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Adenosine A_3 receptor-mediated regulation of p38 and extracellular-regulated kinase ERK1/2 *via* phosphatidylinositol-3′-kinase

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Abstract

The adenosine A_3 receptor generally couples to the G_i class of heterotrimeric G proteins, thereby decreasing cAMP levels and also mediating signaling *via* release of βγ subunits. Here we describe the central role of phosphatidylinositol-3′-kinase (PI3K) for adenosine A_3 receptor-induced intracellular signaling to the stress-activated protein kinase p38 and the extracellular signal-regulated protein kinases ERK1/2. We used Chinese hamster ovary cells expressing the human adenosine A_3 receptor, phospho-specific antibodies and different pharmacological tools to dissect the signaling pathways involving PI3K. The adenosine receptor agonist 5′*N*-ethylcarboxamidoadenosine induced a time- and dose-dependent increase in p38 and ERK1/2 phosphorylation, two signaling pathways that appeared also to be activated in the immortalized microglia cell line N13, which expressed endogenous adenosine A_3 receptors. The 5′*N*-ethylcarboxamidoadenosine-induced effects on p38 and ERK1/2 in CHO cells were blocked by pertussis toxin pretreatment and were sensitive to pharmacological inhibition of PI3K. In addition, inhibition of Rac/Cdc42, small GTPases of the Rho family, by clostridium toxin B, diminished p38 phosphorylation but did not affect ERK1/2. Furthermore, we identified the serine 727 site of signal transducer and activator of transcription STAT3 as a probable downstream target of ERK1/2, and thereby provide evidence that adenosine A_3 receptor mediated ERK1/2 activation has functional consequences.

Keywords: Adenosine; Stress-activated protein kinase; Mitogen-activated protein kinase; Wortmannin; CHO cells; Rho GTPases

1. Introduction

The endogenous nucleoside adenosine activates four distinct adenosine receptors, which all belong to the family of G protein-coupled receptors (GPCR): the adenosine A_1, A_{2A}, A_{2B}, and A_3 receptor [1]. The adenosine A_3 receptor is the most recently discovered adenosine receptor [2]. Although it has been suggested to influence apoptosis [3], immune function [4], mast cell degranulation [5], ischemic preconditioning [6], and cellular growth [7] its overall physiological role remains unclear.

Whereas the two A_2 receptors couple to G_s, the adenosine A_3 and the A_1 receptor generally activate inhibitory G proteins [8]. Thus, adenosine A_3 receptors are known to decrease cAMP levels *via* inhibition of adenylyl cyclase through $G_{\alpha i}$ [9], and to act *via* βγ subunits to activate phospholipase C leading to an increase in inositoltrisphosphate and diacylglycerol and finally the release of Ca^{2+} from intracellular stores [10–12]. Adenosine A_3 receptors are capable of activating the family of mitogen-activated protein kinase (MAPK) [12–15], and we recently showed in Chinese hamster ovary cells expressing the human adenosine A_3 receptor (CHO A_3 cells; [16]) that the coupling to the extracellular signal-regulated kinase ERK1/2 is dependent on βγ subunits, PI3K, and Ras [12].

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Abbreviations: CHO A_3 cells, Chinese hamster ovary cells expressing the human adenosine A_3 receptor; Cl-IB-MECA, N^6-(4-amino-3-chlorobenzyl)-*N*-methylcarboxamidoadenosine; CTB, clostridium toxin B; ERK1/2, extracellular signal-regulated kinase 1/2; GPCR, G protein-coupled receptor; JAK, Janus kinase; LY294002, 2-(4-morphonilyl)-8-phenyl-1(4*H*)-benzopyran-4-one hydrochloride; MAPK, mitogen-activated protein kinase; MEK, MAP/ERK kinase; NECA, 5′*N*-ethylcarboxamidoadenosine; p38, stress-activated protein kinase with molecular weight 38 kDa; PI3K, phosphatidylinositol-3-kinase; PD98059, 2′-amino-3′-methoxyflavone; PKB/Akt, protein kinase B; PTX, pertussis toxin; SAPK, stress-activated protein kinase; STAT, signal transducer and activator of transcription.

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At least some of these signaling events are found also in rat mast cells expressing endogenous adenosine A_3 receptors [17].

