Human Adenosine A₁, A₂A, A₂B, and A₃ Receptors Expressed in Chinese Hamster Ovary Cells All Mediate the Phosphorylation of Extracellular-Regulated Kinase 1/2

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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 A₁, A₂A, A₂B, and A₃, 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 A₁ and A₃ receptor-transfected cells, intermediate in A₂A, and low in A₂B receptor-expressing CHO cells. For all receptors the half-maximal ERK1/2 phosphorylation was observed at 19–115 nM NECA. NECA acting on adenosine A₂B receptors was much more potent in stimulating ERK1/2 phosphorylation (EC₅₀ = 19 nM) than cAMP formation (EC₅₀ = 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 A₁, A₂A, A₂B, and A₃ receptors. The different subtypes can be distinguished pharmacologically and differ also in their coupling to second messenger systems (Fredholm et al., 1994, 1996). Adenosine A₁ and A₃ receptors inhibit adenylyl cyclase and stimulate phospholipase Cβ via activation of the pertussis toxin-sensitive G proteins Gᵢ and/or Gₛ. Adenosine A₂A and A₂B 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 (Shneyveys 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, Gₛ/Gᵢ as well as Gₛ 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 A₁ (Dickenson et al., 1998), A₂A (Sexl et al., 1997; Seidel et al., 1999), and A₂B (Feoktistov et al., 1999; Gao et al., 1999) receptors has recently been detected in a variety of systems. However, activation of A₂A 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 A₃ receptors. It seemed

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]phenyl-ethylamino)-5′-N-ethylcarboxamidoadenosine; R-PIA, (R)-N′-phenylisopropyladenosine.

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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 A1, A2A, A2B, or A3 receptor.

**Results**

Stimulation of Human Adenosine A1, A2A, A2B, and A3 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 A1, A2A, or A3 receptor, whereas in cells transfected with the A2B receptor, NECA receptor-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 A1 and A3 receptor-transfected cells showed a much more pronounced increase in ERK1/2 phosphorylation at 5 min compared with the A2A- or A2B-transfected cells (Fig. 1). The maximal stimulation at 5 min in cells transfected with A1, A2A, A2B, and A3 receptor was (mean ± S.E.) 1305 ± 545, 546 ± 165, 158 ± 9, and 988 ± 99% compared with the unstimulated control (100%) (100 nM NECA for A1, A2A, and A3 receptor; 10 μM NECA for A2B 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 EC50 values (mean and 95% CI) for NECA-stimulated ERK1/2 phosphorylation were A1, 115 nM (56 to 236 nM); A2A, 26 nM (12 to 56 nM); A2B, 19 nM (6 to 62 nM); and A3, 65 nM (36 to 119 nM). The effect of NECA in A1-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 A2B-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 A2B 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 A2B receptor, however, remained unchanged (NECA > R-PIA > CGS 21680). Furthermore, this

**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-][5-]quinazolin-5-amine (CGS 15943), 2-(2-carboxyethyl)phenylisothiophenol; 5'-N-ethylcarboxamidoadenosine (CGS 21680), and (R)-N'-phenylisopropyladenosine (R-PIA) were from Research Biochemicals International (Wayland, MA). Polyclonal rabbit 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% CO2, 95% air in Dulbecco’s modified Eagle’s medium/F-12 (1:1), 0.2 mg/ml gentamicin, 2 mM L-glutamine, and 10% fetal calf serum. Untransfected CHO cells expressing the human A1, A2A, A2B, or A3 receptor obtained from Research Biochemicals International (Wayland, MA). Polyvinylidene difluoride membrane by using Towbin’s buffer for transfer.

**Immunoblotting.** The proteins were transferred onto a polyvinylidene difluoride membrane by using Towbin’s buffer for transfer. After fixing and then incubation with the phosphospecific rabbit anti-P-ERK1/2 1:1000 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% Na2SO4 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.

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**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). Sigmodial dose-response curves were calculated by using nonlinear regression.

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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 \(\mu\)M 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 \( EC_{50} = 1.4 \text{ mM} \) (0.7 to 2.7 \text{ mM}). 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 \text{ mM}, 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).

### 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 analogue 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 DDT1 MF-2 \[110,000 \text{ receptors/cell} \] (Gerwins et al., 1990). Previously, it was found that the number of \( A_2 \) 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\text{ZM241385}, \text{which does bind to adenosine A}_{2B} \text{ receptors with appreciable affinity (Ji and Jacobson, 1999). The results}

<table>
<thead>
<tr>
<th>Receptor Subtype</th>
<th>( EC_{50} ) Value for ERK1/2 Phosphorylation</th>
<th>( K_D ) or ( K_I )</th>
<th>( EC_{50} ) Value for cAMP Production</th>
<th>Total Receptor Number per Cell</th>
</tr>
</thead>
<tbody>
<tr>
<td>( A_1 )</td>
<td>115.4 \text{ nM}</td>
<td>13.6 \text{ nM} (( K_D ), high affinity)</td>
<td>( 26 \text{ nM} )</td>
<td>~40,000 (see Discussion)</td>
</tr>
<tr>
<td>( A_{2A} )</td>
<td>26.4 \text{ nM}</td>
<td>29.1 \text{ nM}</td>
<td>( 26.1 \text{ nM} )</td>
<td>~20,000 (Kull et al., 1999a)</td>
</tr>
<tr>
<td>( A_{2B} )</td>
<td>19.4 \text{ nM}</td>
<td>N.D.</td>
<td>( 1.4 \text{ \mu M} )</td>
<td>&lt;200,000 (see Discussion)</td>
</tr>
<tr>
<td>( A_3 )</td>
<td>65.4 \text{ nM}</td>
<td>6.18 \text{ nM}</td>
<td>( 129 \text{ nM} )</td>
<td>Similar to ( A_1 ) (Klotz et al., 1998)</td>
</tr>
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\( \text{N.D.}, \text{not determined.} \)
Adenosine Receptor Stimulation of ERK1/2: A Comparison

Adenosine receptor stimulation of ERK1/2 phosphorylation in transfected CHO cells.

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. A1, A2A, A2B, and A3 receptor expressing cells were stimulated with 100 nM and 3 μM adenosine. The immunoblots show one representative experiment of 2 to 3 per receptor 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 A1, A2A, A2B, and A3 receptors, respectively.

suggest that there are fewer than 200,000 A2B 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 A1 and A2A 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 A3 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) A3 receptor by using cAMP generation as the assay. However, in A2B 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 inhibition adenylyl cyclase have suggested that the A1,A2A, and A3 receptors may be activated by concentrations of the endogenous agonist adenosine that occur under basal physiological conditions, whereas A2B receptors may be activated by lower, physiologically normal concentrations (30–300 nM) as the assay. However, in A2B receptor-transfected CHO cells, this may have fundamental consequences for understanding the role of adenosine A2B 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.

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acterization of stably transfected receptors in CHO cells. Naunyn-Schmiedeberg’s Arch Pharmacol 357:1–9
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