**A₂B Adenosine and P₂Y₂ Receptors Stimulate Mitogen-activated Protein Kinase in Human Embryonic Kidney-293 Cells**

**CROSS-TALK BETWEEN CYCLIC AMP AND PROTEIN KINASE C PATHWAYS**

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Mitogen-activated protein kinase (MAPK) cascades underlie long-term mitogenic, morphogenic, and secretory activities of purinergic receptors. In HEK-293 cells, N-ethylcarboxamidoadenosine (NECA) activates endogenous A₂B receptors that signal through G_s and G_q/11. UTP activates P₂Y₂ receptors and signals only through G_s/11. The MAPK isoforms, extracellular-signal regulated kinase 1/2 (ERK), are activated by NECA and UTP. H-89 blocks ERK activation by forskolin, but weakly affects the response to NECA or UTP. ERK activation by NECA or UTP is unaffected by a tyrosine kinase inhibitor (genistein), attenuated by a phospholipase C inhibitor (U73122), and is abolished by a MEK inhibitor (PD908059) or dominant negative Ras. Inhibition of protein kinase C (PKC) by GF 109203X failed to block ERK activation by NECA or UTP, however, another PKC inhibitor, Ro 31-8220, which unlike GF 109203X, can block the ω-isofrom, and prevents UTP- but not NECA-induced ERK activation. In the presence of forskolin, Ro 31-8220 loses its ability to block UTP-stimulated ERK activation. PKA has opposing effects on B-Raf and c-Raf-1, both of which are found in HEK-293 cells. The data are explained by a model in which ERK activation is modulated by differential effects of PKC ω and PKA on Raf isoforms.

Diverse physiological effects of purines, adenosine, and ATP are mediated through cell surface purinergic receptors. To date, four subtypes of P₁ or adenosine receptors (ARs) have been cloned: A₁, A₂A, A₂B, and A₃. They all belong to the G protein-coupled receptor superfamily (1). P₂ (ATP) receptors are divided into two major subfamilies, the P₂X receptors that are ligand-gated channels, and the P₂Y receptors that are G protein-coupled (2). The activation of G protein-coupled purinergic receptors has acute functional effects on all tissues that can be attributed to G protein-mediated effects on enzymes and ion channels. In addition, recent evidence indicates that purinergic receptor activation produces more slowly developing mitogenic, morphogenic, and secretory activities (3, 4).

Recent studies have suggested that A₂BARs, in addition to coupling to G_s and cyclic AMP accumulation, appear to be responsible for triggering acute Ca²⁺ mobilization and degranulation of canine mast cells (5) as well as a delayed interleukin-8 release from human HMC-1 mast cells (6). A role for mast cell A₂B ARs in asthma is suggested by the therapeutic efficacy of theophylline and enprofylline. Both of these xanthines were found to block human A₂B ARs in the therapeutic dose range, and enprofylline was found to be a selective antagonist of human A₂B ARs (7). Stimulation of adenyl cyclase probably cannot account for A₂BAR-mediated degranulation and stimulation of interleukin-8 synthesis from human HMC-1 mast cells, and in fact cyclic AMP has been found to be inhibitory to rodent mast cell degranulation (8, 9). In mast cells, activation of IgE receptors and adenosine receptors produces a synergistic interaction to trigger degranulation (10). IgE receptors are known to activate MAPK in mast cells (11, 12), but little is known about the regulation of this signaling pathway by adenosine receptors. The study of mast cell adenosine receptors is complicated by the fact that individual cells express multiple adenosine receptor subtypes. In addition, different adenosine receptor subtypes appear to be functionally predominant in different mast cell lines (5, 13, 14). For this reason we decided to initially characterize functional effects of the endogenous A₂B AR in HEK-293 cells where it is the only adenosine receptor expressed.

ERK1/2 are 44- and 42-kDa isofrom members of the MAPK family that regulate gene expression, protein synthesis, cell growth, secretion, and differentiation (15, 16). MAP kinase signaling was initially shown to be activated by single-transmembrane receptor protein tyrosine kinases, such as the EGF and platelet-derived growth factor receptors. In recent years, a number of mitogenic G protein-coupled receptor (GPCR) agonists including lysophosphatidic acid (17), angiotensin II (18), endothelin (19), thromboxane A₂ (20), and bombesin (21) have been shown to be capable of potently activating ERK. In contrast to receptor tyrosine kinases, the intermediate steps linking GPCRs to the activation of ERK are poorly understood, and significant heterogeneity and complexity exist in the signaling pathways utilized by various GPCRs (22). It is now widely believed that the mechanism of ERK activation by GPCRs varies among cell types and individual receptors (23).
In the present study we show that activation of HEK-293 cell adenosine receptors stimulates adenyl cyclase, Ca^{2+} mobilization, and ERK1/2 activation. ERK activation is Ras-dependent, but is not blocked by inhibitors of protein kinase C (PKC) or tyrosine kinases, and differs from ERK activation elicited by UTP acting on a P2Y_{2} receptor. We also demonstrate that A_{2B}ARs are principally responsible for initiating a sustained ERK activation in canine mastocytoma cells.

