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Role of p38 Mitogen-Activated Protein Kinase and Extracellular Signal-Regulated Protein Kinase Kinase in Adenosine A_{2B} Receptor-Mediated Interleukin-8 Production in Human Mast Cells

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ABSTRACT

The endogenous nucleoside adenosine is thought to play a role in the pathophysiology of asthma by stimulating mast cells. We previously showed that the human mast cell line HMC-1 expresses A_{2A} and A_{2B} receptors, and that both receptors activate adenylate cyclase via G_s-protein but that only A_{2B} receptors are also coupled to phospholipase C via G_q proteins. Stimulation of A_{2B} but not A_{2A} receptors induced production of interleukin-8 (IL-8) from HMC-1 cells. The mechanism by which adenosine promotes IL-8 synthesis has not been defined. In this study, we tested the hypothesis that mitogen-activated protein kinase (MAPK) signaling pathways are involved in this process. Stimulation of HMC-1 with the stable adenosine analog NECA (5′-N-ethylcarboxamidoadenosine) activated $p21^{ras}$ and both p42 and p44 isoforms of extracellular signal-regulated kinase (ERK). NECA (10 μM) induced a 1.9 $\pm$ 0.06-fold increase in ERK activity, whereas 10 μM of the selective A_{2A} agonist CGS 21680 (4-((*N*-ethyl-5′-carbamoyladenos-2-yl)-aminoethyl)-phenylpropionic acid) had no effect. NECA, in parallel with the activation of ERK, also stimulated the p46 isoform of c-Jun N-terminal kinase (MEK) and p38 MAPK. Furthermore, the selective MAPK/ERK kinase 1 inhibitor PD 98059 (2′-amino-3′-methoxyflavone), and p38 MAPK inhibitors SB 202190 (4-(4-fluorophenyl)-2-(4-hydroxyphenyl)-5-(4-pyridyl)1H-imidazole) and SB 203580 (4-(4-fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)1H-imidazole) blocked A_{2B} receptor-mediated production of IL-8. These results indicate that extracellular adenosine can regulate ERK, c-Jun N-terminal kinase, and p38 MAPK signaling cascades and that activation of ERK and p38 MAPK pathways are essential steps in adenosine A_{2B} receptor-dependent stimulation of IL-8 production in HMC-1.

The endogenous nucleoside adenosine can be released to or formed in the extracellular space under hypoxic and inflammatory conditions. Once generated, adenosine acts as an autocoid by interacting with adenosine receptors belonging to the seven transmembrane G protein-coupled group of cell surface receptors. Among other sources, activated mast cells, platelets, and neutrophils have been shown to release adenosine and adenine nucleotides. Adenosine has diverse effects on inflammatory process. The activation of A_{2A} receptors inhibits oxidative burst, degranulation, and adhesion of neutrophils (Cronstein et al., 1992) and also inhibits platelet aggregation (Cristalli et al., 1994). Adenosine at A_1 receptors can increase neutrophil chemotaxis (Cronstein et al., 1992), whereas activation of A_3 receptors inhibits eosinophil chemotaxis (Walker et al., 1997).

The ability of extracellular adenosine to modulate mast cell function has long been recognized (Marquardt et al., 1978; Church and Hughes, 1985; Peachell et al., 1988). Activation of mast cells by adenosine has been implicated in the pathophysiology of asthma (Church and Holgate, 1986; Feoktistov et al., 1998). Inhaled adenosine, or its precusor AMP, provokes bronchoconstriction in asthmatic patients via activation of mast cells (Cushley and Holgate, 1985). Whereas adenosine A_3 receptor has been shown to modulate rat mast cell function (Ramkumar et al., 1993), it appears that the A_{2B} adenosine receptor subtype regulates mouse (Marquardt et al., 1994), canine (Auchampach et al., 1997), and human mast cell activation (Feoktistov and Biaggioni, 1995).

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ABBREVIATIONS: NECA, 5′-N-ethylcarboxamidoadenosine; CGS 21680, 4-((*N*-ethyl-5′-carbamoyladenos-2-yl)-aminoethyl)-phenylpropionic acid; IL-8, interleukin-8; MAPK, mitogen-activated protein kinase; ERK, extracellular signal-regulated kinase; JNK, Jun N-terminal kinase; SB 202190, 4-(4-fluorophenyl)-2-(4-hydroxyphenyl)-5-(4-pyridyl)1H-imidazole; SB 203580, 4-(4-fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)1H-imidazole; MEK, MAPK/ERK kinase; RBD, minimal $p21^{ras}$-binding domain of Raf-1; GST, glutathione *S*-transferase; PD 98059, 2′-amino-3′-methoxyflavone; Ro 32-0432, [2-{8-[(dimethylamino)methyl]-6,7,8,9-tetrahydropyrido[1,2-a]indol-3-yl}-3-(1-methylindol-3-yl)maleimide, hydrochloride]; PMA, phorbol 12-myristate 13-acetate.

We previously showed that the human mast cell line HMC-1 expresses functional A_{2A} and A_{2B} receptors (Feoktistov and Biaggioni, 1995; Feoktistov and Biaggioni, 1998). Both A_2 subtypes of adenosine receptors activate adenylate cyclase via G_s-protein. However, only the A_{2B} receptor has been shown to also be coupled to phospholipase C in mast cells via a GTP-binding protein of the G_q family. Furthermore, the nonselective A_{2A}/A_{2B} adenosine receptor agonist 5′-N-ethylcarboxamidoadenosine (NECA), but not the selective A_{2A} agonist 4-((*N*-ethyl-5′-carbamoyladenos-2-yl)-aminoethyl)-phenylpropionic acid (CGS 21680), induced secretion of interleukin-8 (IL-8) from HMC-1 cells (Feoktistov and Biaggioni, 1995). IL-8 is not stored preformed in HMC-1 and requires synthesis de novo (Selvan et al., 1994), suggesting that stimulation of adenosine A_{2B} receptors can trigger transcription and synthesis of this cytokine. However, the mechanism by which adenosine promotes biosynthesis of IL-8 is not clear.

