

Signaling Pathway from the Human Adenosine A₃ Receptor Expressed in Chinese Hamster Ovary Cells to the Extracellular Signal-Regulated Kinase 1/2

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Received April 8, 2002; accepted July 31, 2002

This article is available online at <http://molpharm.aspetjournals.org>

ABSTRACT

Adenosine activates four different receptors, the A₁, A_{2A}, A_{2B}, and the A₃ receptors, all of which are G protein-coupled. We have previously shown that stimulation of the human adenosine A₃ receptor can induce phosphorylation of extracellular signal-regulated kinase (ERK1/2). Here we show that the adenosine receptor agonist 5'-N-ethylcarboxamidoadenosine (NECA) induces phosphorylation and activation of ERK1/2 in Chinese hamster ovary (CHO) cells expressing the human adenosine A₃ receptor (CHO A₃ cells) with the same potency. Pretreatment with pertussis toxin abolished the effect, which also could be blunted by overexpressing the $\beta\gamma$ -sequestering peptide β -adrenergic receptor kinase-ct, implicating the involvement of $\beta\gamma$ subunits released from G_{i/o} proteins. Activation of phosphatidylinositol-3-kinase (PI3K) by adenosine A₃ receptors is inferred from a dose-dependent Ser-phosphorylation of the protein kinase B (Akt). Furthermore the ERK1/2 phosphorylation was sensitive to the PI3K inhibitors wortmannin and LY294002 (2-(4-morpholinyl)-8-

phenyl-1(4H)-benzopyran-4-one hydrochloride) and the MEK inhibitor PD98059 (2'-amino-3'-methoxyflavone), whereas chelation of Ca²⁺ with 1,2-bis(2-aminophenoxy)ethane-*N,N,N',N'*-tetraacetic acid tetrakis (acetoxymethyl ester) and long-term treatment with phorbol dibutyrate did not decrease the adenosine A₃ receptor-mediated ERK1/2 phosphorylation. Thus, Ca²⁺ mobilization and conventional and novel protein kinase C (PKC) isoforms are not involved in this pathway. The atypical PKC ζ was not activated by NECA and thus not involved in the A₃ receptor-mediated ERK1/2 phosphorylation. NECA stimulation of CHO A₃ cells activated the small G protein Ras and the dominant negative mutant RasS17N prevented the phosphorylation of ERK1/2. In conclusion, the adenosine A₃ receptor recruits a pathway that involves $\beta\gamma$ release from G_{i/o}, PI3K, Ras, and MEK to induce ERK1/2 phosphorylation and activation, whereas signaling is independent of Ca²⁺, PKC, and c-Src.

The adenosine A₃ receptor is the adenosine receptor discovered most recently (Zhou et al., 1992; Salvatore et al., 1993), and its physiological role is poorly understood. Although it has been stated that the adenosine A₃ receptor is a "low-affinity" receptor, adenosine can be shown to activate adenosine A₃ receptors in concentrations occurring physiologically (30–300 nM) (Schulte and Fredholm, 2000; Fredholm et al., 2001). Adenosine A₃ receptors are known to

couple to the G proteins G_{i1-3} and G_{q11} (Palmer et al., 1995), thereby inhibiting adenylyl cyclase and activating phospholipase C β (PLC β). The A₃ receptor has been implicated in cell cycle progression and cell growth (Brambilla et al., 2000), modulation of apoptosis (Abbracchio et al., 1997), mast cell degranulation (Jin et al., 1997), ischemic preconditioning in the heart (Strickler et al., 1996), neuroprotection (Fredholm, 1997), and pro- and anti-inflammatory modulation (Salvatore et al., 2000). The family of mitogen-activated protein kinases (MAPK) may play an important role in such phenomena, as well as in proliferation, differentiation, and cell death.

The MAPK family consists of three main subgroups—the

Supported by the Swedish Medical Research Council (2553), the European Commission (EURCAR), and Karolinska Institutet.

Parts of the results were presented in abstract form at the 51st Mosbacher Kolloquium, Gesellschaft für Biochemie und Molekularbiologie (GBM), Mosbach, Germany, April 2–5, 2000.