**EXPERIMENTAL PROCEDURES**

**Materials**—c-AMP, NECA, CGS21680, theophylline, and enprofylline were purchased from Research Biochemicals (Natick, MA). IB-MECA was from Dr. Saul Kadin (Pfizer, Groton, CT) and WRC0571 from Dr. Pauline Martin (Discovery Therapeutics, Richmond, VA). Ro 20-1724 is from BIOMOL Research Laboratories (Plymouth Meeting, PA); phorbol 12-myristate 13-acetate, PD088059, U73122, GF 109203X, genistein, Ro 31-8220, and H-8 were from Calbiochem (San Diego, CA). A23187, pertussis toxin, and UTP from Sigma. Fura-2/AM is from Molecular Probes (Eugene, OR); adenosine deaminase from Boehringer-Mannheim; cell culture medium and LipofectAMINE were from Life Technologies, Inc. (Gaithersburg, MD). Rabbit anti-phospho-MAP kinase antibodies were raised against a synthetic peptide corresponding to the MAP kinase phosphorylation site (CTGFLT(p)RY(p)AVTR) conjugated to keyhole hemocyanin (Pierce, Rockford, IL) and affinity purified negatively against the unphosphorylated peptide and positively against the phosphopeptide (24). Mouse monoclonal anti-ERK2 antibody was from Upstate Biotechnology (Lake Placid, NY). Anti-Raf-1 (C-19) and anti-Raf-1 (C-20) were from Santa Cruz Biotechnology (Santa Cruz, CA). Pan-Ras antibody (F111) was purchased from Santa Cruz. pcDNA3 was from Invitrogen. FLAG-tagged ERK2 was provided by Dr. S. T. Elen. pcDNA-Ras(N17) was constructed by ligating a 0.9-kilobase XbaI/StuI fragment from pAT-Ras(N17) (25) into NheI/Pmel-digested pcDNA3.1(+) vector (Invitrogen, Carlsbad, CA).

**Cell Culture and Transfection**—HEK-293 cells were obtained from the American Type Culture Collection and maintained in Dulbecco’s modified Eagle’s medium/F-12 medium supplemented with 10% fetal calf serum, 100 units/ml penicillin, 100 μg/ml streptomycin, and 2% donor calf serum, 1.5 mM KCl, 1 mM MgSO_{4}, 1 mM KH_{2}PO_{4}, 25 mM NaHCO_{3}, 0.5 mM CaCl_{2}, 2.7 g/liter MgCl_{2}, 20 mM HEPES, pH 7.4, and 0.25% bovine serum albumin for 45 min. Cells were washed and resuspended in the same buffer without bovine serum albumin, plus 1 unit/ml adenosine deaminase, and 20 μM of the phosphodiesterase inhibitor, Ro 20-1724, and then aliquoted into test tubes. Compounds in 50-μl aliquots were added to 200 μl of cell suspension and transferred to a 37 °C shaker bath for 15 min. Assays were terminated by the addition of 500 μl of 0.15 N HCl. Cyclic AMP in the acid extract (500 μl) was acetylated and quantified by automated radioimmunoassay (28).

**Measurement of Intracellular Ca^{2+}**—Monolayers of HEK-293 cells were loaded with 1 μM Fura-2/AM in buffer containing 100 mM NaCl, 5 mM KCl, 1 mM MgSO_{4}, 1 mM KHPO_{4}, 25 mM NaHCO_{3}, 0.5 mM CaCl_{2}, 2.7 g/liter MgCl_{2}, 20 mM HEPES, pH 7.4, and 0.25% bovine serum albumin for 45 min. Cells were washed and resuspended in the same buffer without bovine serum albumin, plus 1 unit/ml adenosine deaminase. Fluorescence was monitored at an emission wavelength of 510 nm and excitation wavelengths of 340 and 380 nm using an SLM spectrofluorimeter in a thermostable cuvette.