Recently, several mitogen-activated protein kinase (MAPK) signaling pathways have been demonstrated to play a central role in mediating intracellular signal transduction from the cell surface to the nucleus. At least three MAPK subfamilies are present in mammalian cells and form distinct signaling cascades. These include extracellular signal-regulated kinases (ERK), c-Jun N-terminal kinases (JNK), and p38 MAPKs. There is recent evidence that the p38 MAPK pathway regulates synthesis of IL-8 in neutrophils stimulated with tumor necrosis factor-α and *N*-formylmethionine leucyl-phenylalanine, whereas the ERK cascade does not play any role in this process (Zu et al., 1998). Less is known about the coupling of adenosine receptors to MAPK pathways. It has been shown that adenosine A_{2A} receptors expressed in Chinese hamster ovary cells inhibit thrombin-induced ERK activation via increases in cAMP (Hirano et al., 1996). Similarly, the selective A_{2A} agonist CGS 21680 inhibited ERK activation by cAMP-dependent mechanism in mast cells derived from human umbilical cord blood (Suzuki et al., 1998). In contrast, in human endothelial cells A_{2A} adenosine receptors stimulated ERK pathway independently from cAMP (Sexl et al., 1997). The coupling of A_{2B} adenosine receptors with MAPK signaling pathways, to our knowledge, has not been described and the coupling of A_2 adenosine receptors to JNK and p38 MAPKs has not been reported. The present study was undertaken to elucidate the role of MAPK signaling pathways in A_{2B}-mediated IL-8 secretion in the human mast cell line HMC-1. We report that stimulation of adenosine A_2 receptors triggers activation of $p21^{ras}$-ERK, JNK, and p38 MAPK signaling pathways. Using highly selective inhibitors of extracellular signal-regulated protein kinase kinase (MEK) and p38 MAPK we found that stimulation of ERK and p38 MAPK pathways are essential steps in the mechanism of adenosine A_{2B} receptor-mediated IL-8 secretion in HMCs.

Materials and Methods

Cell Culture and Reagents. HMC-1 cells were a generous gift from Dr. J. H. Butterfield (Mayo Clinic, Rochester, MN). HMC-1 cells were maintained in suspension culture at a density between 3 and 9×10^5 cells/ml by dilution with Iscove's medium supplemented with 10% (vol/vol) fetal bovine serum, 2 mM glutamine, antibiotics, and 1.2 mM α-thioglycerol. The cells were kept under a humidified atmosphere of air/CO_2 (19:1) at 37°C.

CGS 21680 and NECA were purchased from Research Biochemicals, Inc. (Natick, MA). Forskolin, wortmannin, genistein, herbimycin A, thapsigargin, calcium ionophore A23187, and phorbol 12-myristate 13-acetate (PMA) were obtained from Sigma Chemical Co. (St. Louis, MO). 2′-Amino-3′-methoxyflavone (PD 98059), 4-(4-fluorophenyl)-2-(4-hydroxyphenyl)-5-(4-pyridyl)1H-imidazole (SB 202190), 4-(4-fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)1H-imidazole (SB 203580), and [2-{8-[(dimethylamino)methyl]-6,7,8,9-tetrahydropyrido[1,2-a]indol-3-yl}-3-(1-methylindol-3-yl)maleimide, hydrochloride] (Ro 32–0432) were purchased from Calbiochem-Novabiochem Corp. (San Diego, CA).

Measurement of IL-8 Secretion. HMC-1 cells were harvested and resuspended to a concentration of 10^6 cells/ml in serum-free Iscove's media containing 1 U/ml adenosine deaminase. Cells were incubated for 2.5 h (or for the times indicated in *Results*) under a humidified atmosphere of air/CO_2 (19:1) at 37°C with the reagents indicated in *Results*. At the end of this incubation period, the culture media were collected by centrifugation at 12,000*g* for 1 min at 4°C. IL-8 concentrations were measured using an enzyme-linked immunosorbent assay kit (American Laboratory Products Co. Ltd, Windham, NH).

Stimulation of Mast Cells. HMC-1 cells were harvested and resuspended to a concentration of 10^7 cells/ml in a buffer, pH 7.4, containing 150 mM NaCl, 2.7 mM KCl, 0.37 mM NaH_2PO_4, 1 mM $MgSO_4$, 1 mM $CaCl_2$, 5 g/liter D-glucose, 10 mM HEPES-NaOH, and 1 U/ml adenosine deaminase. After 15 min preincubation at 37°C, 200- to 1000-μl aliquots of cell suspension were incubated for various times at 37°C with the reagents indicated in *Results*.

Assay of Tyrosine Phosphorylation of ERK. To evaluate phosphorylation of ERK at tyrosine residues, following each stimulation, 2×10^6 HMC-1 cells were collected by centrifugation for 15 s at 12,000*g* and lysed by addition of 100 μl of boiling 1% SDS. After boiling for 15 min, samples were diluted with 900 μl of ice-cold buffer containing 10 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1% Triton X-100, 0.5% Nonidet P-40, 1 mM EDTA, 1 mM EGTA, 2 mM sodium orthovanadate, 10 μg/ml leupeptin, 10 μg/ml aprotinin and 1 mM phenylmethylsulfonyl fluoride (lysis buffer A). Unsoluble material was removed by centrifugation for 10 min at 16,000*g* at 4°C and supernatant was precleared with 50 μl of agarose for 1 h at 4°C. Proteins phosporylated on tyrosine residues were precipitated after incubation for 4 h at 4°C with antiposphotyrosine monoclonal antibody (clone PT-66) covalently coupled to agarose (Sigma Chemical Co.). The immune complexes were washed three times with the lysis buffer A, and then 50 μl of sample buffer (250 mM Tris-HCl, pH 6.8, 10% SDS, 10% glycerol, 5% β-mercaptoethanol, 0.5% bromophenol blue) was added. After boiling for 5 to 10 min, the samples (20 μl) were loaded onto 0.75-mm 10% SDS-polyacrylaminde gel electrophoresis (PAGE) gels, and discontinuous electrophoresis was performed as described by Laemmli (1970). Proteins on the gel were transferred to Immobilon-*P* polyvinilidene fluoride 0.45-μm membrane (Millipore, Bedford, MA). Nonspecific protein binding sites on the membrane were blocked by incubation for 2 h at room temperature or overnight at 4°C in 5% (w/v) skimmed milk powder, 0.2% (v/v) Tween-20, 100 mM Tris-HCl, pH 7.5, and 0.9% (w/v) NaCl. ERK1/ERK2-specific antiserum (M5670, Sigma Chemical Co.) was incubated with the membrane for 1 h at room temperature at a dilution of 1:1000 in a fresh blocking solution. The blot was then washed five times with 0.2% (v/v) Tween-20, 100 mM Tris-HCl, pH 7.5, and 0.9% (w/v) NaCl (10 min/wash) and then incubated for 1 h with horseradish peroxidase-conjugated anti-rabbit IgG (Sigma Chemical Co.) in the blocking solution. The membrane was washed again as described above and the bands were visualized with an enhanced chemiluminescence method (Nesbitt and Horton, 1992).

Alternatively, tyrosine phosphorylation of ERK was assayed as described below for JNK and p38 MAPK activation. Phospho-specific ERK antibody (Calbiochem-Novabiochem Corp.) was used to detect phosphorylated tyrosine in the TEY motif of p44 and p42 ERK isoforms.