The MAPK family consists of mainly three different groups of serine-threonine kinases: the extracellular signal-regulated kinases, and the stress-activated protein kinases (SAPKs) p38 and c-*jun* N-terminal kinase (JNK). MAPK signaling represents an important pathway for GPCRs to modify gene transcription by direct phosphorylation of a series of transcription factors [18]. In addition, ERK1/2 was reported to affect the transcriptional activity of signal transducer and activator of transcription 3 (STAT3) *via* serine phosphorylation [19,20].

In this report we show the central role of PI3K in signaling through the G_i-coupled adenosine A_3 receptor not only to ERK1/2 but also to p38. Indeed, these pathways were not only activated by adenosine A_3 receptor stimulation in CHO A_3 cells but also in mouse microglia cells that endogenously express this receptor. In addition we identify serine site phosphorylation of STAT3 as a possible downstream target of adenosine A_3 receptor-mediated ERK1/2 activation.

2. Materials and methods

2.1. Materials

Cell culture media, fetal calf serum and supplies were from Gibco-Life Technologies. 5′*N*-Ethylcarboxamidoadenosine (NECA) was from Research Biochemicals International. Wortmannin and N^6-(4-amino-3-chlorobenzyl)-*N*-methylcarboxamidoadenosine (Cl-IB-MECA) were from Sigma. PVDF-Immobilon P® membrane was from Millipore Corp. Clostridium toxin B (CTB) was from Calbiochem-Novabiochem Corporation. Rabbit anti-ERK1/2, phosphospecific rabbit anti-phosphoThr202/Tyr204-ERK1/2, rabbit anti-p38, rabbit anti phospho-Thr180/Tyr182-p38, rabbit anti phosphoSer727 STAT3, rabbit anti phosphoTyr705-STAT3 and 2′-amino-3′-methoxyflavone (PD98059) were from Cell Signaling Technology. Rabbit anti STAT3 was from Santa Cruz Biotechnology, Inc. LY294002 (2-(4-morphonilyl)-8-phenyl-1(4*H*)-benzopyran-4-one hydrochloride) was from Tocris Cookson Inc. Goat anti-rabbit horseradish peroxidase coupled antibody was from Pierce. Enhanced chemiluminescence detection (ECL®) kit was from Amersham International Plc.

2.2. Cell culture

CHO cells transfected with the human adenosine A_3 receptor [16] were grown adherent at 37°, 5% CO_2/95% air in Dulbecco's modified Eagle's medium (DMEM)/F-12 (1:1), geneticin (G-418), 0.2 mg/mL, penicillin (50 U/mL), streptomycin (50 μg/mL), L-glutamine (2 mM), 10% fetal calf serum (FCS). N13 microglia cells were grown as described elsewhere [21].

2.3. Protein phosphorylation and immunoblotting

ERK1/2, p38, and STAT3 phosphorylation were examined using the standard polyacrylamide gelelectrophoresis and immunoblotting technique as described before [13]. Briefly, cells were serum deprived overnight (0.5% FCS, v/v) and stimulated at 37° (NECA) for 5 min, inhibitors of PI3K and MAP/ERK kinase (MEK) were added 20 min before NECA stimulation. After two washes in ice-cold PBS, cells were lysed in lysis buffer (70 mM β-glycerophosphate, 0.5% Triton X-100, 2 mM $MgCl_2$, 1 mM dithiothreitol, 1 mM NaF, 1 mM Na_3VO_4, 20 μg/mL aprotinin, 5 μg/mL leupeptin) and cellular debris was removed by centrifugation. Samples were denatured with Laemmli buffer and analyzed by PAGE (12% resolving gel for MAPKs, 8% resolving gel for STAT3). After transfer onto PVDF membrane, protein phosphorylation was detected with rabbit phospho-specific ERK1/2, phospho-specific p38, or phospho-specific STAT3 antibodies, goat anti-rabbit horseradish peroxidase-coupled secondary antibody and the ECL detection method. To confirm equal loading in each lane, parallel immunoblots were run to detect the unphosphorylated ERK1/2 or STAT3 (not shown).

2.4. Data analysis

For quantitative analysis immunoblots were analyzed with the Scion Image software and dose–response curves were generated with GraphPad Prism3 using nonlinear regression and a Hill slope fixed at unity.