**ERK1/2 Activation Assay**—Prior to stimulation, HEK-293 cells or canine BR cells were serum-starved for about 18 h. Assays were carried out on monolayers of HEK-293 cells in serum-free Dulbecco’s modified Eagle’s medium/F-12 medium in a 57 °C, 5% CO_{2} incubator or on suspended canine BR cells in complete Tyrode’s buffer in a 37 °C shaking water bath. The reactions were terminated by placing the cells on ice and washing with ice-cold phosphate-buffered saline. The cells were then lysed in Triton lysis buffer (50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 50 mM sodium fluoride, 5 mM EDTA, 1% (v/v) Triton X-100, 40 mM β-glycerophosphate, 40 mM p-nitrophosphothiol, 200 μM sodium orthovanadate, 100 μM phenylmethylsulfonyl fluoride, 1 μg/ml leupeptin, 1 μg/ml pepstatin A, 1 μg/ml aprotinin). The lysate was mixed and clarified by centrifugation (15 min, 14,000 rpm, 4 °C) in an Eppendorf microcentrifuge. The supernatant was subjected to SDS-polyacrylamide gel electrophoresis followed by transfer to nitrocellulose and immunoblotting. For co-transfection experiments, FLAG-tagged ERK2 was immunoprecipitated from the cell lysate (~400 μg) using the anti-FLAG M2 gel according to the manufacturer’s instruction (Kodak) before resolution by SDS-polyacrylamide gel electrophoresis. Phosphorylation and activation of ERK1/2 was detected by immunoblotting using rabbit polyclonal anti-phospho-ERK1/2 antibody and visualized by enhanced chemiluminescence with horseradish peroxidase-conjugated goat anti-rabbit IgG as the secondary antibody (1:10,000 dilution). The membranes were then stripped by incubating in stripping buffer (62.5 mM Tris-HCl, 2% SDS, and 100 mM β-mercaptoethanol, pH 6.7, at 65 °C) in a shaking water bath, and re-probed with mouse monoclonal anti-ERK2 antibody to quantify the total ERK2 loaded onto each lane. For quantification of ERK1/2 phosphorylation, films were scanned by a laser densitometry (Molecular Dynamics) and volume integration was performed using Image QuantTM software (Molecular Dynamics).

**RESULTS**

**Endogenous A_{2B}ARs Evoke cAMP Accumulation and Ca^{2+} Mobilization in HEK-293 Cells**—G_{s}-coupled A_{2B}ARs are widely expressed in tissue culture lines. To detect A_{2B}ARs in cultured HEK-293 cells, we performed cAMP accumulation assays using the non-selective adenosine receptor agonist NECA. As shown in Fig. 1A, NECA produces a concentration-dependent increase in intracellular levels of cyclic AMP with an EC_{50} of 2.7 ± 0.9
Fig. 1B shows that the response to NECA (1 μM) is substantially attenuated by the A2BAR-selective antagonist enprofylline (100 μM) as well as by the non-selective AR antagonist theophylline (100 μM), but not by the A1AR-selective antagonist, WRC0571. In binding assays both enprofylline and theophylline block recombinant human A2B with Ki values of 7 μM (7). When added at 1 μM, CPA, IB-MECA, or CGS21680, agonists that are selective for A1, A3, or A2A adenosine receptors, respectively, had little effect on intracellular cAMP in HEK-293 cells (Fig. 1B). Only at a very high concentration (100 μM) did the A2A-selective compound CGS21680 induce a small increase in intracellular cAMP (Fig. 1B). Also, we could not detect A1, A2A, or A3 receptors by subtype-selective radioligand binding to HEK-293 cell membranes (data not shown). These findings are consistent with the observation that mRNA transcripts for A2B, but not for A1, A2A, or A3 adenosine receptor subtype have been detected in HEK-293 cells by Northern analysis (27). Collectively, these data suggest that the predominant endogenous adenosine receptors found on HEK-293 cells are A2BARs that are functionally coupled to Gs to stimulate adenylyl cyclase.

