0026-895X/00/030477-00$3.00/0
MOLECULAR PHARMACOLOGY
Copyright © 2000 The American Society for Pharmacology and Experimental Therapeutics
Mol Pharmacol 58:477–482, 2000

Vol. 58, No. 3
13171/847055
Printed in U.S.A.

Human Adenosine A_1, A_{2A}, A_{2B}, and A_3 Receptors Expressed in Chinese Hamster Ovary Cells All Mediate the Phosphorylation of Extracellular-Regulated Kinase 1/2

GUNNAR SCHULTE and BERTIL B. FREDHOLM

Karolinska Institutet, Department of Physiology and Pharmacology, Section of Molecular Neuropharmacology, Stockholm, Sweden

Received December 14, 1999; accepted May 22, 2000

This paper is available online at http://www.molpharm.org

ABSTRACT

The known diverse effects of adenosine on mitogenesis may be related to changes in mitogen-activated protein kinases. In this study we therefore compared the phosphorylation of extracellular-regulated kinase 1/2 (ERK1/2) via the four known human adenosine receptors A_1, A_{2A}, A_{2B}, and A_3, stably transfected into Chinese hamster ovary (CHO) cells. The adenosine analog 5′-*N*-ethylcarboxamidoadenosine (NECA), known to act on all subtypes, had no effect on untransfected CHO cells, but did cause a substantial time- and dose-dependent phosphorylation in CHO cells transfected with each of the receptors. The maximal phosphorylation was highest in A_1 and A_3 receptor-transfected cells, intermediate in A_{2A} and low in A_{2B} receptor-expressing CHO cells. For all receptors the half-maximal ERK1/2 phosphorylation was observed at 19–115 nM NECA. NECA acting on adenosine A_{2B} receptors was much more potent in stimulating ERK1/2 phosphorylation (EC_{50} = 19 nM) than cAMP formation (EC_{50} = 1.4 μM). Stimulation with the endogenous ligand adenosine resulted in the same pattern of ERK1/2 phosphorylation as NECA. Concentrations of adenosine that occur physiologically caused an increased phosphorylation after 5 min in CHO cells transfected with any one of the four adenosine receptors. Adenosine at levels reached during ischemia (3 μM) induced a more pronounced, but still transient, activation of ERK1/2. In conclusion, this study shows that all the human adenosine receptors transfected into CHO cells are able to activate ERK1/2 at physiologically relevant concentrations of the endogenous agonist.

The endogenous nucleoside adenosine binds to and activates four different subtypes of G protein-coupled receptors (GPCR): adenosine A_1, A_{2A}, A_{2B}, and A_3 receptors. The different subtypes can be distinguished pharmacologically and differ also in their coupling to second messenger systems (Fredholm et al., 1994, 1996). Adenosine A_1 and A_3 receptors inhibit adenylyl cyclase and stimulate phospholipase Cβ via activation of the pertussis toxin-sensitive G proteins G_i and/or G_o. Adenosine A_{2A} and A_{2B} receptors are positively coupled to adenylyl cyclase, but also may activate alternative signaling pathways (Feoktistov et al., 1994; Feoktistov and Biaggioni, 1995; Mirabet et al., 1997).

This diversity in intracellular signaling downstream of adenosine receptors and the fact that adenosine has been reported, depending on the receptor subtype activated, to both enhance and inhibit mitogenesis (Jonzon et al., 1985; Neary et al., 1996), postischemic cell death (Fredholm, 1997), and apoptosis (Shneyvays et al., 1998; Vitolo et al., 1998) led us to examine the effects of adenosine receptor stimulation on extracellular regulated kinases 1/2 (ERK1/2). ERK1/2 belong to the family of mitogen-activated protein kinases (MAPKs), which can be activated by receptor tyrosine kinases via the classical MAPK cascade, and mediate proliferation, differentiation, cell survival, and cell death (Seger and Krebs, 1995). G protein-coupled receptors, G_i/G_o as well as G_s coupled, also have been shown to activate the MAPK cascade (Sugden and Clerk, 1997; Gutkind, 1998; Luttrell et al., 1999).

The activation of ERK1/2 via adenosine A_1 (Dickenson et al., 1998), A_{2A} (Sexl et al., 1997; Seidel et al., 1999), and A_{2B} (Feoktistov et al., 1999; Gao et al., 1999) receptors has recently been detected in a variety of systems. However, activation of A_{2A} receptors constitutively expressed in the rat pheochromocytoma cell line PC12 (Arslan et al., 1997) or stably transfected into Chinese hamster ovary (CHO) cells (Hirano et al., 1996) inhibited ERK activation by other stimuli. Little or nothing is known about A_3 receptors. It seemed

This study was supported by the Swedish Medical Research Council (2553), by the European Commission (EURCAR), and by Karolinska Institutet. Some of these results have been presented in abstract form at the 1999 International Society for Neurochemistry/European Society for Neurochemistry meeting in Berlin, Germany.