Assay of ERK Enzymatic Activity. To measure activity of ERK, following each stimulation, 2×10^6 HMC-1 cells were collected by centrifugation for 15 s at 12,000*g* and lysed by adding 70 μl of the lysis buffer A, containing also 5 μg/ml pepstatin and 2 μM microcystin. After incubation in an ice bath for 1.5 h, insoluble material was removed from lysates by centrifugation at 16,000*g* for 10 min at 4°C. Affinity-purified polyclonal ERK1/ERK2-specific antibody R2 Erk 1-CT (Upstate Biotechnology, Lake Placid, NY) was preincubated with protein A-agarose (Pharmacia Biotech Inc., Piscataway, NJ) at a ratio of 1 μg IgG per 30 μl agarose in the lysis buffer A for 1 h at 4°C, then washed three times with the same buffer and combined with 50 μl of lysates. Enzymatically active ERK was precipitated after incubation for 2 h at 4°C. The immune complexes were washed twice with the lysis buffer A and once with assay dilution buffer, containing 20 mM 3-(*N*-morpholino)propanesulfonic acid, pH 7.2, 25 mM β-glycerol phosphate, 5 mM EGTA, 1 mM sodium orthovanadate, and 1 mM dithiothreitol. ERK activity was evaluated with a MAPK assay kit (Upstate Biotechnology) according to the manufacturer's protocol.

Evaluation of JNK and p38 MAPK Activation. Dual phosphorylation of MAPKs at both threonine and tyrosine residues in a regulatory Thr-Xaa-Tyr site has been shown to be an accurate indicator of their activation (Burack and Sturgill, 1997; Moriguchi et al., 1996; Raingeaud et al., 1995). To evaluate the dual phosphorylation of JNK and p38 MAPKs, following each stimulation, 2×10^6 HMC-1 cells were collected by centrifugation for 15 s at 12,000*g* and lysed by addition of 100 μl sample buffer (250 mM Tris-HCl, pH 6.8; 10% SDS, 10% glycerol, 5% β-mercaptoethanol, and 0.5% bromophenol blue). After 2 s of sonication, samples were heated to 95 to 100°C for 5 min and insoluble material was removed by centrifugation at 16,000*g* for 10 min at 4°C. The samples (20 μl) were loaded onto 0.75-mm 10% SDS-PAGE gels, and discontinuous electrophoresis was performed as described by Laemmli (1970). Western blotting was done as described above. Affinity-purified antibody specific for human p38 MAPK phosphorylated at Thr^{180} and Tyr^{182} (Calbiochem-Novabiochem Corp.) or monoclonal IgG_1 antibody specific for human JNK phosphorylated at Thr^{183} and Tyr^{185} (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) was incubated with the membrane for 1 h at room temperature in a fresh blocking solution. The blot was then washed five times with 0.2% (v/v) Tween-20, 100 mM Tris-HCl, pH 7.5, 0.9% (w/v) NaCl (10 min/wash) and then incubated for 1 h with horseradish peroxidase-conjugated anti-rabbit IgG (Sigma Chemical Co.) or with horseradish peroxidase-conjugated anti-mouse IgG (Sigma Chemical Co.) in the blocking solution. The membrane was washed again as described above and the bands were visualized with an enhanced chemiluminescence method (Nesbitt and Horton, 1992).

Evaluation of $p21^{ras}$ Activation. The active GTP-bound form of $p21^{ras}$ was detected using the minimal $p21^{ras}$-binding domain of Raf-1 (RBD) according to previously published technique (de Rooij and Bos, 1997). pGEX 2T RBD prokaryotic expression vector was kindly provided by Dr. A. Wittinghoffer (Max-Planck Institute, Dortmund, Germany). Glutathione *S*-transferase (GST)-RBD was produced and isolated as described (Herrmann et al., 1995). Following each stimulation, 10^7 HMC-1 cells were collected by centrifugation for 15 s at 12,000*g* and lysed by addition of 200 μl 6 mM Na_2HPO_4, 4 mM NaH_2PO_4, 1% NP-40, 150 mM NaCl, 2 mM EDTA, 50 mM NaF, 0.1 mM Na_3VO_4, 4 μg/ml leupeptin, and 2 mM benzamidine, containing Complete Mini protease inhibitor cocktail (Boehringer Mannheim, Mannheim, Germany) (lysis buffer B). GST-RBD, precoupled to glutathion-agarose beads in the lysis buffer B, was added and lysates were incubated at 4°C for 30 min. Beads then were washed five times with the lysis buffer B by centrifugation and resuspended in 40 μl of sample buffer (250 mM Tris-HCl, pH 6.8, 10% SDS, 10% glycerol, 5% β-mercaptoethanol, and 0.5% bromophenol blue). After boiling for 5 to 10 min, the supernatant was collected by centrifugation and the protein samples (20 μl) were separated on a 15% SDS-PAGE gel and subsequently transferred to polyvinilidene fluoride membrane by Western blotting. $p21^{ras}$ was detected by incubating the membrane with the rat monoclonal antibody Y13–259 (Calbiochem-Novabiochem Corp.) overnight at 4°C. Horseradish peroxidase-conjugated anti-rat IgG (Sigma Chemical Co.) was used as a second antibody (1 h at room temperature). The bands on the membrane were visualized with an enhanced chemiluminescence method (Nesbitt and Horton, 1992).

Measurement of Intracellular Calcium. Cytosolic free calcium concentrations were determined by fluorescent dye techniques. HMC-1 cells (2×10^6 cells/ml) were loaded with 1 μM fura-2/acetoxymethyl ester in a buffer containing 150 mM NaCl, 2.7 mM KCl, 0.37 mM NaH_2P0_4, 1 mM $MgSO_4$, 1 mM $CaCl_2$, 5 g/liter D-glucose, 10 mM HEPES-NaOH, pH 7.4, and 0.35% BSA. After incubation for 1 h at room temperature, cells were washed to remove excess of fura-2 and were resuspended (2×10^5 cells/ml) in the same buffer containing 1 U/mL adenosine deaminase and no BSA. Fluorescence was monitored at an emission wavelength of 510 nm and excitation wavelengths of 340 and 380 nm. Maximal fluorescence was determined after addition of 0.004% digitonin. Minimal fluorescence was then determined in the presence of 20 mM EGTA. The intracellular calcium was calculated using previously described formulae (Grynkiewicz et al., 1985), assuming a K_d of 224 nM. Fluorescence was measured with a spectrofluorimeter (Fluorolog 2; Spex Industries, Inc., Edison, NJ) in a thermostated cuvette (37°C).

