, 2004). Incubation of HMC-1 cells with 1 μg/ml *P. multocida* toxin resulted in stimulation of phosphoinositide turnover that was approximately 60% of that induced by 10 μM NECA (Fig. 7B).

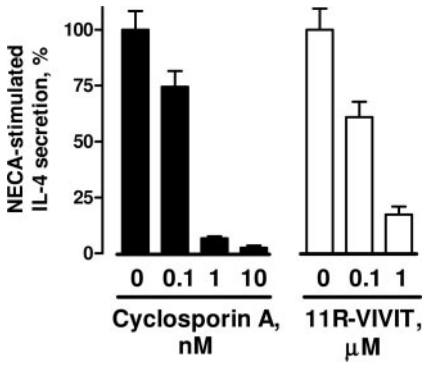

Fig. 5. Effect of calcineurin inhibition on IL-4 production in HMC-1. Effect of cyclosporin A (left) and 11R-VIVIT peptide (right) on IL-4 secretion from cells stimulated with 10 μM NECA. In the absence of inhibitors, 10 μM NECA increased concentrations of IL-4 in the medium from 5.3 ± 0.1 to 78.4 ± 6.2 pg/ml. Values are presented as mean ± S.E.M. (n = 3).

As seen in Fig. 7C, *P. multocida* toxin stimulated IL-4 secretion by 1.9 ± 0.2 fold. Forskolin had no effect on its own but potentiated the effect of *P. multocida* toxin on IL-4 secretion, resulting in 2.8 ± 0.2-fold stimulation ($p < 0.05$, unpaired, two-tailed t test, compared with stimulation with *P. 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, Fig. 7C). These data indicate that cross-talk between G_s- and G_q-linked pathways occurs downstream from IP_3-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 Fig. 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- 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

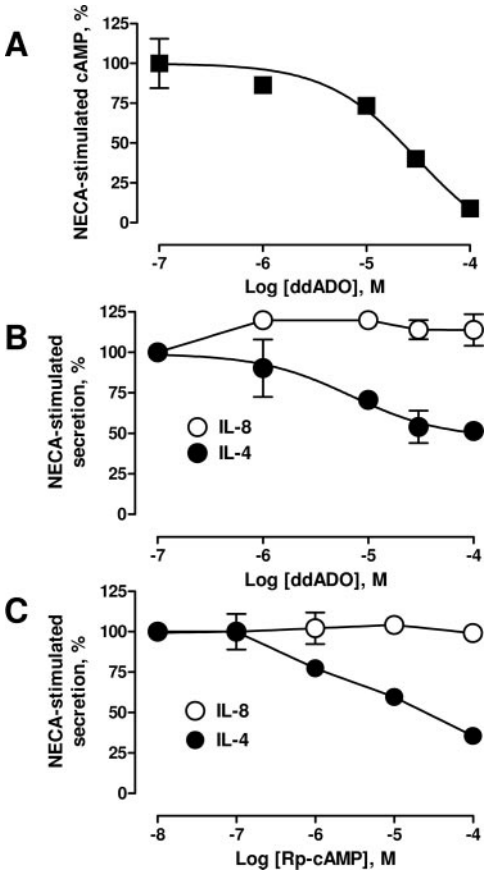

Fig. 6. Effects of adenylate cyclase and protein kinase A inhibition on cytokine production in HMC-1. A, effect of adenylate cyclase inhibitor 2′,5′-dideoxyadenosine on cAMP accumulation in cells stimulated with 10 μM NECA. In the absence of 2′,5′-dideoxyadenosine, 10 μM NECA increased cAMP levels from 3.2 ± 0.6 to 19.4 ± 2.9 pmol/10^6 cells. Values are presented as mean ± S.E.M. (n = 3) of NECA-stimulated response. B, effect of adenylate cyclase inhibitor 2′,5′-dideoxyadenosine on IL-4 (●) or IL-8 (○) 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 1209 ± 27 pg/ml. Values are presented as mean ± S.E.M. (n = 3) of NECA-stimulated response. C, effect of protein kinase A inhibitor Rp-cAMPs on IL-4 (●) or IL-8 (○) secretion from cells stimulated with 10 μM NECA. Values are presented as mean ± S.E.M. (n = 3) of NECA-stimulated response.