ABBREVIATIONS: ERK, extracellular-regulated kinase; MAPK, mitogen-activated protein kinase; CHO, Chinese hamster ovary; NECA, 5′-*N*-ethylcarboxamidoadenosine; CGS 15943, 9-chloro-2-(2-furyl)[1,2,4]triazolo[1,5-*c*]quinazolin-5-amine; CGS 21680, 2-[*p*-(2-carboxyethyl)phenylethylamino]-5′-*N*-ethylcarboxamidoadenosine; R-PIA, (*R*)-N^6-phenylisopropyladenosine.

important to compare activation of MAPKs mediated by all the human adenosine receptor subtypes against an identical cellular background. Transfection of CHO cells with the different adenosine receptors presents a useful model to compare the intracellular events after receptor stimulation in a cellular environment with an identical signaling machinery. Such receptor-expressing cells have been previously characterized biochemically as well as pharmacologically (Klotz et al., 1998). Our results show a transient time- and dose-dependent phosphorylation of ERK1/2 after 5′-*N*-ethylcarboxamidoadenosine (NECA) and adenosine stimulation of CHO cells expressing the human A_1, A_{2A}, A_{2B}, or A_3 receptor.

Experimental Procedures

Materials. All cell culture media, fetal calf serum, and supplies were from Life Technologies (Täby, Sweden). NECA, 9-chloro-2-(2-furyl)[1,2,4]triazolo[1,5-*c*]quinazolin-5-amine (CGS 15943), 2-[*p*-(2-carboxyethyl)phenylethylamino]-5′-*N*-ethylcarboxamidoadenosine (CGS 21680), and (*R*)-N^6-phenylisopropyladenosine (R-PIA) were from Research Biochemicals International (Wayland, MA). Polyvinylidene difluoride-Immobilon P membrane was from Millipore Corp. (Bedford, MA). Phosphospecific rabbit anti-phospho-Thr-202/Tyr-204-ERK1/2 was from New England Biolabs, Inc. (Beverly, MA) and rabbit polyclonal anti-human cyclophilin A was from Upstate Biotechnology (Lake Placid, NY). Goat anti-rabbit horseradish peroxidase antibody was from Pierce (Rockford, IL). Enhanced chemiluminescence detection (ECL) kit was from Amersham International (Buckinghamshire, England).

Cell Culture. CHO cells transfected with the four human adenosine receptors (Klotz et al., 1998) were grown adherent at 37°C and 5% CO_2, 95% air in Dulbecco's modified Eagle's medium/F-12 (1:1), 0.2 mg/ml geneticin (G-418), 100 U/ml penicillin, 100 U/ml streptomycin, 2 mM L-glutamine, and 10% fetal calf serum. Untransfected control cells were maintained under the same conditions, but without G-418. All cells were split three times a week at a ratio of 1:20. For detection of ERK1/2 phosphorylation 1.5×10^6 cells were plated in 10-cm Petri dishes and grown overnight. After serum deprivation (growth medium with 0.5% fetal calf serum without G-418) overnight, the cells were washed two times with Dulbecco's modified Eagle's medium, 20 mM HEPES, pH 7.4, at 37°C and then stimulated with drugs as described in the figure legends. The reaction was stopped at 0, 5, 10, 15, or 30 min with two quick washes with 2 ml of ice-cold PBS and lysis in 500 μl of 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, and 5 μg/ml leupeptin on ice. Time points earlier than 5 min have not been studied because the method used for cell lysis did not allow us to stop the reaction precisely. The cell lysate was centrifuged for 5 min in a microfuge and the supernatant was denatured with Laemmli buffer. Equal amounts of each sample were analyzed by polyacrylamide gel electrophoresis (4% stacking gel; 12% resolving gel) with the Hoefer Mini VE system.

Immunoblotting. The proteins were transferred onto a polyvinylidene difluoride membrane by using Towbin's buffer for transfer. After blocking for 1 h in 3% dry milk powder dissolved in 50 mM Tris, 150 mM NaCl, 0.05% Tween 20, the membrane was incubated with the phosphospecific rabbit anti-P-ERK1/2 1:1000 in 50 mM Tris, 150 mM NaCl, 0.05% Tween 20, 0.01% BSA, 0.01% NaN_3 either for 2 h at room temperature or overnight at 4°C. As an internal standard for normalization, the membrane was simultaneously incubated with a rabbit anti-human cyclophilin A antibody, which detects a protein of about 18 kDa. P-ERK1/2 and cyclophilin A were visualized with a goat anti-rabbit horseradish peroxidase antibody (1:10,000) in 10 mM Tris, 500 mM NaCl, pH 7.6, 0.5% Tween 20 for 1 h at room temperature and the enhanced chemiluminescence method according to the manufacturer's protocol.

cAMP Assay. Intracellular cAMP levels were determined by using a competitive protein-binding assay and [^{3}H]cAMP. Briefly, cells were plated into 24-well microplates, grown overnight, and stimulated at 37°C. The reaction was stopped by addition of perchloric acid to a final concentration of 0.4 M and incubation on ice. After lysis and neutralization with KOH in 50 mM Tris, the supernatant was examined for cAMP content as described elsewhere (Kull et al., 1999a).