Results

Adenosine Induces Tyrosine Phosphorylation and Activation of ERK. The activation of ERK1/ERK2 is accompanied by the phosphorylation of Tyr^{185} residue at the TEY motif (Burack and Sturgill, 1997), which can be detected using immunoprecipitation with antiphosphotyrosine antibody following by immunoblotting with specific anti-ERK1/ERK2 antibody. To determine whether adenosine induces tyrosine phosphorylation of ERK isoforms, HMC-1 cells were incubated for 5 or 30 min with the stable adenosine analog NECA at a concentration of 100 μM. Cells were incubated in the presence of 1 U/ml of adenosine deaminase to remove endogenously produced adenosine. PMA, a potent activator of protein kinase C, was used as a positive control at the concentration of 10 nM. As seen in Fig. 1, NECA, and to a greater extent PMA, induced phosphorylation of both the p42 and p44 isoforms of ERK. This effect was evident after 5 min of incubation. After 30 min of incubation in the presence of NECA, the tyrosine phosphorylation of ERK was completely reversed, and it was also considerably reduced in the presence of PMA.

The enzymatic activity of ERK1/ERK2 immunoprecipi-

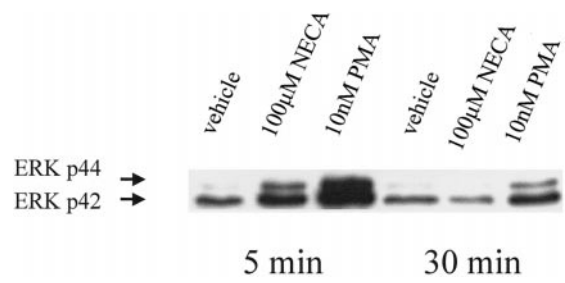

Fig. 1. Tyrosine phosphorylation of ERK isoforms in HMC-1. Cells were incubated in the absence or presence of 100 μM NECA for 5 and 30 min; 10 nM PMA was used as a positive control. Proteins phosphorylated on tyrosine residues were immunoprecipitated from cell lysates with a phosphotyrosine antibody. After separation on 10% SDS-PAGE, phosphorylated ERK isoforms were identified by immunoblotting with antiserum against p42/p44 ERK.

tated from HMC-1 lysates was assayed by its ability to phosphorylate myelin basic protein. In preliminary experiments we determined that stimulation of ERK activity reached a peak within the first minute of incubation with 10 μM NECA. Therefore, we chose this time point to study ERK activation by various agents. The enzymatic activity of nonstimulated ERK1/ERK2 was 277 ± 51 fmol P_i/min per 10^6 cells in four separate experiments performed in triplicates. As seen in Fig. 2, incubation of HMC-1 cells for 1 min in the presence of the nonselective A_{2A}/A_{2B} adenosine agonist NECA at a concentration of 10 μM induced a 1.9 ± 0.06-fold increase in ERK activity, whereas the selective A_{2A} adenosine receptor agonist CGS 21680 had no effect (1.03 ± 0.07). By comparison, activation of protein kinase C with 10 nM PMA produced a 1.8 ± 0.1-fold increase in ERK activity, but stimulation of adenylate cyclase with 100 μM forskolin had no significant effect (1.2 ± 0.07 from baseline, $P = .1$ by Student's t test). In ancillary studies, we verified that forskolin at this concentration induces cAMP accumulation to levels similar to those produced by NECA (from 2.8 ± 0.1 pmol/10^6 cells to 38.4 ± 3.0 and 22.3 ± 3.2 pmol/10^6 cells respectively). These data indicate the involvement of A_{2B} adenosine receptor in adenosine-induced stimulation of ERK cascade, because the nonselective A_{2A}/A_{2B} adenosine agonist NECA, but not the selective A_{2A} agonist CGS 21680, activated ERK. On the other hand, the stimulation of adenylate cyclase with forskolin, which mimics the effect on cAMP shared by both A_{2A} and A_{2B} receptors, had no effect on ERK activity.

We showed previously that stimulation of A_{2B} receptors results in calcium mobilization in HMC-1 cells (Feoktistov and Biaggioni, 1995). We asked, therefore, whether an increase in intracellular calcium levels can mimic the effect of NECA on ERK activation in HMC-1 cells. For this purpose we chose calcium ionophore A23187 and thapsigargin, both of which increase intracellular calcium but through different mechanisms; calcium ionophore acts primarily through calcium influx, whereas thapsigargin induces mobilization of calcium from internal stores. After 1 min incubation with 10 nM A23187 or 10 nM thapsigargin, the intracellular calcium in HMC-1 cells was increased by 426 ± 25 nM and 320 ± 26 nM, respectively. By comparison, 10 μM NECA increased intracellular calcium by 190 ± 7 nM. In parallel studies, the activation of ERK at the same time point was determined by immunoblotting of extracted proteins with a phospho-specific ERK antibody. As seen in Fig. 3, the increase of intracellular calcium levels produced by A23187 or thapsigargin did not mimic the effect of NECA on ERK activation.

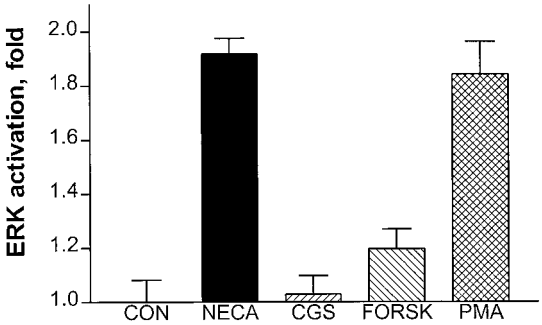

Fig. 2. Stimulation of ERK activity in HMC-1. Cells were incubated for 1 min in the absence (CON) or presence of 10 μM NECA (NECA), 10 μM CGS 21680 (CGS), 100 μM forskolin (FORSK), or 10 nM PMA (PMA). ERK was immunoprecipitated from cell lysates with an antibody against p42/p44 ERK, and enzymatic activity was determined by phosphorylation of myelin basic protein. Normalized values are presented as mean ± SEM of four independent experiments done in triplicate.

Stimulation of p21^{ras} by Adenosine. Increase in guanine nucleotide exchange on p21^{ras} results in binding of this small G protein to Raf protein kinase with subsequent stimulation of MEK and ERK activation. To determine whether adenosine induces formation of GTP-bound active form of p21^{ras}, we incubated cells with the stable adenosine analog NECA (10 μM) in the presence of 1 U/ml adenosine deaminase. Samples were collected at different time points and the extracted proteins were incubated with bacterially expressed GST-p21^{ras}-binding domain of Raf coupled to glutathion-agarose. The absorbed proteins were then analyzed by immunoblotting with anti-p21^{ras} antibody. As seen in Fig. 4, the nonselective A_{2A}/A_{2B} agonist NECA induced maximal formation of active p21^{ras} during the first minute, whereas the selective A_{2A} agonist CGS 21680 produced virtually no effect.