3. Results

CHO cells expressing the human adenosine A_3 receptor are a suitable model for studying signaling pathways *via* this receptor since pathways activated in these cells appear to resemble those activated in cells that express endogenous adenosine A_3 receptors. NECA-induced effects in CHO A_3 cells are entirely mediated by the transfected human receptor since untransfected control cells do not respond to even very high concentrations of NECA as previously shown [12,13]. Thus, the non-selective, but water-soluble adenosine receptor agonist NECA was preferred for experiments on CHO cells, whereas the more insoluble, receptor subtype-selective agonist Cl-IB-MECA was used to stimulate the endogenously expressed A_3 receptor in microglia cells that co-express different adenosine receptor subtypes.

3.1. Time- and dose-dependent phosphorylation of p38 and ERK1/2

In accordance with previous findings [12–14] we found a time- and dose-dependent increase of ERK1/2 phosphorylation upon stimulation of CHO A_3 cells with NECA

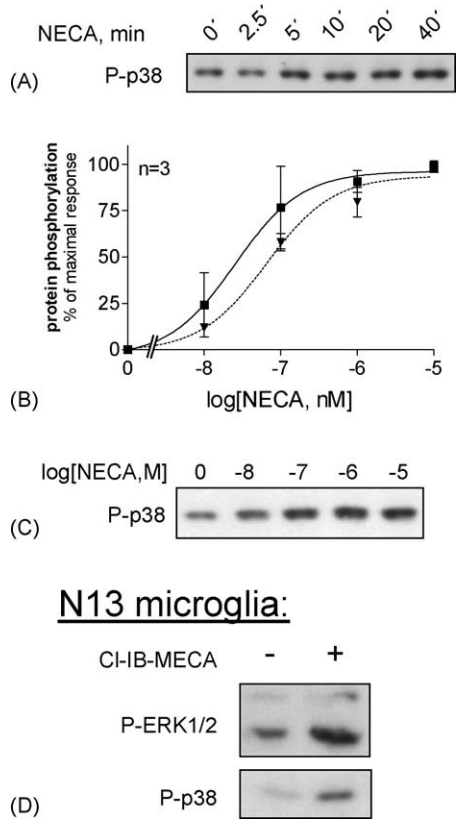

Fig. 1. Time- and dose-dependent effects of NECA on phosphorylation of p38 (A–C). CHO A_3 cells were stimulated with 100 nM NECA for the indicated amount of time (N = 3). For dose–response experiments CHO A_3 cells were stimulated for 5 min. Data were normalized (P-p38, solid line; P-ERK1/2, dashed line): the unstimulated control was set to 0% and maximal phosphorylation was set to 100% (B). For comparison, data on P-ERK1/2 from a previous study [13] were added. Error bars give standard deviation of three independent experiments. The immunoblots show one representative experiment for each protein. (D) N13 microglia cells were stimulated with 10 nM Cl-IB-MECA (5 min) and analyzed for ERK1/2 and p38 phosphorylation (N = 5).