Data Analysis. We used the NIH Scion Image software for quantitative analysis of the immunoblots. For the graphical presentation and the statistical analysis of data (one-way ANOVA) we used GraphPad Prism2 software (GraphPad, San Diego, CA). Sigmoidal dose-response curves were calculated by using nonlinear regression.

Results

Stimulation of Human Adenosine A_1, A_{2A}, A_{2B}, and A_3 Receptors Expressed in CHO Cells Led to Phosphorylation of the MAPK ERK1/2. The immunoblot signal seen using the dual phosphorylation site-selective antibody increased in a time- (Fig. 1) and dose-dependent (Fig. 2) manner. In cells not transfected with any adenosine receptor, NECA was virtually without effect on CAMP production and ERK1/2 phosphorylation (Figs. 3 and 4). After stimulation with the nonselective adenosine receptor agonist NECA, ERK1/2 phosphorylation reached a maximal value at 5 min in cells transfected with each type of the receptor. After 30 min NECA stimulation the levels of ERK1/2 phosphorylation had returned to control (184 ± 47, 147 ± 39, and 145 ± 10%, respectively) in cells transfected with A_1, A_{2A}, or A_{2B} receptor, whereas in cells transfected with the A_3 receptors phospho-ERK1/2 remained almost 4-fold (394 ± 137%) above control levels at this time point.

There appeared to be differences in the maximal stimulation of ERK1/2 phosphorylation between cells transfected with different adenosine receptors. Thus, adenosine A_1 and A_3 receptor-transfected cells showed a much more pronounced increase in ERK1/2 phosphorylation at 5 min compared with the A_{2A}- or A_{2B}-transfected cells (Fig. 1). The maximal stimulation at 5 min in cells transfected with A_1, A_{2A}, A_{2B}, and A_3 receptor was (mean ± S.E.) 1305 ± 545, 546 ± 165, 158 ± 9, and 988 ± 99% compared with the unstimulated control (100%) (100 nM NECA for A_1, A_{2A}, and A_3 receptor; 10 μM NECA for A_{2B} receptor).

The potency of NECA on the adenosine receptors was similar and in the nanomolar range for all of the receptor subtypes (Fig. 2). The EC_{50} values (mean and 95% CI) for NECA-stimulated ERK1/2 phosphorylation were A_1, 115 nM (56 to 236 nM); A_{2A}, 26 nM (12 to 56 nM); A_{2B}, 19 nM (6 to 62 nM); and A_3, 65 nM (36 to 119 nM).

The effect of NECA in A_{2B}-transfected cells was modest. It was therefore important to show that it is indeed receptor mediated. The results in Fig. 4 show that ERK1/2 phosphorylation in untransfected CHO cells was not increased and that the NECA effects in A_{2B}-transfected cells was abolished by a receptor antagonist (CGS 15943, 10 μM). In addition, we performed experiments with CGS 21680 and R-PIA as agonists on adenosine A_{2B} receptor-transfected CHO cells (data not shown). The dose-response curves for ERK1/2 phosphorylation of these compounds, compared with those reported for cAMP production (Klotz et al., 1998), were shifted to the left as well. The order of potency for the agonists tested at the human adenosine A_{2B} receptor, however, remained unchanged (NECA > R-PIA > CGS 21680). Furthermore, this