12 ± 1- to 32 ± 6-fold ($p < 0.05$, t test, Fig. 8B). These data indicate that interaction between G_s- and G_q-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 G_s-adenylate cyclase-dependent processes seems unlikely. We hypothesized then that stimulation of G_s-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 G_q-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 Fig. 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 A_{2B} 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 A_{2B} receptor antagonists as a new therapeutic approach to asthma (Feoktistov et al., 1998, 2001; Kim et al., 2000; Hayallah et al., 2002; Cacciari et al., 2005; Holgate, 2005; Varani et al., 2005; Zablocki et al., 2005). We have 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 A_{2A}, A_{2B}, and A_3 adenosine receptors (Meade et al., 2002; Feoktistov et al., 2003b). There is, however, no evidence of functional coupling of A_3 receptors to adenylate cyclase or phospholipase Cβ in HMC-1 (Feoktistov et al., 2003b), whereas both A_{2A} and A_{2B} are linked to stimulation of adenylate cyclase (Feoktistov and Biaggioni, 1995). In addition, A_{2B}

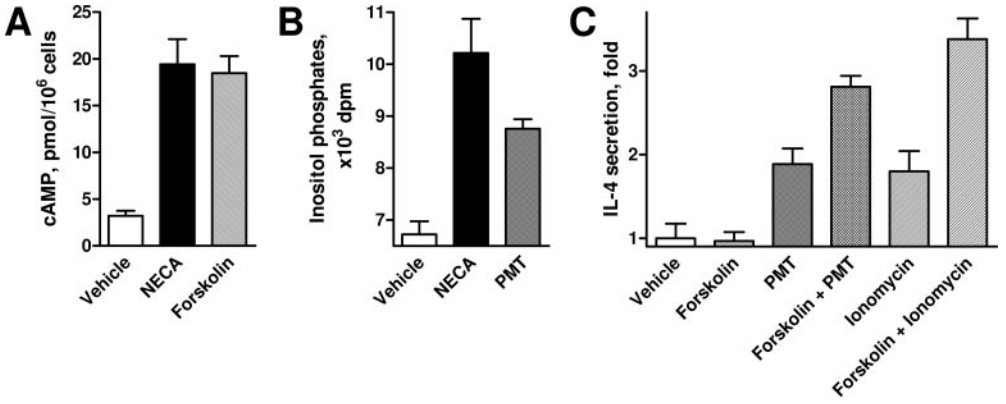

Fig. 7. Potentiation of *P. multocida* toxin- and ionomycin-induced stimulation of IL-4 by forskolin. A, effects of 10 μM NECA, 1 μM forskolin or their vehicle on cAMP accumulation in HMC-1. Values are presented as mean ± S.E.M. ($n = 3$). B, effects of 10 μM NECA, 1 μg/ml *P. multocida* toxin (PMT) or their vehicle on accumulation of inositol phosphates in HMC-1. Values are presented as mean ± S.E.M. ($n = 4$). C, effects of 1 μM forskolin, 1 μg/ml *P. multocida* toxin (PMT), combination of 1 μM forskolin and 1 μg/ml *P. multocida* toxin, 1 μM ionomycin, combination of 1 μM forskolin and 1 μM ionomycin, or their vehicle on IL-4 secretion from HMC-1 cells. Values are presented as mean ± S.E.M. ($n = 3$).

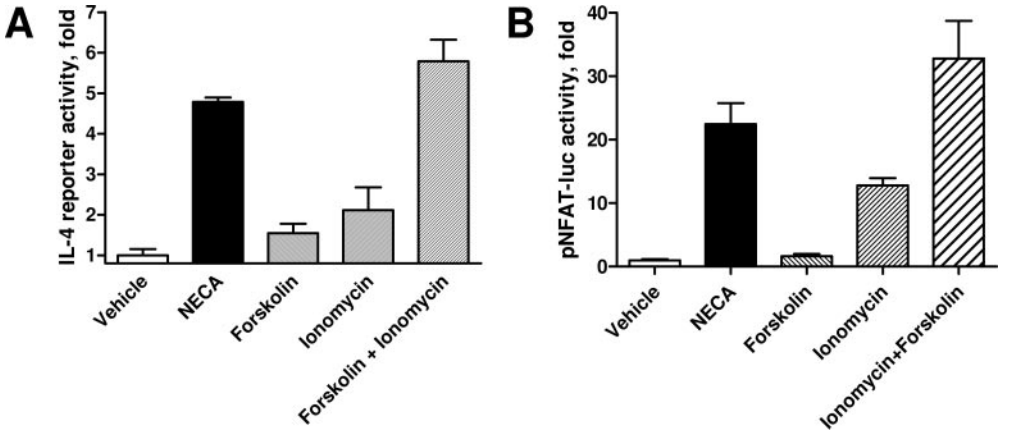

Fig. 8. Interaction of cAMP and Ca^{2+}-dependent pathways at the NFAT-binding site and IL-4 promoter. Effects of 10 μM NECA, 1 μM forskolin, 1 μM ionomycin, combination of 1 μM forskolin and 1 μM ionomycin, or their vehicle, on IL-4 reporter activity (A), or activity of pNFAT-luc reporter driven by a minimal promoter under control of 4× NFAT binding sequences (B), in HMC-1 cells. Values are presented as mean ± S.E.M. ($n = 3$).