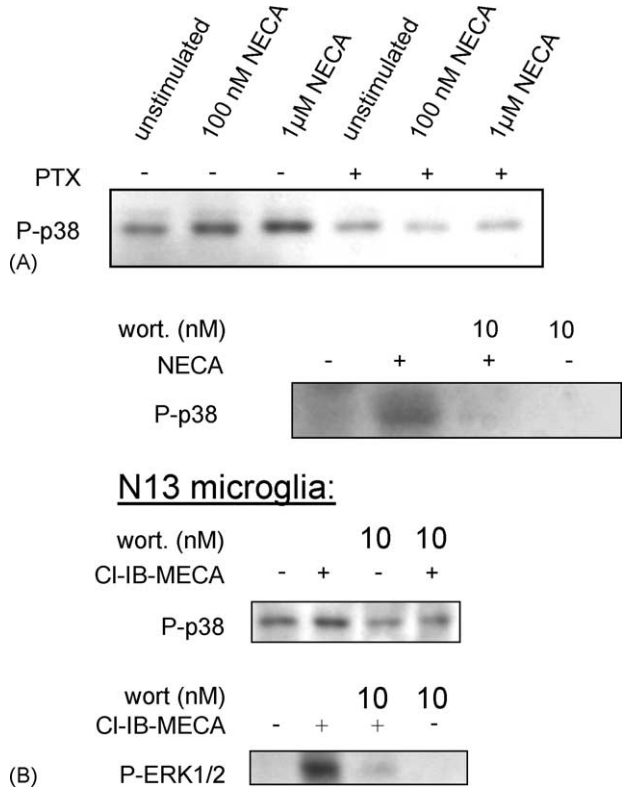

Fig. 2. (A) PTX-sensitive phosphorylation of p38 and STAT3. CHO A_3 cells were pretreated overnight with 200 ng/mL PTX to downregulate G_i proteins. After 5 min NECA stimulation cells were lysed, and lysates were analyzed by immunoblotting. One of three experiments is shown for each protein. (B) The PI3K inhibitor wortmannin (wort.) inhibited the NECA-induced phosphorylation of p38 in CHO A_3 cells (N = 3). Wortmannin was added 20 min prior to NECA (100 nM) stimulation. N13 microglia cells were pretreated with 10 nM wortmannin prior to Cl-IB-MECA stimulation (10 nM for 5 min) and analyzed for ERK1/2 and p38 phosphorylation.

(not shown). In comparison to the time course of ERK1/2 phosphorylation, with a maximum level at 5 min and a return to basal levels after 20 min [13], the phosphorylation of p38 (Fig. 1A) was as rapid but more prolonged. p38 phosphorylation was increased by NECA with an EC_{50} value of 25 nM (Fig. 1B; 95% confidence interval, CI, 11–61 nM) comparable to the previously published induction of P-ERK1/2 (EC_{50} (CI) = 65 nM (36–119 nM)) [13] and P-PKB (EC_{50} (CI) = 61 nM (31–117 nM)) [12].

Signaling from adenosine A_3 receptors to ERK1/2 and p38 was not only detected in CHO cells heterologously expressing the human adenosine A_3 receptor, but also in the immortalized mouse microglia cell line N13 (Fig. 1D; [21]). Stimulation with 10 nM of the selective adenosine A_3 receptor agonist Cl-IB-MECA for 5 min induced an increase in both ERK1/2 and p38 phosphorylation, implicating that these pathways are also activated in cells that express endogenous adenosine A_3 receptors.

3.2. The role of G_i proteins

Adenosine A_3 receptors mediate ERK1/2 activation *via* the release of βγ subunits from pertussis toxin (PTX)-sensitive G_i proteins [12]. Here we show that also the SAPK p38 is phosphorylated upon NECA stimulation of CHO A_3 cells *via* G_i proteins, since NECA (100 nM, 1 μM) had no effect on p38 phosphorylation in CHO A_3 cells pretreated overnight with 200 ng/mL PTX (Fig. 2A).

3.3. PI3K mediates phosphorylation of p38 and ERK1/2

The role of PI3K in adenosine A_3 receptor signaling was investigated using the PI3K inhibitors wortmannin (Fig. 2A and B) and LY294002 (not shown), which produced similar results. Pretreatment of CHO A_3 cells with increasing concentration of PI3K inhibitors dose-dependently reduced the NECA-induced phosphorylation of both ERK1/2 [12,14] and p38 (Fig. 2A). Effective

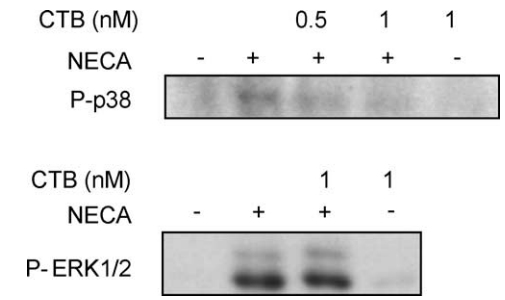

Fig. 3. Inhibition of Cdc42/Rac by CTB inhibited p38 but not ERK1/2 phosphorylation. CHO A_3 cells were treated with CTB (0.5 and 1 nM) for 18 hr prior to NECA stimulation. Cells were analyzed for ERK1/2 and p38 phosphorylation as described in Section 2.

concentrations of wortmannin were in the low nanomolar range, thus indicating a specific inhibition. Similar results were obtained in the microglia celline N13 stimulated with the adenosine A_3 receptor agonist Cl-IB-MECA (Fig. 2B).