We next sought to identify and characterize other signaling pathways mediated by A2BARs in HEK-293 cells. We found that NECA (1 μM) triggers transient intracellular Ca2+ mobilization (Fig. 2A), which is blocked by both enprofylline and theophylline but not by WRC0571, whereas 1 μM CPA, IB-MECA, or CGS21680 failed to provoke such a response (Fig. 2B). Ca2+ mobilization also is elicited in response to UTP (via

Fig. 2. Ca2+ mobilization in HEK-293 stimulated with AR agonists. Intracellular Ca2+ concentrations were measured in suspended cells loaded with FURA-2/AM. A, NECA (1 μM)-stimulated Ca2+ mobilization in the absence or presence of 100 μM enprofylline, 100 μM theophylline, or 1 μM WRC0571. B, Ca2+ mobilization in response to selective AR agonists, 1 μM CPA (A1AR), IB-MECA (A3AR), or CGS21680 (A2AAR). C, blockade of NECA or UTP-stimulated Ca2+ mobilization by the phospholipase C inhibitor U73122. HEK-293 cells loaded with Fura-2 were treated with either dimethyl sulfoxide (vehicle) or U73122 (10 μM) for 15 min prior to the stimulation with 10 μM NECA or 100 μM UTP. The data shown are representative of at least three independent experiments.
Many G\textsubscript{i}, G\textsubscript{q}, and some G\textsubscript{s}-coupled receptors have been shown to couple to cAMP production. As shown in Fig. 3, NECA evokes a time- and dose-dependent ERK1/2 activation. This activation is transient, peaks at 5 min, and gradually decreases to the baseline level in 15 min. The estimated EC\textsubscript{50} for NECA-induced ERK1/2 activation is 0.7 \textmu M. CPA, CGS21680, or IB-MECA (1 \textmu M) are only weak activators of ERK1/2 compared with NECA (Fig. 4A), and the response to NECA (0.5 \textmu M) is blocked by enprofylline (100 \textmu M) or theophylline (100 \textmu M) (Fig. 4B). These data suggest that NECA-induced ERK1/2 activation in HEK-293 cells is mediated by the endogenous A\textsubscript{2B}AR.

A\textsubscript{2B}AR-induced ERK1/2 Activation Is MEK1/2- and Ras-dependent—To investigate the mechanism of A\textsubscript{2B}AR activation of ERK1/2, we first examined the effect of a highly specific inhibitor of MEK1/2, PD098059 (30). The ERK activation cascade is thought to proceed through Raf, which phosphorylates and activates MEK1/2. The MEKs phosphorylate ERK1/2 on both Thr and Tyr residues. PD098059 inhibits the activation of both MEK1 (IC\textsubscript{50} = 5–10 \textmu M) and MEK2 (IC\textsubscript{50} = 50 \textmu M). As shown in Fig. 5A, ERK1/2 activation in response to 10 \textmu M NECA stimulation was completely abolished by pretreatment for 20 min with 50 \textmu M PD098059, suggesting that MEK1/2 are involved in A\textsubscript{2B}AR-mediated ERK1/2 activation.

Next, we investigated the involvement of p21\textsuperscript{ras} (Ras) in NECA-induced ERK1/2 activation. Both Ras-dependent and independent pathways have been reported for GPCR-mediated ERK activation (31). HEK-293 cells were transiently transfected with FLAG-tagged ERK2 together with either dominant-negative Ras-N17 or empty vector pcDNA3. Consistent with the well known involvement of Ras in receptor protein tyrosine kinase-mediated ERK activation, overexpression of Ras-N17...
ERK activation in response to forskolin (10 μM) was about 35% lower than the activation induced by NECA (Fig. 7). The increase in intracellular cAMP in response to a 5-min simulation with forskolin (10 μM) is about 2-fold higher than that induced by NECA (10 μM, data not shown). These data indicate that cAMP accumulation can contribute to but may not fully account for the NECA-stimulated ERK activation. On the other hand, we investigated the effect of the protein kinase A inhibitor, H-89, on NECA- or forskolin-stimulated ERK1/2 activation. In a series of experiments, pretreatment of cells with H-89 (10 μM, 30 min) abolished the forskolin-induced ERK1/2 activation, whereas it only slightly decreased NECA- or UTP-induced ERK1/2 activation (Fig. 7). Taken together, these data are consistent with the hypothesis that cyclic AMP may have both stimulatory and inhibitory inputs on A2Bmediated ERK activation.