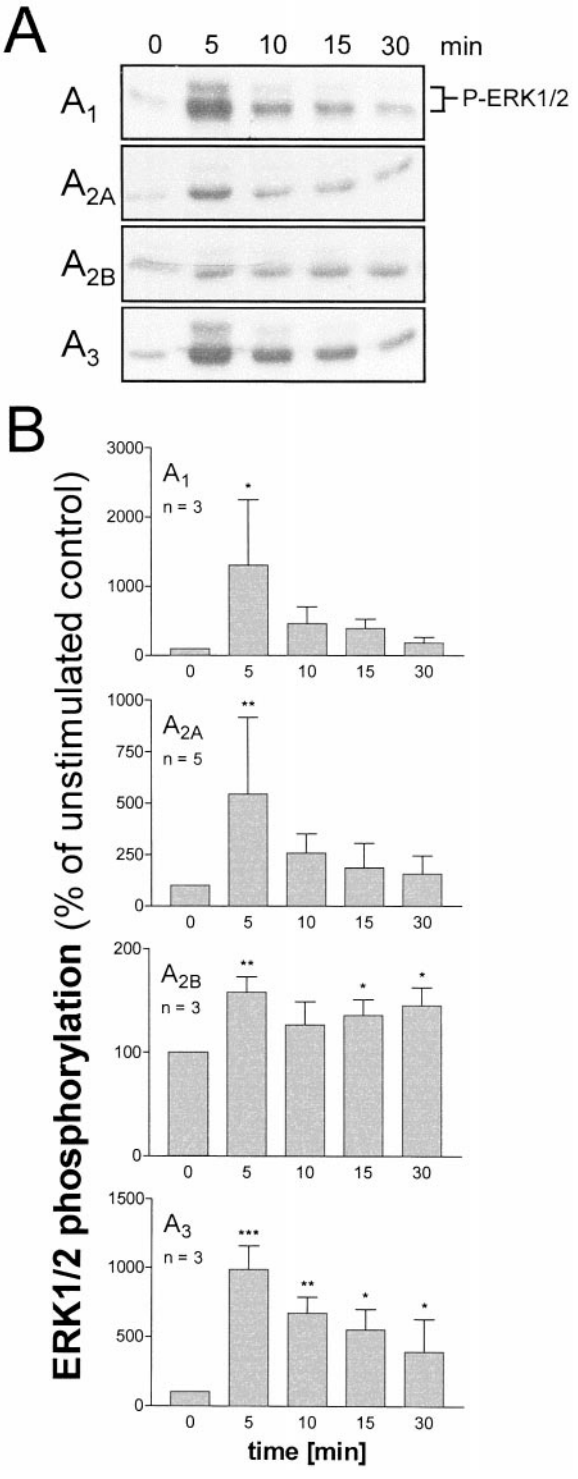

Fig. 1. Time course of ERK1/2 phosphorylation in quiescent CHO cells stably transfected with the human adenosine A_1, A_{2A}, A_{2B}, or A_3 receptor after NECA stimulation (A_1, A_{2A}, and A_3 stimulated with 100 nM NECA; A_{2B} stimulated with 10 μM NECA). For further details see *Experimental Procedures*. The immunoblots (A) are representative of at least three independent experiments per transfected cell line as summarized in the bar graphs (B). Error bars show S.D. *P* values for the statistical analysis (one-way ANOVA) with $^*P < .05$, $^{**}P < .01$, $^{***}P < .001$.