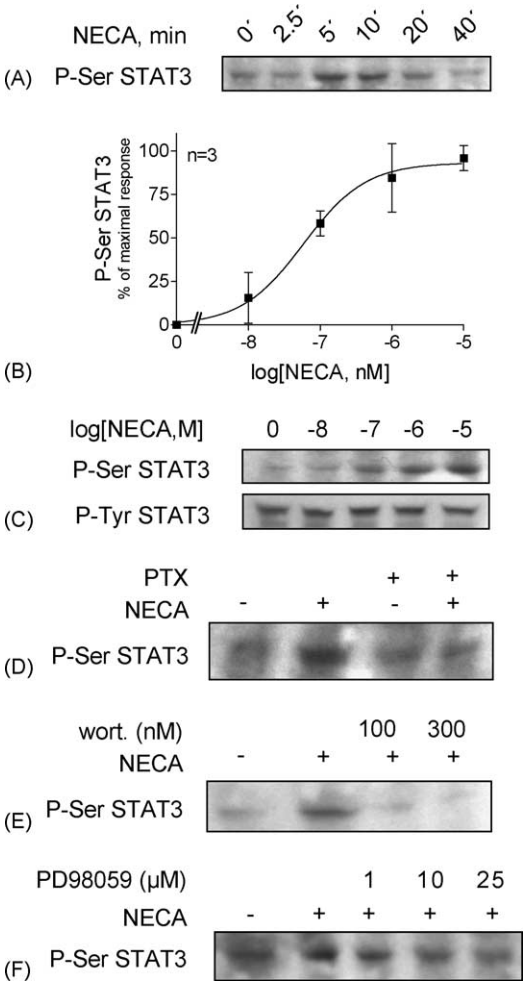

Fig. 4. Ser727 STAT3 is a possible downstream target of ERK1/2. In CHO A_3 cells, NECA induced a time-dependent (A) and dose-dependent (B) increase in Ser727 STAT3 phosphorylation (P-Ser STAT3), whereas it did not affect Tyr705 STAT3 phosphorylation. The NECA-induced Ser727 STAT3 phosphorylation was sensitive to PTX (D), wortmannin (E, wort.), and PD98059 (F) treatment (N = 3).

3.4. Rac/Cdc42 is necessary for p38 but not ERK1/2 phosphorylation

In order to investigate the role of small GTPases of the Rho family in the activation of NECA-induced p38 activation, we used the bacterial toxin B from Clostridium difficile (CTB), which has been shown to inhibit the small GTPases Rho/Rac/Cdc42 by glycosylation of specific threonine residues [22]. Pretreatment of CHO A_3 cells with CTB (500 pg to 1 ng/mL; 18 hr) decreased the NECA-induced p38 phosphorylation, whereas ERK1/2 phosphorylation was unaffected (Fig. 3).

3.5. Serine 727 phosphorylation of STAT3 as a possible downstream target of ERK1/2

In Fig. 4, we show that both time-course (Fig. 4A) and the dose–effect curve (Fig. 4B) for NECA-induced Ser727 STAT3 phosphorylation—as examined by immunoblotting—followed the pattern of ERK1/2 phosphorylation [13] with an EC_{50} value (CI) of 59 nM (26–131 nM). Furthermore, Ser727 STAT3 phosphorylation, comparable to ERK1/2 phosphorylation [12,14], was dependent on $G_{i/o}$ proteins, PI3K and MEK, as investigated by treatment with PTX (Fig. 4D; 200 ng/mL for 18 hr), wortmannin and PD98059, respectively (Fig. 4E and F). NECA stimulation of CHO A_3 cells, however, did not affect Tyr705 STAT3 phosphorylation (Fig. 4C).

4. Discussion

The signaling pathway from GPCRs to p38 is not as well known as that leading to ERK1/2 [18,23]. Although small G proteins and different upstream kinases have been implicated to be involved in signaling from GPCR to p38 [23,24], the role of PI3K in p38 signaling has not been previously established. Adenosine A_3 receptor stimulation directly activates PI3K as demonstrated by the fact that the downstream target PKB/Akt is dose-dependently phosphorylated upon adenosine A_3 receptor activation in CHO cells [12]. Furthermore this activation of PI3K is a necessary step in signaling from the adenosine A_3 receptor to ERK1/2 as demonstrated previously for other G_i-coupled receptors [18,23].