ABBREVIATIONS: PLC β , phospholipase C β ; MAPK, mitogen-activated protein kinase; ERK1/2, extracellular signal-regulated kinase 1/2; MEK, MAP/ERK kinase; NECA, 5'-N-ethylcarboxamidoadenosine; PTX, pertussis toxin; PI3K, phosphatidylinositol-3-kinase; PDBu, phorbol 12,13 dibutyrate; PD98059, 2'-amino-3'-methoxyflavone; BAPTA-AM, 1,2-bis(2-Aminophenoxy)ethane-*N,N,N',N'*-tetraacetic acid tetrakis (acetoxymethyl ester); LY294002, 2-(4-morpholinyl)-8-phenyl-1(4H)-benzopyran-4-one hydrochloride; PP2, 4-amino-5-(4-chlorophenyl)-7-(*t*-butyl)pyrazolo[3,4-*d*]pyrimidine; PP3, 4-amino-7-phenylpyrazolo[3,4-*d*]pyrimidine; Ro 31-8220, 3-[1-[3-(amidinothio)propyl]-1H-indol-3-yl]-3-(1-methyl-1H-indol-3-yl)maleimide (Bisindolylmaleimide IX), methanesulfonate; CREB, cAMP response element binding protein; CHO A₃ cells, Chinese hamster ovary cells expressing the human adenosine A₃ receptor; FCS, fetal calf serum; PKB/Akt, protein kinase B; bFGF, basic fibroblast growth factor; CI, confidence interval; PKC, Ca²⁺-dependent protein kinase; aPKC, atypical PKC; MBP, myelin basic protein; GPCR, G protein-coupled receptor.

extracellular signal-regulated kinases ERK1/2, the c-jun N terminal kinases, and the stress-activated protein kinase p38—as well as several distantly related MAPKs, for example ERK5 or BMK, ERK7, and MOK (Miyata and Nishida, 1999). MAPKs are activated via the classic MAPK cascade pathway downstream of receptor tyrosine kinases, such as the epidermal growth factor receptor, which couples via an array of adapter molecules to small Ras-like GTPases. This in turn leads to the activation of the MAPK kinase Raf that phosphorylates and activates MAP/ERK kinase (MEK), the upstream kinase of ERK1/2 (Seger and Krebs, 1995). We have recently shown that 5'-N-ethylcarboxamidoadenosine (NECA) stimulation of Chinese hamster ovary cells expressing the human adenosine A₃ receptor (referred to hereafter as CHO A₃ cells) induces ERK1/2 phosphorylation in a time- and dose-dependent manner (Schulte and Fredholm, 2000). This was recently confirmed (Graham et al., 2001). The precise pathway leading from receptor activation to ERK activation is incompletely understood. However, G_{i/o}-coupled G protein-coupled receptors (GPCRs) other than the adenosine A₃ receptor have been shown to be able to use several different transduction pathways to activate MAPKs such as the ERK1/2 (Gutkind, 1998; Marinissen and Gutkind, 2001).

The aim of this study was to delineate the events downstream of human adenosine A₃ receptor stimulation in CHO A₃ cells, leading to ERK1/2 activation. This was investigated by specifically blocking key events in G protein signaling with pharmacologically well-described tools and by overexpression of dominant-negative mutants of crucial signaling intermediates. Our results describe the signaling pathway from the human adenosine A₃ receptor to ERK1/2 being dependent on $\beta\gamma$ -subunits released from pertussis toxin (PTX)-sensitive G_{i/o} proteins, phosphatidylinositol-3-kinase (PI3K), the small GTP binding protein Ras, and MEK.

Materials and Methods

Materials. All cell culture media, fetal calf serum, and supplies were from Invitrogen (Täby, Sweden). NECA was from Sigma/RBI (Natick, MA). Chelerythrine, wortmannin, phorbol 12,13-dibutyrate (PDBu), probenecid, bovine serum albumin, phosphatidylserine, thrombin, and protein A Sepharose were from Sigma. PKC ϵ peptide substrate was from BioSource International (Camarillo, CA). [³²P]ATP was from ICN Biomedicals (Asse-Relegem, Belgium). Polyvinylidene difluoride-Immobilon P membrane was from Millipore Corp. (Bedford, MA). Rabbit anti-ERK1/2 (for immunoblotting), phosphospecific rabbit anti-phosphoThr202/Tyr204-ERK1/2, rabbit anti-phosphoSer217/221-MEK1/2, rabbit anti-phosphoSer473-PKB/Akt, and PD98059 were from New England Biolabs, Inc. (Beverly, MA). LY294002 was from Tocris Cookson Inc. (Ballwin, MO). BAPTA-AM and Fura-2 AM were from Molecular Probes (Leiden, Netherlands). PP2 (AG 1879; 4-amino-5-(4-chlorophenyl)-7-(*t*-butyl)pyrazolo[3,4]pyrimidine), PP3, (4-amino-7-phenylpyrazolo[3,4-d]pyrimidine), and Ro 31-8220 were from Calbiochem-Novabiochem Corporation (San Diego, CA). Mouse monoclonal anti-pan Ras antibody was from Oncogene Research Products (Cambridge, MA). Dr. P. Gerwins (Rudbeck Laboratory, Uppsala, Sweden) kindly provided anti-ERK1/2 antibody (for immunoprecipitation). Mouse anti-phospho-myelin basic protein, rabbit anti-phospho-Y416-Src, rabbit anti-phospho-cAMP response element binding protein (CREB) and rabbit anti-PKC ζ were from Upstate Biotechnology, Inc. (Lake Placid, NY). Goat anti-rabbit and goat anti-mouse horseradish peroxidase-coupled antibodies were from Pierce (Rockford, IL). Enhanced chemiluminescence detection (ECL) kit and glutathione-Sepharose 4B