We showed previously that adenosine A_{2B} receptors can stimulate protein kinase C via a G_q -dependent mechanism in HMC-1 cells (Feoktistov and Biaggioni, 1995). In this study we asked whether stimulation of protein kinase C activity with forbol ester would mimic the effect of NECA on p21^{ras}. Figure 4 shows virtually no formation of the active p21^{ras} within the first minute after incubation with 10 nM PMA. However, activation of p21^{ras} with PMA was evident at later time points, with a maximum stimulation observed at 15 min. These data demonstrate that the kinetics of NECA- and PMA-dependent activation of p21^{ras} in HMC-1 cells are different.

Adenosine Activates JNK and p38 MAPK. To determine whether adenosine stimulation of HMC-1 cells would trigger activation of other MAPK cascades, we incubated cells with the stable adenosine analog NECA (10 μM) in the presence of 1 U/ml adenosine deaminase. Samples were collected at different time points and immunoblotted either with an antibody specific for human p38 MAPK phosphorylated at

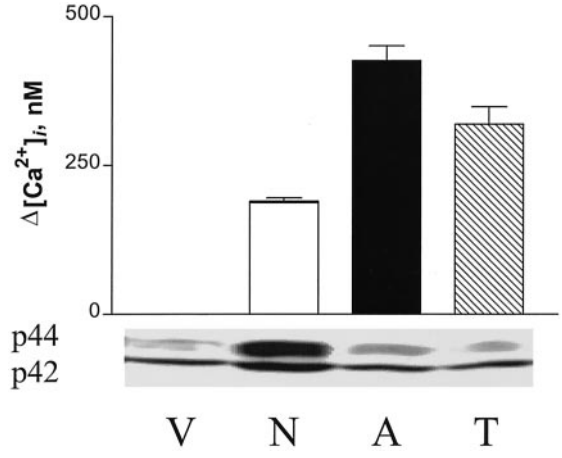

Fig. 3. Effect of 10 μM NECA, 10 nM calcium ionophore A23187, and 10 nM thapsigargin on free intracellular calcium and ERK activation in HMC-1. Upper panel, rise in inracellular calcium was determined by fura-2 technique within 1 min after addition of vehicle (V), NECA (N), A23187 (A), or thapsigargin (T). Results are presented as mean ± SEM of four experiments. Lower panel, activation of ERK was evaluated by immunoblotting with phospho-specific ERK antibody within 1 min after stimulation. These results are representative of three similar experiments.

Thr^{180} and Tyr^{182} or with an antibody specific for human JNK phosphorylated at Thr^{183} and Tyr^{185}. The double phosphorylation of the TGY motif in p38 MAPK or TPY in JNK is considered to be an accurate indicator of their stimulation (Raingeaud et al., 1995; Moriguchi et al., 1996). As seen in Fig. 5, the stable adenosine analog NECA stimulated both JNK and p38 MAPK. It should be noted, however, that stimulation of these kinases followed different kinetics; maximal stimulation of p38 MAPK was observed within the first minute, whereas JNK activation reached a maximum only within 10 to 15 min of incubation with NECA. The JNK antibody used in these experiments recognizes the phosphorylated p55 and p46 isoforms of JNK, but with NECA we consistently observed an increase in the band corresponding to p46 JNK only. In some but not all gels we observed an appearance of a faint band of a lower molecular weight, which may be explained by phosphorylation of a product of partial proteolytic degradation of JNK. The data, presented here, confirm that adenosine, in parallel with the activation of ERK cascade shown above, can also stimulate JNK and p38 MAPK pathways in HMC-1 cells.

Stimulation of ERK and p38 MAPK Cascades Is Required for Adenosine A_{2B} Receptor-Mediated IL-8 Secretion. We previously showed that stimulation of adenosine A_{2B} receptors in HMC-1 cells induces secretion of IL-8 (Feoktistov and Biaggioni, 1995). Figure 6A shows that there is a 1-h delay between stimulation with 100 μM NECA and secretion of IL-8, the time probably needed to initiate protein synthesis. IL-8 production increased linearly between 1 and 3 h of incubation with NECA and reached maximal levels of 33 $\pm$ 0.5 pmol/ml after 4 h. In a control experiment spontaneous release of IL-8 reached 4 $\pm$ 0.2 pmol/ml after 4 h of incubation in the absence of NECA (Fig 6A). Incubation of HMC-1 cells with increasing concentrations of NECA for 2.5 h revealed a sigmoid curve for IL-8 production with an EC_{50} of 3 μM (Fig. 6B). In parallel experiments we also made an approximate estimate of the effects of increasing concentrations of NECA on activation of ERK and p38 MAPK. As seen in the inset of Fig. 6B, NECA in micromolar concentrations produced a dose-dependent phosphorylation of the TXY motif, measured as an increase in the intensity of the immunoreactive p44 ERK and p38 MAPK bands in corresponding immunoblots. The relatively low potency of NECA agrees with previous reports of A_{2B} receptor-mediated IL-8 production in HMC-1 cells (Feoktistov and Biaggioni, 1995).

Based on these results we chose 2.5-h incubation with 3 μM NECA in further studies to analyze the signaling pathways involved in adenosine-induced IL-8 production. First, we evaluated a potential role of protein kinase C in NECA-induced synthesis of IL-8. In ancillary studies we found that a 2.5-h incubation with PMA produced a sigmoidal dose-dependent increase in IL-8, with an EC_{50} of 3 nM. We preincubated HMC-1 cells for 1 h in the absence or in the presence of increasing concentrations of the cell-permeable selective protein kinase C inhibitor Ro 32–0432. We then stimulated cells with 3 μM NECA or with 3 nM PMA as a

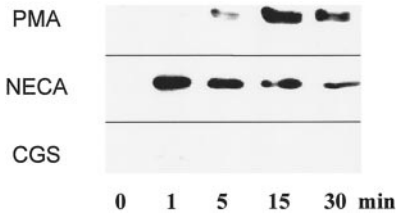

Fig. 4. Time course of activation of $p21^{ras}$ produced by 10 nM PMA, 10 μM NECA, and 1 μM CGS 21680 (CGS) in HMC-1 cells. Effects of PMA and NECA were determined at indicated periods of time, and effect of CGS 21680 was determined at 1 min only. Analysis of $p21^{ras}$ activation was performed by Western blotting of proteins absorbed to GST-$p21^{ras}$-binding domain of Raf as described in *Materials and Methods*. These results are representative of three similar experiments.