Signaling *via* PI3K and the activation of PKB may be the molecular basis for the reported apoptosis-modulating effects of the adenosine A_3 receptor [3,17,25,26] since that pathway is known to counteract apoptosis by direct phosphorylation and inhibition of the Bcl-2 family member BAD [27]. Whereas PI3K and ERK1/2 were suggested to act in an anti-apoptotic way, activation of the stress-activated p38 pathways is generally thought to be pro-apoptotic [28,29]. Thus, parallel activation of ERK1/2, PI3K and p38, and dependence of p38 activity on PI3K signaling represents an apparent conundrum. This is emphasized by

the previous findings that adenosine A_3 receptor-mediated effects on apoptosis are biphasic with regard to the degree of receptor stimulation and its effect on apoptosis. Depending on the cellular system used, low agonist concentrations may protect cells from apoptosis whereas strong receptor stimulation may induce apoptotic cell death [17,30]. In contrast to these biphasic effects on apoptosis, phosphorylation of relevant proteins as described in this study and others [12–14] is mainly monophasic, and occurs over the same concentration range (see, however, Ref. [21]). It is therefore probable that the steps between PI3K and ERK1/2 activation and between PI3K and p38 activation are not only quite divergent but also differentially regulated by other stimuli.

Another signaling pathway that has been implicated to modulate apoptosis is the JAK/STAT pathway [28]. Activation of STATs in general is accomplished by tyrosine phosphorylation, for example, by Janus kinases (JAK), while serine phosphorylation of STATs has a more modulatory role. It is, however, unclear, whether serine phosphorylation of STAT3 leads to an increase or decrease in STAT3 transcriptional activity (for a review, see Refs. [31,32]). Tyrosine phosphorylation, and thus activation of STATs can occur, for example, downstream of cytokine receptors such as the interferon γ receptor (for a review, see Ref. [33]), but also upon activation of GPCRs [34–37]. Adenosine A_3 receptor stimulation, however, did not change basal tyrosine phosphorylation of STAT3 but it increased its serine (Ser727) phosphorylation. Results from a previous study suggest that STAT3 is not constitutively active in CHO cells since leptin induced an increase in Tyr705 phosphorylation of STAT3 [38]. Even though other GPCRs have been shown to activate the JAK/STAT pathway *via* the induction of JAK-mediated tyrosine phosphorylation of STAT3, this appears not to be the case in NECA-stimulated CHO A_3 cells. The fact that Ser727 STAT3 phosphorylation is completely blocked by 1 and 10 μM of the MEK inhibitor PD98059 (Fig. 4), which completely blocks ERK1/2 phosphorylation, strongly suggests that Ser727 phosphorylation is mediated by ERK1/2 rather than p38. Thus, we conclude that Ser727 STAT3 phosphorylation might be a possible downstream target of adenosine A_3 receptor-mediated ERK1/2 activation through which adenosine A_3 receptors might affect the JAK/STAT pathway in a modulatory fashion. Future studies will have to elucidate the role of adenosine A_3 receptor-induced Ser727 STAT3 phosphorylation in mediating, for example, changes in transcriptional activity of STAT3. The results do, however, indicate that the observed ERK1/2 activation is associated with functional consequences.

Since NECA is very potent in inducing phosphorylation of ERK1/2, Ser727 STAT3 as well as p38 phosphorylation, we suggest that these pathways are activated by physiological adenosine concentrations, at least in regions where the adenosine A_3 receptor is abundant. In regions where the receptor density is lower adenosine levels elevated by, for example, hypoxia may suffice to activate A_3 receptor signaling. As adenosine concentrations increase when energy consumption and supply are imbalanced, the activation of stress-activated and mitogenic pathways suggests that they are part of a protective or alerting program. This hypothesis is, indeed, strengthened by our observation that at least some of these pathways are also activated by functional adenosine A_3 receptors expressed in mouse microglia cells (Figs. 1 and 2; [21]), a cell type that is a sensor, for example, for hypoxia/ischemia or inflammation in the central nervous system. This implicates that MAPK phosphorylation and PI3K signaling downstream of adenosine receptors is physiologically relevant.

Our findings add to the growing body of information on the intracellular signaling pathways that are activated by the adenosine A_3 receptors [15]. Apparently, intracellular signaling *via* this adenosine receptor subtype is not restricted to the inhibition of cAMP production and phospholipase-dependent signaling: other important signaling relays, such as the small GTPase Ras [12] and PI3K are also recruited in order to activate a network of kinase cascades, such as the ERK1/2 and p38 cascade (Fig. 4). Furthermore, it is important to note that modulatory effects may appear intracellularly rather than intercellularly, as implicated by the NECA-induced Ser727 STAT3 phosphorylation and the cross talk between the MAPK and the JAK/STAT pathway. The understanding of the intracellular network activated by adenosine A_3 receptors may help us to examine the physiological and pathophysiological roles of the A_3 receptor.

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