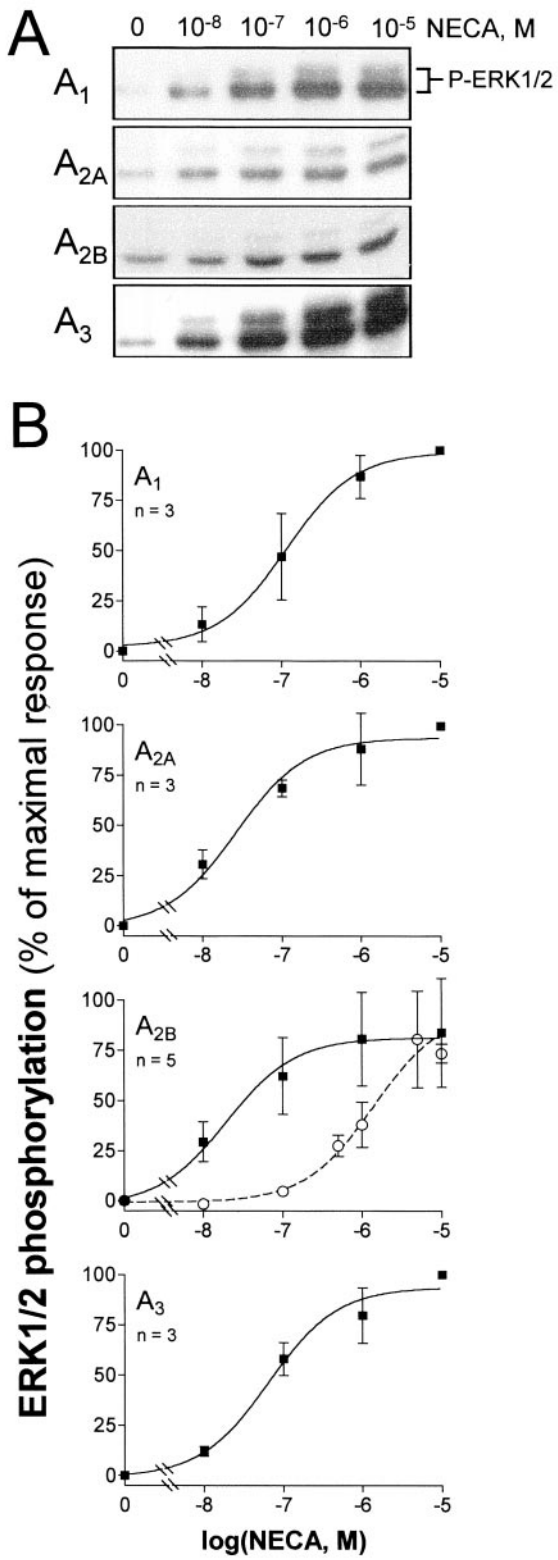

Fig. 2. ERK1/2 phosphorylation in serum-deprived CHO cells stably transfected with the human adenosine A_1, A_{2A}, A_{2B}, or A_3 receptor is dependent on NECA concentration. Cells were stimulated for 5 min. The immunoblots (A) show representative results of at least three experiments. These data are summarized in B that shows the normalized concentration-response curves. In each experiment the maximal level of ERK1/2 phosphorylation was set to 100% and that seen in unstimulated cells to 0% (■). The graph for adenosine A_{2B} receptor-transfected CHO cells also contains a concentration-response curve for the production of cAMP after NECA stimulation (○). The error bars give S.D.

high potency of agonists on ERK1/2 phosphorylation in these cells prompted the examination of NECA's ability to stimulate cAMP production. As seen in Fig. 2 there is a close to 100-fold difference in EC_{50} = 1.4 μM (0.7 to 2.7 μM). A similar discrepancy does not exist in the other transfected cells (Table 1).

The rather high potency of the adenosine analogs NECA, CGS 21680, and R-PIA suggested that the endogenous agonist adenosine also might exert effects in a physiological range of concentrations. We therefore tested the effect of adenosine on ERK1/2 phosphorylation in CHO cells at 100 nM, a concentration that occurs under basal physiological conditions in many body fluids, and at 3 μM, a concentration that is reached under hypoxic conditions. As seen in Fig. 5, in all the adenosine receptor-transfected cells, adenosine mimicked the response to NECA in time as well as dose dependence. Thus, at the high physiological concentration a clear-cut increase in ERK1/2 phosphorylation was seen in all adenosine receptor transfected cells after 5 min. This effect was smaller after 30 min. The lower adenosine concentration produced small, but clear effects in cells transfected with any of the four adenosine receptors. As shown for NECA, adenosine stimulation showed differing efficacy on the different receptor subtype-transfected cells, with A_{2B} mediating the smallest changes in ERK1/2 phosphorylation (Fig. 5).

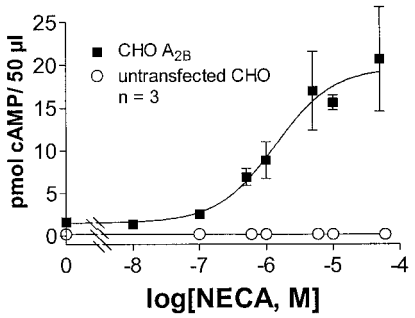

Fig. 3. NECA-dependent changes in cAMP levels detected in CHO cells transfected with the human adenosine A_{2B} receptor and in untransfected CHO cells. The experiment was performed in triplicate. Error bars show S.D.

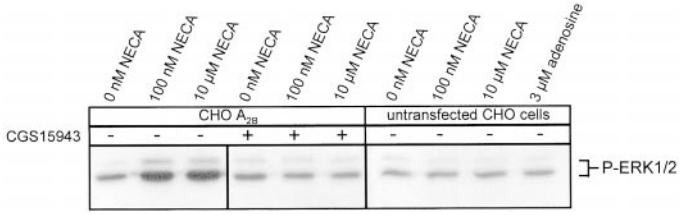

Fig. 4. Receptor-mediated ERK1/2 phosphorylation induced by NECA in A_{2B} receptor-transfected CHO cells. The left part shows that the adenosine receptor antagonist CGS 15943 (10 μM) abolishes the NECA-induced changes in ERK1/2 phosphorylation in CHO A_{2B} cells, thus confirming the adenosine receptor dependence of the effect. The right part shows that neither NECA nor adenosine stimulates ERK1/2 phosphorylation in untransfected CHO cells.

Discussion

Our results show that adenosine or the adenosine analog NECA can increase phosphorylation of the MAPK ERK1/2 in CHO cells expressing each of the human adenosine receptors A_1, A_{2A}, A_{2B}, or A_3, but not in untransfected CHO cells. Although the two A_2 receptors have been the subject of most previous studies (see Introduction) the maximal increase of ERK1/2 phosphorylation was lower in cells expressing these receptors than in cells with the A_1 or A_3 receptor. In fact, the adenosine analog produced the highest level of phosphorylation in cells expressing A_3 receptors, a receptor that has not previously been studied.