Effect of the Phospholipase C Inhibitor U73122 on A2BAR-mediated ERK1/2 Activation—Since A2B receptors appear to couple to adenylyl cyclase, we set out to determine if increased cAMP contributes to A2BAR-induced ERK1/2 activation. Depending on the cell type, cAMP can have either a stimulatory (via B-Raf) or an inhibitory (via c-Raf-1) impact on ERK activation (33). Western analysis reveals the presence of both B-Raf and c-Raf in HEK-293 cells (data not shown). Forskolin (10 μM) increased cyclic AMP and induced a transient ERK1/2 activation in HEK-293 cells with a time course similar to that produced by NECA (data not shown). However, the magnitude of ERK activation in response to forskolin (10 μM) was about 35% lower than the activation induced by NECA (Fig. 7). The increase in intracellular cAMP in response to a 5-min simulation with forskolin (10 μM) is about 2-fold higher than that induced by NECA (10 μM, data not shown). These data indicate that cAMP accumulation can contribute to but may not fully account for the NECA-stimulated ERK activation. On the other hand, we investigated the effect of the protein kinase A inhibitor, H-89, on NECA- or forskolin-stimulated ERK1/2 activation. In a series of experiments, pretreatment of cells with H-89 (10 μM, 30 min) abolished the forskolin-induced ERK1/2 activation, whereas it only slightly decreased NECA- or UTP-induced ERK1/2 activation (Fig. 7). Taken together, these data are consistent with the hypothesis that cyclic AMP may have both stimulatory and inhibitory inputs on A2BAR-mediated ERK activation.

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to G\textsubscript{s}/11 and activation of this pathway stimulates phospholipase C activity, we next set out to determine if phospholipase C is involved in NECA-stimulated ERK1/2 activation. As shown in Fig. 8, preincubation of HEK-293 cells with the specific phospholipase C inhibitor, U73122 (10 \(\mu\)M for 15 min), significantly attenuates (>50%) but does not eliminate NECA- and UTP-stimulated ERK1/2 activation, suggesting NECA- and UTP-induced ERK1/2 occurs at least in part via phospholipase C activation.

**Differential Effects of PKC Inhibitors on A\textsubscript{2B}AR- and UTP-mediated ERK1/2 Activation**—To assess the involvement of PKC in NECA-induced ERK1/2 activation, HEK-293 cells were pretreated with the specific PKC inhibitors GF 109203X (2 \(\mu\)M, 15 min)(34) or Ro 31-8220 (10 \(\mu\)M, 15 min)(35). Whereas both inhibitors completely block phorbol 12-myristate 13-acetate-induced ERK1/2 activation, neither inhibited NECA-induced ERK1/2 activation (Fig. 9). In fact, Ro 31-8220 somewhat enhanced NECA-mediated ERK1/2 activation. UTP or the calcium ionophore A23187 induced ERK1/2 activation and showed differential sensitivity to GF 109203X and Ro 31-8220. Whereas GF 109203X had no effect on UTP or A23187-mediated ERK1/2 activation, Ro 31-8220 inhibited both.

We were particularly struck by the differential effect of Ro 31-8220 on NECA- and UTP-stimulated ERK activation. Since A\textsubscript{2B}ARs signal through G\textsubscript{s} and G\textsubscript{s}/11, and P2Y\textsubscript{2} receptors signal through G\textsubscript{s}/11 only, we set out to determine if the differential effect of Ro 31-8220 is due to the elevated cAMP accompanied by G\textsubscript{s} activation. We reasoned that through simultaneous application of both UTP and forskolin to the cell, it would be possible to mimic the cellular effect of A\textsubscript{2B}AR activation by NECA. Fig. 9C shows that in fact the combination of forskolin and UTP does mimic the NECA response and is not inhibited by Ro 31-8220. In addition, ERK activation in response to forskolin alone is enhanced by Ro 31-8220 pretreatment. These data suggest that the lack of an apparent inhibitory effect of Ro 31-8220 on A\textsubscript{2B}AR-induced ERK1/2 activation may be due to the enhancement of cyclic AMP-mediated responses by Ro 31-8220.

**A\textsubscript{2B}ARs Initiate Sustained ERK1/2 Activation in Canine BR Mast Cells**—A\textsubscript{2B}ARs have recently been shown to play an important role in regulating degranulation and cytokine release from canine and human mast cells (5). We next determined if the A\textsubscript{2B}AR-mediated ERK1/2 activation that occurs in HEK-293 can also be observed in canine BR mast cells. As shown in Fig. 10, NECA elicited ERK1/2 activation in canine BR mast
**DISCUSSION**