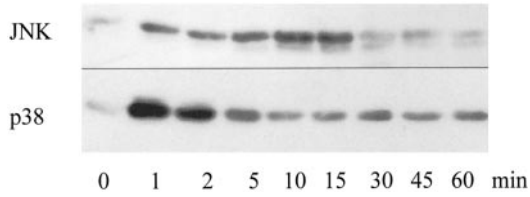

Fig. 5. Time course of JNK and p38 MAPK activation by NECA in HMC-1. Cells were incubated with 10 μM NECA for indicated periods of time. Analysis of JNK and p38 MAPK activation was performed concomitantly in the same samples. Proteins from cell lysates were separated on 10% SDS-PAGE. MAPKs activation was determined by immunoblotting with antibody specific for phosphorylated JNK or for phosphorylated p38 MAPK. These results are representative of four similar experiments.

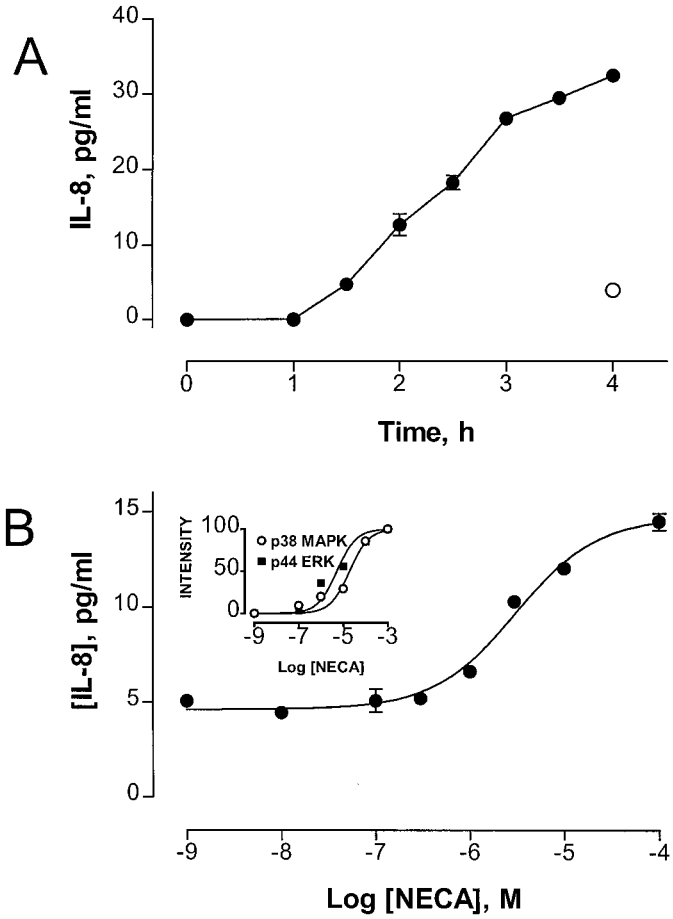

Fig. 6. Release of IL-8 from HMC-1 cells. A, time course of IL-8 release from the cells stimulated with 100 μM NECA (●). ○, Spontaneous release of IL-8 after 4 h of incubation in the absence of NECA. B, effect of increasing concentrations of NECA on IL-8 release from the cells incubated for 2.5 h. Values are presented as mean $\pm$ SEM of four experiments. Inset, effect of increasing concentrations of NECA on activation of p38 MAPK and p44 ERK. MAPK activation was evaluated by increase in intensity of phospho-specific immnoreactive bands in corresponding Western blots, using SigmaScan/Image software (Jandel Scientific, San Rafael, CA). Results represent an average of two experiments.

positive control. As shown in Fig. 7, 1 μM Ro 32–0432 completely blocked PMA-induced production of IL-8, whereas the NECA-dependent IL-8 production was decreased by 48 ± 7%. These data suggest that, although protein kinase C activation may contribute to IL-8 production, it is not an absolute requirement for NECA-dependent stimulation.

We then used two different protein tyrosine kinase inhibitors to investigate whether signaling pathways connecting adenosine receptors and IL-8 secretion involve tyrosine phosphorylation. Genistein is a competitive inhibitor of ATP binding at the catalytic domain of various tyrosine kinases. Herbimycin A produces irreversible inactivation of tyrosine kinases, possibly by binding to reactive sulfhydryl groups. Both inhibitors blocked IL-8 secretion induced by 3 μM NECA (Fig. 8, A and B and Table 1). The potencies of genistein (IC_{50}, 33 μM) and herbimycin A (IC_{50}, 0.23 μM) are in good agreement with their reported potencies as inhibitors of tyrosine kinases (White and Lee, 1993).

It was demonstrated recently that stimulation of phosphatidylinositol 3-kinase is critical for secretion of preformed granule-associated mediators in mast cells, but is not required for IL-6 production to occur (Marquardt et al., 1996). In agreement with this observation, wortmannin, a selective inhibitor of phosphatidylinositol 3-kinase, did not block IL-8 secretion in HMC-1 cells induced by 3 μM NECA (Fig. 8C). We then used PD 098059, a highly selective inhibitor of MEK, to evaluate the role of ERK activation in adenosine-induced IL-8 production. As seen in Fig. 8D, PD 098059 blocked the NECA-induced IL-8 production with an IC_{50} of 3 μM (Table 1), which is in good agreement with its reported potency as inhibitor of MEK (Dudley et al., 1995; Pang et al., 1995). To investigate the role of p38 MAPK, we used two highly selective inhibitors, SB 202190 and SB 203580. Both inhibitors blocked IL-8 secretion induced by 3 μM NECA (Fig. 8, E and F and Table 1). The potencies of SB 202190 (IC_{50}, 0.3 μM) and SB 203580 (IC_{50}, 0.3 μM) are in good

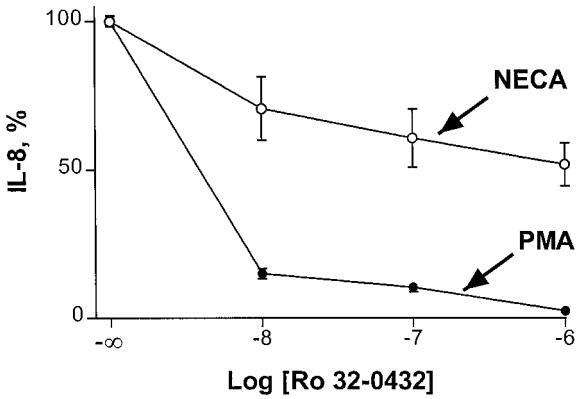

Fig. 7. Effect of selective protein kinase C inhibitor Ro 32–0432 on IL-8 release stimulated by 3 μM NECA or by 3 nM PMA for 2.5 h. Values are presented as mean ± SEM of three experiments.