Differences in efficacy may be caused by differences in receptor expression and it has been pointed out that overexpression of receptors can lead to detection of signaling mechanisms that do not occur in cells where the receptors are expressed at a normal level (Kenakin, 1997). However, in the experimental model used in our study the transfected receptors are expressed at levels that resemble normal expression (Klotz et al., 1998) as shown by ligand binding for A_1, A_{2A}, and A_3 receptors. The number of A_1 receptors per A_1-transfected CHO cell is on the order of 40,000 (L. V. Lopes, B. Kull, and B. B. Fredholm, unpublished data), which is lower than the number reported, e.g., on the hamster smooth muscle cell line DDT_1 MF-2 [110,000 receptors/cell (Gerwins et al., 1990)]. Previously, it was found that the number of A_1 and A_3 receptors in CHO cells is similar (Klotz et al., 1998). We also have shown that the number of A_{2A} receptors in the CHO cells transfected with the human A_{2A} receptor is lower than the number of receptors expressed in striatopallidal neurons in the intact rat striatum (Kull et al., 1999b). It is difficult to estimate the number of A_{2B} receptors expressed in CHO cells because specific ligands for ligand-binding studies are not available. In unpublished experiments we have used [^{3}H]ZM241385, which does bind to adenosine A_{2B} receptors with appreciable affinity (Ji and Jacobson, 1999). The results

TABLE 1

Comparison of NECA-mediated effects on ERK1/2 phosphorylation/cAMP production and NECA binding between the four human adenosine receptor subtypes

Summary of EC_{50} values for ERK1/2 activation after NECA stimulation in CHO cells stably transfected with the human adenosine A_1, A_{2A}, A_{2B}, or A_3 receptor. For a comparison, K_D or K_i values and EC_{50} values from other sources have been used.

Receptor Subtype	EC_{50} Value for ERK1/2 Phosphorylation	K_D or K_i	EC_{50} Value for cAMP Production	Total Receptor Number per Cell
	nM			
A_1	115.4	13.6 nM (K_D high affinity) (Klotz et al., 1998)	26 nM (Bevan et al., 1999)	~40,000 (see *Discussion*)
A_{2A}	26.4	20.1 nM (Klotz et al., 1998)	26.1 nM (Kull et al., 1999a)	~20,000 (Kull et al., 1999a)
A_{2B}	19.4	N.D.	1.4 μM (present study)	<200,000 (see *Discussion*)
A_3	65.4	6.18 nM (Klotz et al., 1998)	129 nM (Salvatore et al., 1993)	Similar to A_1 (Klotz et al., 1998)

N.D., not determined.

suggest that there are fewer than 200,000 A_{2B} receptors per transfected CHO cell. For all these reasons we conclude that there is no major risk that we have studied signal pathways that only operate when receptors are expressed at levels dramatically higher than those occurring naturally.

The potency of NECA on the adenosine A_1 and A_{2A} receptor resembles the values reported previously in the same cells for radioligand binding or cyclic AMP production (Klotz et al., 1998; Kull et al., 1999a). The situation is somewhat similar for the A_3 receptor: half-maximal ERK1/2 phosphorylation was observed at 65 nM NECA, which lies in the same range as described for the rat (Zhou et al., 1992) and the human (Salvatore et al., 1993) A_3 receptor by using cAMP generation as the assay. However, in A_{2B} receptor-transfected CHO cells, half-maximal stimulation of ERK1/2 phosphorylation occurred at concentrations two orders of magnitude lower than those required to half maximally activate cAMP production (Fig. 2). Indeed, NECA was more potent in causing ERK1/2 phosphorylation via A_{2B} receptors than via any of the other subtypes. The adenosine receptor antagonist CGS 15943 abolished the A_{2B}-mediated effect, and the order of potency of different agonists, such as NECA, CGS 21680, and R-PIA, was similar to the one reported earlier, thus confirming that the increase in ERK1/2 phosphorylation in A_{2B}-transfected CHO cells is really mediated by this receptor subtype.

Previous studies of the potency of agonists to activate/inhibit adenylyl cyclase have suggested that the A_1, A_{2A}, and A_3 receptors may be activated by concentrations of the endogenous agonist adenosine that occur under basal physiological conditions, whereas A_{2B} receptors may be activated by levels that occur only pathophysiologically (Fredholm et al., 1994). The present data suggest that this conclusion may not be as valid if other signaling pathways than the cAMP/cAMP-dependent protein kinase cascade, phospholipase C/inositol-trisphosphate formation or ion channel activation are considered. Consistent with this hypothesis is the finding that adenosine mediates the phosphorylation of ERK1/2 via any one of the human adenosine receptor subtypes already at physiologically normal concentrations of 100 nM.