receptors are also linked to stimulation of phospholipase Cβ through coupling to pertussis toxin-insensitive $G_{q/11}$ proteins (Feoktistov and Biaggioni, 1995). In agreement with previous results (Ryzhov et al., 2004), only stimulation of A_{2B} receptors, not A_{2A} or A_3 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 (Fig. 10) revealed their essential role in A_{2B} receptor-dependent IL-4 generation. Activation of G_q with *P. multocida* toxin stimulated phospholipase Cβ and IL-4 secretion. Overexpression of the preferential G_q inhibitor RGS2 significantly reduced A_{2B} receptor-dependent stimulation of IL-4 reporter. Stimulation of IL-4 secretion by NECA, mediated via A_{2B} 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, IP_3 and diacylglycerol, stimulate Ca^{2+} mobilization and protein kinase C, respectively. Our results indicate that Ca^{2+} mobilization, but not protein kinase C stimulation, contribute to IL-4 up-regulation by A_{2B} receptors; IL-4 secretion was stimulated by increasing intracellular calcium with ionomycin; conversely, chelation of intracellular Ca^{2+} 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 A_{2B} receptor-dependent IL-4 secretion. Inhibition of calcineurin downstream from calcium mobilization with cyclosporin A blocked A_{2B} receptor-dependent IL-4 secretion. Furthermore, 11R-VIVIT peptide, a selective blocker of calcineurin-NFAT interaction, also inhibited this process.

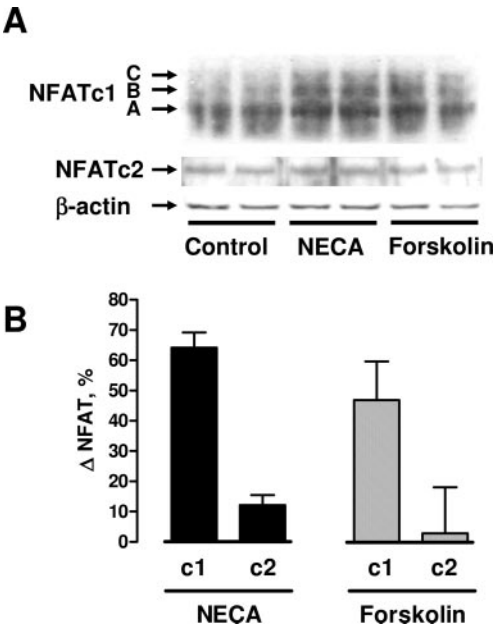

Fig. 9. Effects of NECA and forskolin on NFAT protein levels. A, Western blot analysis of NFATc1 and NFATc2 protein levels in resting HMC-1 (control) and cells stimulated with 10 μM NECA or 1 μM forskolin. Arrows A, B, and C indicate positions of NFATc1 splice variants commonly present in various cells (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.

The results of our study delineated a signal transduction pathway from A_{2B} receptors (via G_q, phospholipase Cβ, IP_3, mobilization of intracellular calcium, calcineurin, and NFAT) to IL-4 production. This is in agreement with the reported property of other G_q-coupled receptors to stimulate NFAT in PC12 and Jurkat cells (Boss et al., 1996). These data also explain why stimulation of A_{2B} receptors coupled to G_s and G_q proteins, but not A_{2A} receptors coupled only to G_s, induced IL-4 secretion in HMC-1.

Our study also revealed the existence of cross-talk between G_s- and G_q-dependent pathways stimulated by A_{2B} adenosine receptors. We demonstrated for the first time that G_s-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 granulocyte macrophage–colony-stimulating factor, IL-5 and MIP-1α production in cord blood-derived mast cells (Shichijo et al., 1999). We have reported previously that A_{2B} adenosine receptors stimulated generation of IL-8 in HMC-1 independently from cAMP (Feoktistov and Biaggioni, 1995). In the current study, we found that A_{2B}