The activation of purinergic receptors produces various acute G protein-mediated responses, e.g. changes in muscle tone, neuronal firing, immune function, and secretion of various hormones and cytokines. Recent studies also suggest that purines may trigger more slowly acting signal transduction cascades to mediate changes in cellular proliferation (36, 37), growth and differentiation (38), and apoptosis (39, 40). MAPK cascades may regulate the latter responses. In the present study we show that stimulation of endogenous A2BARs in HEK-293 cells evokes three responses: cyclic AMP accumulation, Ca\(^{2+}\) mobilization, and activation of ERK1/2. This newly characterized A2B AR-mediated ERK1/2 activation and a P2Y\(_2\) receptor-mediated response elicited by UTP are dependent on Ras and MEK1/2. Both responses are attenuated by U73122, an inhibitor of phospholipase C, are completely insensitive to genistein, an inhibitor of certain tyrosine kinases, and are only minimally affected by the PKA inhibitor, H-89. In this study, we have demonstrated for the first time the existence of an interesting interaction between PKA and PKC in regulating ERK activity by endogenous GPCRs in HEK-293 cells. We also have discovered that both B-Raf and c-Raf-1 exist in HEK-293 cells, and we discuss below how these Raf isoforms may participate in cross-talk between the PKA and PKC pathways to influence ERK activity. These results provide a novel mechanistic insight into pathways linking GPCRs to ERK activation.

Our data with endogenous purinergic receptors show both similarities and differences (discussed below) with previous studies that utilized transiently overexpressed receptors. A caveat to the use of overexpression is the possibility that it may result in abnormal coupling.

One interesting observation in this study is the differential effects of the two closely related bisindolylmaleimide PKC inhibitors, GF 109203X and Ro 31-8220. Whereas both inhibitors effectively blocked ERK activation by phorbol 12-myristate 13-acetate, only Ro 31-8220 attenuated P2Y\(_2\) and A23187-mediated responses. Both compounds have been reported to be potent and selective PKC inhibitors. Nevertheless, it has been recently noted that these two PKC inhibitors have differential actions and distinct pharmacological properties (41–44). Ro 31-8220 is a much more potent inhibitor of PKC \(\zeta\) (106–169 nM versus 5800 nM) than is GF 109203X (45–48), whereas they are almost equipotent as inhibitors of other PKC isozymes. This raises the interesting possibility that G\(_{i1}\)-coupled receptors may selectively activate PKC \(\zeta\) to regulate ERK1/2 activity in HEK-293 cells. The presence of PKC \(\zeta\) in HEK-293 cell membranes has recently been demonstrated by Western blotting (49). Of note, in this regard, is the finding that in rat astrocytes, ERK activation by endogenous P2Y receptors, was also inhibited by Ro 31-8220, but not by Go 6976, an inhibitor of PKC \(\alpha\) and \(\beta1\) isoforms which does not affect PKC \(\delta\), \(\varepsilon\), or \(\zeta\) (50). It has been reported that in vascular smooth muscle cells, PKC \(\zeta\), mediates Ras-dependent ERK1/2 activation induced by Ang-II (51). Involvement of Ras in PKC \(\zeta\) activation both in vivo and in vitro has also been reported (52). In a previous study (23), it has been concluded that GPCRs in HEK-293 cells do not activate ERK via PKC because these responses are insensitive to GF 109203X. Based on our data, we propose that PKC \(\zeta\) is the primary PKC isozyme that contributes to ERK stimulation due to activation of A\(_{2B}\) AR and P2Y\(_2\) receptors in HEK-293 cells (Fig. 11).

Another interesting aspect of this study is evidence of cross-talk between PKA and PKC \(\zeta\) in modulating ERK activation by A2BARs. We show that ERK activation by the P2Y\(_2\) receptor, but not by A2BAR, is inhibited by Ro 31-8220. The major difference between P2Y\(_2\) receptors and A2BARs is that only A2BARs couple via G\(_{i1}\) to activate adenylyl cyclase. This suggests that a common G\(_{i1}\) and PKC \(\zeta\) pathway utilized by A2BARs and P2Y\(_2\) receptors may be modulated by cyclic AMP. We show that cyclic AMP has several effects on ERK signaling in HEK-293 cells (Fig. 11). ERK is activated by forskolin and this response is abolished by the PKA inhibitor, H-89, and enhanced by the PKC inhibitor Ro 31-8220. The addition of forskolin converts UTP-induced activation of ERK from being attenuated, to being unaffected by Ro 31-8220. According to our model (Fig. 11) Ro 31-8220 can inhibit PKC \(\zeta\)-mediated activation of ERK, but enhance a cyclic AMP-dependent B-Raf-induced activation. Hence, when cyclic AMP is elevated by NECA or UTP plus forskolin, Ro 31-8220 has little net effect on ERK activation.