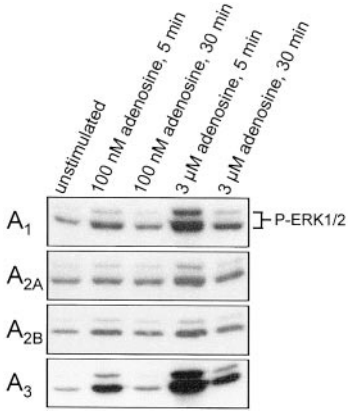

Fig. 5. Adenosine induces ERK1/2 phosphorylation in a time- and dose-dependent manner similar to NECA in quiescent CHO cells transfected with the four human adenosine receptor subtypes. A_1, A_{2A}, A_{2B}, and A_3 receptor expressing cells were stimulated with 100 nM and 3 μM adenosine. The immunoblots show one representative experiment of 2 to 3 per cell type. The increase in ERK1/2 phosphorylation after 5 min 100 nM adenosine compared with the unstimulated control was 2.92 ± 0.10-fold (n = 2), 1.34 ± 0.06-fold (n = 2), 2.14 ± 0.83-fold (n = 3), and 11.35 ± 0.21-fold (n = 2) (error in S.D.) for the A_1, A_{2A}, A_{2B}, and A_3 receptor, respectively.

Adenosine A_{2B} receptors have been suggested to play an important role during conditions such as hypoxia and ischemia when the adenosine concentration rises dramatically and can reach levels where A_{2B} receptors are activated. Our results show an activation of ERK1/2 at NECA concentrations in the lower nanomolar range in A_{2B} receptor-transfected CHO cells. This may have fundamental consequences for understanding the role of adenosine A_{2B} receptors. It may be that not only cAMP and calcium signals initiated by adenosine levels above 500 nM but also MAPK signaling at much lower, physiologically normal concentrations (30–300 nM) turn out to be important. In vitro studies have suggested that adenosine receptor-activated MAPK is important in the regulation of endothelial cell (Sexl et al., 1997) and astrocyte (Neary et al., 1998) proliferation, and in mast cell activation (Feoktistov et al., 1999), but the physiological role of adenosine receptor-mediated ERK1/2 activation remains to be studied.

Acknowledgments

Giulia Arslan and Björn Kull are acknowledged for fruitful discussions and for sharing unpublished information.

References

Arslan G, Kontny E and Fredholm BB (1997) Down-regulation of adenosine A_{2A} receptors upon NGF-induced differentiation of PC12 cells. *Neuropharmacology* **36:**1319–1326.

Bevan N, Palmer T, Drmota T, Wise A, Coote J, Milligan G and Rees S (1999) Functional analysis of a human A_1 adenosine receptor/green fluorescent protein/G_{i1} alpha fusion protein following stable expression in CHO cells. *FEBS Lett* **462:**61–65.

Dickenson JM, Blank JL and Hill SJ (1998) Human adenosine A_1 receptor and $P2Y_2$-purinoceptor-mediated activation of the mitogen-activated protein kinase cascade in transfected CHO cells. *Br J Pharmacol* **124:**1491–1499.

Feoktistov I and Biaggioni I (1995) Adenosine A_{2b} receptors evoke interleukin-8 secretion in human mast cells. An enprofylline-sensitive mechanism with implications for asthma. *J Clin Invest* **96:**1979–1986.

Feoktistov I, Goldstein AE and Biaggioni I (1999) 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. *Mol Pharmacol* **55:**726–734.

Feoktistov I, Murray JJ and Biaggioni I (1994) Positive modulation of intracellular Ca^{2+} levels by adenosine A_{2b} receptors, prostacyclin, and prostaglandin E1 via a cholera toxin-sensitive mechanism in human erythroleukemia cells. *Mol Pharmacol* **45:**1160–1167.

Fredholm BB (1997) Adenosine and neuroprotection. *Int Rev Neurobiol* **40:**259–280.

Fredholm BB, Abbracchio MP, Burnstock G, Daly JW, Harden TK, Jacobson KA, Leff P and Williams M (1994) Nomenclature and classification of purinoceptors. *Pharmacol Rev* **46:**143–156.

Fredholm BB, Arslan G, Kull B, Kontny E and Svenningsson P (1996) Adenosine (P1) receptor signalling. *Drug Dev Res* **39:**262–268.

Gao Z, Chen T, Weber MJ and Linden J (1999) A_{2B} adenosine and $P2Y_2$ receptors stimulate mitogen-activated protein kinase in human embryonic kidney-293 cells. Cross-talk between cyclic AMP and protein kinase c pathways. *J Biol Chem* **274:**5972–5980.

Gerwins P, Nordstedt C and Fredholm BB (1990) Characterization of adenosine A_1 receptors in intact DDT1 MF-2 smooth muscle cells. *Mol Pharmacol* **38:**660–666.

Gutkind JS (1998) The pathways connecting G protein-coupled receptors to the nucleus through divergent mitogen-activated protein kinase cascades. *J Biol Chem* **273:**1839–1842.