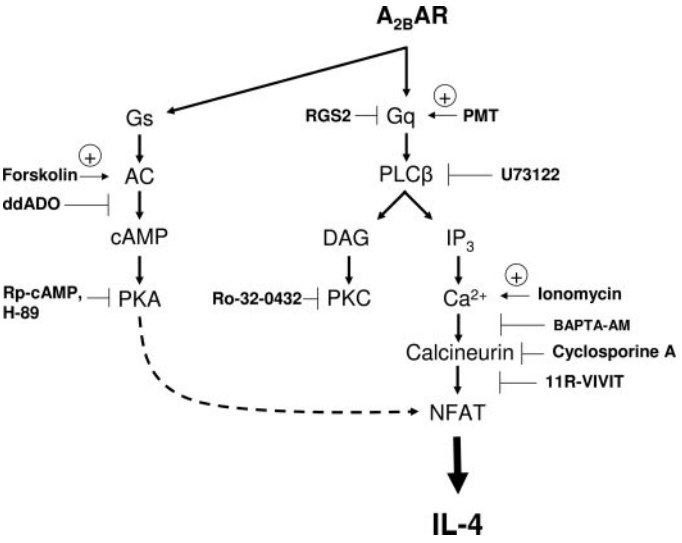

Fig. 10. Schematic representation of A_{2B} receptor-stimulated intracellular pathways involved in regulation of IL-4 production in HMC-1. These cells express functional A_{2B} receptors (A_{2B}AR) coupled to adenylate cyclase (AC) via G_s-protein. Activation of this pathway results in accumulation of cAMP and stimulation of protein kinase A (PKA). A_{2B}AR are coupled also to phospholipase Cβ (PLCβ) via a GTP-binding protein of the G_q family. Activation of this pathway results in increase in diacylglycerol (DAG) and IP_3. DAG stimulates protein kinase C (PKC). IP_3 activates mobilization of calcium from intracellular stores (Feoktistov and Biaggioni, 1995). In this study, we present evidence that A_{2B}AR stimulate IL-4 production via G_q-mediated stimulation of phospholipase Cβ, IP_3-mediated mobilization of intracellular Ca^{2+} and activation of NFAT by calcineurin. This process was blocked by the G_q inhibitor RGS2, phospholipase C inhibitor U73122, the calcium chelator BAPTA-AM, the calcineurin inhibitor cyclosporin A, the calcineurin-NFAT interaction inhibitor 11R-VIVIT peptide, but not by the PKC inhibitor Ro-32-0432. A_{2B}AR also modulates IL-4 production via G_s-mediated stimulation of adenylate cyclase and activation of protein kinase A. A_{2B}AR-stimulated IL-4 production was attenuated by the adenylate cyclase inhibitor 2′,5′-dideoxyadenosine (ddADO), and the protein kinase A inhibitors Rp-cAMPs and H-89. Stimulation of G_s-adenylate cyclase pathways with forskolin did not have an effect on its own, but potentiated IL-4 production associated with stimulation of G_q-phospholipase Cβ with *Pasteurella multocida* toxin (PMT) or mobilization of intracellular Ca^{2+} with ionomycin. The broken arrow in the diagram signifies the potentiating effect of G_s-adenylate cyclase-protein kinase A stimulation on IL-4 production.

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-cAMPs and H-89. The inhibition produced by these compounds was partial, suggesting that the G_s-adenylate cyclase-protein kinase A pathway is not obligatory for IL-4 secretion, but it is rather important for modulation of signal transduction via G_q-phospholipase Cβ pathway. Indeed, stimulation of G_q-phospholipase Cβ pathways with *P. multocida* toxin was associated with increased IL-4 secretion, and stimulation of G_s-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.

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 G_s-adenylate cyclase-protein kinase A pathway has no effect on IL-4 secretion in the absence of G_q-phospholipase Cβ-dependent stimulation of NFAT. Therefore, it is unlikely that G_s-adenylate cyclase-protein kinase A pathway stimulates NFAT directly; rather, it probably facilitates stimulation mediated via G_q-phospholipase Cβ-dependent pathways. One possible explanation could involve up-regulation of NFAT by G_s-adenylate cyclase-protein kinase A-dependent mechanisms, thus increasing the pool of NFAT available for stimulation via G_q-phospholipase Cβ-dependent pathways. Our results support this possibility; both stimulation of A_{2B} receptors with NECA and stimulation of G_s-adenylate cyclase pathway with forskolin up-regulated NFATc1 protein levels. Our results do not exclude, however, the possibility that there could be other protein kinase A-dependent pathways involved in potentiation of IL-4 secretion stimulated via G_q-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 G_q-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, and both positive and negative interactions have been 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 A_{2B} receptors to G_s/G_q proteins with concurrent stimulation of diverse intracellular pathways for adenosine-dependent regulation of IL-4 production in human mast cells (Fig. 10). A_{2B} adenosine receptors induce IL-4 generation via G_q-mediated stimulation of phospholipase Cβ, IP_3-mediated mobilization of intracellular Ca^{2+}, and activation of NFAT by calcineurin. This process is potentiated via G_s-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 G_q-phospholipase Cβ and G_s-adenylate cyclase signaling pathways in regulation of IL-4 secretion enables A_{2B} receptors, coupled to both G_q and G_s, to effectively stimulate IL-4 production in mast cells and contribute to the allergic inflammatory response associated with asthma.

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