According to this scenario, one would expect that cyclic AMP should contribute to ERK activation mediated by A\(_{2B}\) ARs. However, the PKA inhibitor, H-89, although inhibitory to forskolin-induced activation of ERK, only marginally affects NECA-induced responses. To account for this, our model draws on previous studies which show cyclic AMP can have either an inhibitory (e.g. in Rat-1 or NIH3T3 cells) or stimulatory effect (in PC12 cells) on ERK activation depending on the cell type involved. Work by Vossler et al. (33) suggested that cyclic AMP decreases ERK stimulation by inhibiting c-Raf-1 activation, whereas it increases ERK activity by activating B-Raf. Consistent with this notion, we have detected by Western blotting both B-Raf and c-Raf-1 in the HEK-293 cells used in this study. Our demonstration that forskolin can activate ERK in HEK-293
cells, likely via B-Raf, is confirmatory of previous work by Daaka et al. (53). The lack of an effect of elevated cAMP on A2AR-mediated ERK activation might result from a balance between opposing effects of cyclic AMP on ERK activation, i.e. stimulation via B-Raf activation and inhibition via c-Raf-1 inactivation. We note that the magnitude of cyclic AMP accumulation induced by NECA (10 μM) is lower than that induced by forskolin (10 μM). This may also contribute to the small apparent contribution of cAMP to A2AR-mediated ERK activation, along with the fact that PKCζ activation by NECA counteracts the effect of PKA on B-Raf.

In the present study, we show that ERK activation by both A2ARs and P2Y2 receptors requires functional Ras. The mechanism of Ras activation by various GPCRs remains poorly characterized, particularly for Gbγ-coupled receptors. By over-expressing the carboxyl terminus of the Gbγ-adrenergic receptor kinases 1 (βARKct), a scavenger of Gbγ released from activated G proteins, it has been shown that Ras activation by Gbγ-coupled receptors is mediated by Gbγ (54). However, in the case of Gbγ-coupled receptors, the involvement of Gbγ is controversial (54, 55). We found that transfection of HEK-293 cells with βARKct has little effect on co-transfected epitope-tagged ERK activation by A2ARs and P2Y2 receptors in HEK-293 cells (data not shown). This suggests that Ga, but not Gbγ, is principally responsible for Ras-dependent MAPK activation following the stimulation of purinergic receptors. It is possible that Gbγ-coupled receptors release more and/or different βγ subunits than Ga or Gbγ.

We also demonstrate herein that Ras-dependent ERK1/2 activation by either A2ARs or UTP receptors in HEK-293 cells does not require genistein-sensitive tyrosine kinases. Although this is somewhat unexpected, it is not without precedent. Gbγ-coupled m2 muscarinic receptors expressed in Rat-1 fibroblasts activate ERK in a Ras- and Raf-dependent manner, and this response is insensitive to inhibition by genistein (56). The same holds true for Gγ-coupled 5-HT1A receptors expressed in Chinese hamster ovary cells (22). In contrast, ERK activation by transiently transfected Gγ-coupled m2 muscarinic receptors in COS cells, Gγ-coupled lysophosphatidic acid receptors in Rat-1 fibroblast (57), or Gγ11-coupled α1β-adrenergic receptors in HEK-293 cells (23) require both Ras and genistein-sensitive tyrosine kinases. It is possible that overexpression of these receptors influences coupling to ERK. Although this discrepancy cannot be definitively explained at present, these findings support the current view that GPCRs modulate ERK activity in a cell-type, receptor-specific, and possibly a receptor-density dependent manner.

The issue of how ERK signaling specificity is achieved, especially in the case of multiple GPCRs apparently coupled to the same type of G-protein in the same cell type, is addressed in a recent study by Mitchell et al. (58) describing the activation of phospholipase D by various Gγ11-coupled receptors. These investigators identified a specific structural motif (NPXY YX XXY) in a subset of Gγ11-coupled receptors, which is important for Rho-mediated phospholipase D activation. A similar scenario is conceivable for ERK activation by GPCRs coupled to the same G proteins. G protein-coupled receptors may couple to additional cellular constituents other than G proteins to transduce signals and influence ERK activation in a receptor-specific way. Daaka and co-workers (59, 60) recently presented evidence to address the importance of GPCRs in the activation of ERKs. In view of the various pathways used for GPCR internalization, it is possible that specific mechanisms of ERK activation by various GPCRs might also reflect differences in receptor endocytosis.