Hirano D, Aoki Y, Ogasawara H, Kodama H, Waga I, Sakanaka C, Shimizu T and Nakamura M (1996) Functional coupling of adenosine A_{2a} receptor to inhibition of the mitogen-activated protein kinase cascade in Chinese hamster ovary cells. *Biochem J* **316:**81–86.

Ji X-D and Jacobson KA (1999) Use of Triazolotriazine [3H]ZM 241385 as a radioligand at recombinant human A_{2B} adenosine receptors. *Drug Des Discovery* **16:**89–98.

Jonzon B, Nilsson J and Fredholm BB (1985) Adenosine receptor-mediated changes in cyclic AMP production and DNA synthesis in cultured arterial smooth muscle cells. *J Cell Physiol* **124:**451–456.

Kenakin T (1997) Differences between natural and recombinant G protein-coupled receptor systems with varying receptor/G protein stoichiometry. *Trends Pharmacol Sci* **18:**456–464.

Klotz KN, Hessling J, Hegler J, Owman C, Kull B, Fredholm BB and Lohse MJ (1998) Comparative pharmacology of human adenosine receptor subtypes -Char-

acterization of stably transfected receptors in CHO cells. *Naunyn-Schmiedeberg's Arch Pharmacol* **357:**1–9
Kull B, Arslan G, Nilsson C, Owman C, Lorenzen A, Schwabe U and Fredholm BB (1999a) Differences in the order of potency for agonists but not antagonists at human and rat adenosine A_{2A} receptors. *Biochem Pharmacol* **57:**65–75.
Kull B, Ferré S, Arslan G, Svenningsson P, Fuxe K, Owman C and Fredholm BB (1999b) Reciprocal interactions between adenosine A_{2A} and dopamine D_2 receptors in Chinese hamster ovary cells co-transfected with the two receptors. *Biochem Pharmacol* **58:**1035–1045.
Luttrell LM, Daaka Y and Lefkowitz RJ (1999) Regulation of tyrosine kinase cascades by G-protein-coupled receptors. *Curr Opin Cell Biol* **11:**177–183.
Mirabet M, Mallol J, Lluis C and Franco R (1997) Calcium mobilization in Jurkat cells via A_{2b} adenosine receptors. *Br J Pharmacol* **122:**1075–1082.
Neary JT, McCarthy M, Kang Y and Zuniga S (1998) Mitogenic signaling from P1 and P2 purinergic receptors to mitogen-activated protein kinase in human fetal astrocyte cultures. *Neurosci Lett* **242:**159–162.
Neary JT, Rathbone MP, Cattabeni F, Abbracchio MP and Burnstock G (1996) Trophic actions of extracellular nucleotides and nucleosides on glial and neuronal cells. *Trends Neurosci* **19:**13–18.
Salvatore CA, Jacobson MA, Taylor HE, Linden J and Johnson RG (1993) Molecular cloning and characterization of the human A_3 adenosine receptor. *Proc Natl Acad Sci USA* **90:**10365–10369.
Seger R and Krebs EG (1995) The MAPK signaling cascade. *FASEB J* **9:**726–735.
Seidel MG, Klinger M, Freissmuth M and Höller C (1999) Activation of mitogen-activated protein kinase by the A_{2A}-adenosine receptor via a rap1-dependent and via a $p21^{ras}$-dependent pathway. *J Biol Chem* **274:**25833–25841.
Sexl V, Mancusi G, Höller C, Gloria-Maercker E, Schütz W and Freissmuth M (1997) Stimulation of the mitogen-activated protein kinase via the A_{2A}-adenosine receptor in primary human endothelial cells. *J Biol Chem* **272:**5792–5799.
Shneyvays V, Nawrath H, Jacobson KA and Shainberg A (1998) Induction of apoptosis in cardiac myocytes by an A_3 adenosine receptor agonist. *Exp Cell Res* **243:**383–397.
Sugden PH and Clerk A (1997) Regulation of the ERK subgroup of MAP kinase cascades through G protein-coupled receptors. *Cell Signal* **9:**337–351.
Vitolo OV, Ciotti MT, Galli C, Borsello T and Calissano P (1998) Adenosine and ADP prevent apoptosis in cultured rat cerebellar granule cells. *Brain Res* **809:**297–301.
Zhou QY, Li C, Olah ME, Johnson RA, Stiles GL and Civelli O (1992) Molecular cloning and characterization of an adenosine receptor: The A_3 adenosine receptor. *Proc Natl Acad Sci USA* **89:**7432–7436.

Send reprint requests to: Gunnar Schulte, Karolinska Institutet, Department of Physiology and Pharmacology, Section of Molecular Neuropharmacology, S-171 77 Stockholm, Sweden. E-mail: gunnar.schulte@fyfa.ki.se