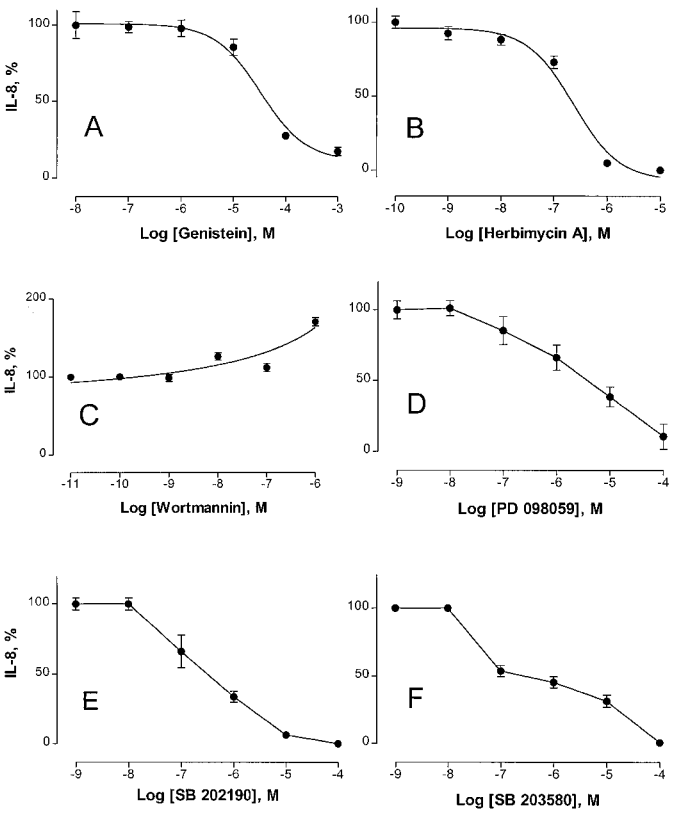

Fig. 8. Effect of tyrosine kinase inhibitors genistein (A) and herbimycin A (B), phosphatidylinositol 3-kinase inhibitor wortmannin (C), selective MEK inhibitor PD 098058 (D), and selective p38 MAPK inhibitors SB 202190 (E) and SB 203580 (F) on A_{2B} adenosine receptor-dependent IL-8 release from HMC-1 cells. HMC-1 cells were incubated with 3 μM NECA for 2.5 h in the absence or presence of increasing concentrations of these inhibitors. Values are presented as mean ± SEM of three or four experiments.

TABLE 1

Comparison between reported potency and selectivity of inhibitors of various kinases and their potency in inhibiting A_{2B}-mediated IL-8 production

To determine IC_{50} for inhibition of A_{2B} adenosine receptor-mediated IL-8 production, HMC-1 cells were incubated in the absence or presence of increasing concentrations of inhibitors and in the presence of 3 μM NECA for 2.5 h. Values are presented as mean ± SEM of three to four experiments.

Inhibitor	IC_{50} for IL-8 Production	Reported IC_{50}	Reference
	μM	*μM and selectivity*	
Genistein	33 ± 24	30 Nonselective tyrosine kinase inhibitor	White and Lee, 1993
Herbimycin A	0.23 ± 0.18	0.87 Nonselective tyrosine kinase inhibitor	White and Lee, 1993
PD 098059	3 ± 1	2–10 Selective MEK 1 inhibitor	Dudley et al., 1995; Pang et al., 1995
Wortmannin	No inhibition	0.005 Selective PI 3-kinase inhibitor	Arcaro and Wymann, 1993
SB 203580	0.3 ± 0.2	0.6 Selective p38 MAPK inhibitor	Cuenda et al., 1995
SB 202190	0.3 ± 0.1	0.4 Selective p38 MAPK inhibitor	Li et al., 1996

agreement with their reported potencies as inhibitors of p38 MAPK (Cuenda et al., 1995; Li et al., 1996). Taken together, these results demonstrate that both the p38 MAPK and ERK cascades play a key role in mediating the adenosine-induced IL-8 production in HMC-1 cells.

Discussion

Four distinct subtypes of adenosine receptors, specifically A_1, A_{2A}, A_{2B}, and A_3, have been pharmacologically characterized and cloned. The classification of adenosine receptors was originally based on their ability to modulate intracellular levels of cAMP. A_2 adenosine receptors stimulate adenylate cyclase through coupling with G_s protein, whereas A_1 and A_3 adenosine receptors are coupled to adenylate cyclase via inhibitory GTP-binding proteins of the $G_{i/o}$ family. Although activation of adenylate cyclase is arguably an important signaling mechanism for A_{2A} receptors, this is not necessarily the case for A_{2B} receptors, because other intracellular pathways have been found to be functionally coupled to A_{2B} receptors in addition to adenylate cyclase. We previously showed that the human mast cell line HMC-1 expresses functional A_{2A} and A_{2B} receptors, whereas no evidence was found for A_1 or A_3 adenosine receptors (Feoktistov and Biaggioni, 1995, 1998). Both A_2 subtypes of adenosine receptors activate adenylate cyclase via G_s protein. However, only A_{2B} receptor was found to couple also to phospholipase C via a GTP-binding protein of the G_q family (Fig. 9). A similar coupling of adenosine A_{2B} receptors to phospholipase C has been also found in mouse bone marrow-derived mast cells (Marquardt et al., 1994) and in the canine BR mast cell line (Auchampach et al., 1997).

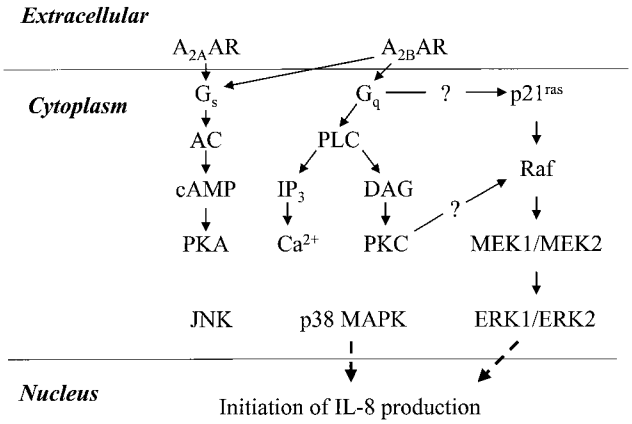

Fig. 9. Schematic representation of intracellular pathways coupled to adenosine receptors in HMC-1. We previously demonstrated that HMC-1 cells express functional A_{2A} (A_{2A}AR) and A_{2B} (A_{2B}AR) receptors. Both subtypes activate adenylate cyclase (AC) via G_s protein. Activation of this pathway results in accumulation of cAMP and stimulation of protein kinase A (PKA). However, only A_{2B} receptors are coupled also to phospholipase C (PLC) via a GTP binding protein of the G_q family. Activation of this pathway results in an increase in diacylglycerol (DAG) and IP_3. Diacylglicerol stimulates PKC. Inositol trisphosphate activates mobilization of calcium from intracellular stores. Stimulation of A_{2B} receptors eventually leads to production of IL-8 (Feoktistov and Biaggioni, 1995). In this study we present evidence that stimulation of A_{2B} receptors activates the small GTP-binding protein $p21^{ras}$. This event triggers ERK signaling pathway with sequential stimulation of Raf, MEK1/2, and ERK1/2 protein kinase activities. The exact mechanism of coupling of A_{2B} receptors with ERK pathway remains to be determined. This may include combination of activation of $p21^{ras}$ via $G_q\alpha$ (Watanabe et al., 1995; Eguchi et al., 1996) and stimulation of Raf via PKC (Hawes et al., 1995) (indicated by a question mark). We also demonstrate the coupling of adenosine receptors to JNK and p38 MAPK signaling pathways, but the upstream events have not been explored. Blockade of ERK pathway with the selective MEK inhibitor PD 098058 or p38 MAPK pathway with the selective p38 MAPK inhibitors SB 202190 and SB 203580 revealed that these MAPK pathways are essential for adenosine A_{2B} receptor-mediated production of IL-8.