In summary, all of our data fit the scheme depicted in Fig. 11. P2Y2 receptors couple via Gγ11 only, and activate ERK via pathways including genistein-insensitive tyrosine kinases, phospholipase C, PKCζ, Ras, and Raf. In contrast, A2ARs couple to both Gα and Gγ11, and the Gγ signaling branch exerts both stimulatory and inhibitory effects on Gγ11-mediated ERK activation via cyclic AMP-dependent PKA. Gγ11-mediated
PKC ζ activation has inhibitory effects on cAMP-dependent ERK activation. Hence, there exists an interesting cross-talk between PKC and PGA signaling pathways in regulating ERK activity by G2 and G11-coupled receptors in HEK-293 cells. It is notable that extracellular ATP release from cells is rapidly broken down to adenosine by ectonucleotidases. Since both P1 and P2 receptors are simultaneously activated, cross-talk between the ERK activation pathways mediated by different purinergic receptors assumes an even broader role in a physiological context.

In the present study, we also demonstrate that A2B receptors trigger sustained ERK activation in canine BR mast cells. Although this response is initiated by A2B adenosine receptors, the sustained phase of activation may not be solely maintained by A2B receptors. The mediators released in response to A2B-AR activation of mast cells may play a role in promoting sustained ERK activation. The cellular implications of transient versus sustained ERK activation are different. In PC12 neuronal cells, sustained ERK activation by NGF leads to differentiation and is associated with ERK translocation from the cytosol to the nucleus, whereas EGF-mediated transient ERK activation leads to cell proliferation but not differentiation or ERK nuclear translocation (16). What might be the function of A2B-mediated ERK activation in mast cells? It was reported that in the rat mast cell line (RBL-2H3), activation of IgE receptors triggers ERK activation, and this signaling is responsible for the release of arachidonic acid and the regulation of cytokine gene expression but not the release of secretory granules (which contains histamine, ATP, etc.) (11, 12). Activation of PKC and an increase in intracellular Ca2+ provide sufficient signals for mast cell degranulation (61). Based on these observations, we hypothesize that ERK activation may be responsible for AR-mediated release of arachidonic acid and promotion of cytokine production (such as A2B-AR-mediated interleukin-8 synthesis in human mast cell line HMC-1 (6)). The sustained phase ERK activation may also be important for AR regulation of mast cell proliferation and differentiation.

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REFERENCES

1. Tucker, A. L., and Linden, J. (1993) Cardiovase. Res. 27, 62–67
2. Schachter, J. B., Sromek, S. M., Nicholas, R. A., and Harden, T. K. (1997) Neuropepharmacology 36, 1181–1187
3. Neary, J. T., Rathbone, M. P., Cattabeni, F., Abbracchio, M. P., and Burnstock, G. (1996) Trends Neurosci. 19, 13–18
4. Feoktistov, I., and Biaggioni, I. (1997) Pharmaco Res. 49, 381–492
5. Auichampach, J. A., Jin, J., Wan, T. C., Caughby, G. H., and Linden, J. (1997) Mol. Pharmacol. 52, 846–860
6. Feoktistov, I., and Biaggioni, I. (1995) J. Clin. Invest. 96, 1979–1986
7. Robeva, A. S., Woodard, R., Jin, X., Gao, Z., Bhattacharya, S., Taylor, H. E., Rosin, D. L., and Linden, J. (1996) Drug Res. 39, 243–252
8. Hughes, P. J., Holgate, S. T., and Church, M. (1984) Biochem. Pharmacol. 33, 3847–3852
9. Hughes, P. J., and Church, M. K. (1986) Agents & Actions 18, 81–84
10. Ali, H., Cunha-Melo, J. R., Saul, W. F., and Beaven, M. A. (1990) J. Biol. Chem. 265, 745–753
11. Hirakawa, S., Scharenberg, A., Yamamura, H., Beaven, M. A., and Kinet, J. P. (1995) J. Biol. Chem. 270, 10960–10967
12. Hirakawa, S., Santini, F., and Beaven, M. A. (1995) J. Immunol. 154, 5391–5402
13. Hoffman, H. M., Walker, L. L., and Marquardt, D. L. (1997) Inflammation 21, 55–68
14. Ramkumar, V., Stiles, G. L., Beaven, M. A., and Ali, H. (1993) J. Biol. Chem. 268, 16858–16860
15. Marshall, C. J. (1994) Curr. Opin. Genet. Dev. 4, 82–89