We previously demonstrated that stimulation of adenosine receptors with the nonselective A_{2A}/A_{2B} agonist NECA but not the selective A_{2A} agonist CGS 21680 induced production of IL-8 from HMC-1 cells (Feoktistov and Biaggioni, 1995). These data indicate that A_{2B} receptors stimulate intracellular pathways eventually leading to transcription and synthesis of IL-8. We hypothesized that among possible candidates for these pathways were MAPK cascades serving as information relays connecting cell-surface receptors to nuclear transcription factors. In favor of this hypothesis it was reported recently that p38 MAPK but not ERK pathway is involved in tumor necrosis factor-α and *N*-formylmethionine leucyl-phenylalanine-stimulated induction of IL-8 in neutrophils (Zu et al., 1998).

The novel finding reported here is that A_{2B} receptors are coupled to the ERK pathway (Fig. 9). This is based on the observation that the nonselective A_{2A}/A_{2B} agonist NECA but not the selective A_{2A} agonist CGS 21680 stimulates $p21^{ras}$ and activates ERK. The current pharmacological characterization of A_{2B} receptors relies on the lack of effectiveness of compounds that are potent and selective agonists of other receptor types (Feoktistov and Biaggioni, 1997). In HMC-1 cells, where only A_{2A} and A_{2B} receptors are present (Feoktistov and Biaggioni, 1995), the lack of effect of the selective A_{2A} agonist CGS 21680 on ERK activity indicates an A_{2B}-mediated mechanism. We also examined whether the second messengers, known to be generated upon activation of A_{2B} receptors, would be responsible for activation of ERK signaling pathway by NECA. Stimulation of both A_{2B} and A_{2A} receptors in HMC-1 cells results in increase of cAMP. However, the increase of cAMP produced by forskolin failed to mimic the effect of NECA on ERK activity. In addition to stimulation of adenylate cyclase in HMC-1 cells, A_{2B} receptors also activate phospholipase C, resulting in mobilization of intracellular calcium and generation of diacylglycerol, a natural activator of protein kinase C (Feoktistov and Biaggioni, 1995). It has been previously reported that thapsigargin and A23187 can stimulate ERK activity in epidermal cells and fibroblasts, but this effect was not observed within the first minute and was obvious only after a 4- to 5-min delay (Chao et al., 1992). Because stimulation of ERK by NECA reached its maximum within 1 min, we focused on the early effects of thapsigargin and A23187 on intracellular calcium and ERK activation in HMC-1 cells in our study. Stimulation of calcium mobilization with thapsigargin or generation of calcium influx with calcium ionophore A23187 followed closely the kinetics of the NECA-dependent calcium increase within the first minute, but they failed to mimic the activation of ERK by NECA. In contrast, stimulation of protein kinase C with PMA produced a strong activation of ERK within the first minute, comparable with the effects of NECA. The mechanism of stimulation of ERK pathway by protein kinase C remains unclear, and may include a $p21^{ras}$-dependent (Marais et al., 1998), or $p21^{ras}$-independent stimulation of Raf (Hawes et al., 1995). We compared the kinetics of $p21^{ras}$ stimulation with PMA and NECA to determine whether stimulation of protein kinase C would mimic the

effect of A_{2B} receptor activation. Our results indicate that PMA does not stimulate $p21^{ras}$ within the first minute, at a time when NECA produced maximal stimulation. On the contrary, formation of the active GTP-$p21^{ras}$ complexes reached a peak by 15 min after incubation with PMA, at a time when the stimulation produced by NECA was on a decline. Taken together, our data provide the first evidence that A_{2B} receptors can stimulate $p21^{ras}$-ERK signaling pathway in HMC-1 cells. The increase in levels of cAMP, intracellular calcium, and protein kinase C activation cannot alone explain the stimulation of $p21^{ras}$-ERK pathway produced by NECA. The events upstream of $p21^{ras}$ activation remain to be delineated. Direct stimulation of $p21^{ras}$ by $G_q\alpha$ subunits is possible, as shown for angiotensin II and prostaglandin $F_{2\alpha}$ receptors (Watanabe et al., 1995; Eguchi et al., 1996).

Our study has also documented that stimulation of adenosine receptors in HMC-1 cells activates JNK and p38 MAPK cascades. This has not been previously shown for A_2 adenosine receptors. Interestingly, the time course was different for activation of these two pathways. NECA-induced activation of p38 MAPK reached its maximum within the first minute, whereas maximal activation of JNK occurred only within 10 to 15 min. The delineation of events that couple adenosine receptors with stimulation of both pathways requires further investigation.

Another novel observation was that simulation of both ERK and p38 MAPK pathways is essential for the A_{2B} receptor-regulated IL-8 production in human mast cells, because inhibition of either MEK, the enzyme that activates ERK, or p38 MAPK completely blocked this process. In contrast, inhibition of phosphatidylinositol 3-kinase did not affect the IL-8 production in HMC-1 cells, which is in agreement with previous observations made in mouse bone marrow mast cells (Marquardt et al., 1996). In this study we found that inhibition of protein kinase C with Ro 32–0432 completely blocked PMA-stimulated IL-8 production, but only partially blunted the response to NECA. These results suggest that stimulation of protein kinase C can contribute to IL-8 production, but this pathway alone cannot explain A_{2B} receptor-dependent IL-8 production.

In summary, we provide the first evidence of coupling of A_2 adenosine receptors to JNK and p38 MAPK signaling pathways, and of A_{2B} receptors to $p21^{ras}$- ERK activation. At least two of these MAPK pathways, namely ERK and p38 MAPK are essential for adenosine A_{2B} receptor-mediated production of IL-8 in the human mast cell line HMC-1. Adenosine uniquely activates mast cells in asthmatics, but not in normal persons, and the processes that explain this differential effects are unknown. The relevance of MAPKs to this phenomenon and to the pathogenesis of asthma remains to be elucidated.

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