were from Amersham Biosciences (Little Chalfont, Buckinghamshire, UK). The transfection reagent Fugene was from Roche Applied Science (Mannheim, Germany). Mouse polyclonal anti-AU5 antibody (raw ascites fluid) was from BabCO (Berkeley, CA). Plasmids were generous gifts: mammalian expression vectors for HA-ERK1 and β ARK-ct were from M. Freissmuth (Universität Wien, Vienna, Austria) and pGEX2T-GST-Raf-RBD was from A. Wittinghofer (Max-Planck-Institut für Molekulare Physiologie, Dortmund, Germany). RasS17N was from J. Toppmaier and U. Rapp (Institut für Medizinische Strahlenkunde und Zellforschung, Würzburg, Germany). The mammalian expression vector for AU5-PKB was from W. F. Simonds (National Institute of Diabetes and Digestive and Kidney Diseases, Bethesda, MD).

Cell Culture. CHO cells transfected with the human adenosine A₃ receptor (Klotz et al., 1998) were grown adherent at 37°C, 5% CO₂/95% air in Dulbecco's modified Eagle's medium/Ham's F-12 medium (1:1), 0.2 mg/ml G-418 (Geneticin), 50 U/ml penicillin, 50 μ g/ml streptomycin, 2 mM L-glutamine, 10% fetal calf serum (FCS). All cells were split three times a week at a ratio of 1:20.

Protein Phosphorylation and Immunoblotting. ERK1/2, MEK 1/2, protein kinase B (PKB/Akt), and c-Src phosphorylation were examined as described previously (Schulte and Fredholm, 2000). Briefly, cells were serum deprived overnight (0.5% FCS, v/v) and stimulated at 37°C [NECA and basic fibroblast growth factor (bFGF) for 5 min, other drugs for 20 min before NECA stimulation]. After two washes in ice-cold phosphate-buffered saline, cells were lysed in lysis buffer (70 mM β -glycerophosphate, 0.5% Triton X-100, 2 mM MgCl₂, 1 mM dithiothreitol, 1 mM NaF, 1 mM Na₃VO₄, 20 μ g/ml aprotinin, 5 μ g/ml leupeptin) and cellular debris was removed by centrifugation. Samples were denatured with Laemmli buffer and analyzed by PAGE. After transfer onto polyvinylidene difluoride membranes, protein phosphorylation was detected with rabbit phosphospecific ERK1/2, phospho-specific MEK 1/2, phospho-specific PKB/Akt, or phosphospecific c-Src antibodies, goat anti-rabbit horseradish peroxidase-coupled secondary antibody, and the enhanced chemiluminescence detection method. To confirm equal loading in each lane, parallel immunoblots were run to detect the unphosphorylated ERK1/2. Equal loading was confirmed running parallel immunoblots using the ERK1/2 antibody (not shown).

ERK 1/2 Activity. After clearance by centrifugation, cell lysates were immunoprecipitated with anti-ERK 1/2 antibody and protein A Sepharose, washed two times with lysis buffer and once with kinase buffer (20 mM HEPES, 20 mM MgCl₂, 2 mM MnCl₂, and 0.2 mM dithiothreitol). Immunoprecipitates were then incubated in 40 μ l of assay buffer (kinase buffer, 10 μ g MBP and 0.1 mM ATP) at room temperature for 15 min. Denaturing with Laemmli buffer and boiling for 5 min stopped the reaction. The protein phosphorylation was analyzed by immunoblotting using a mouse anti-phospho MBP antibody and a horseradish goat anti-mouse peroxidase-coupled secondary antibody, as described above.

Calcium Imaging. CHO A₃ cells were grown on coverslips overnight and loaded with Fura-2 AM (1 μ M) in 145 mM NaCl, 5 mM KCl, 1.3 mM MgCl₂, 1.2 mM CaCl₂, 1.2 mM NaH₂PO₄, 10 mM glucose, 20 mM HEPES, 0.1% bovine serum albumin, and 1 mM probenecid at 37°C for 30 min. Excitation wavelengths were 340 and 380 nm and the images were collected at 510 nm with an intensified charge-coupled device camera and a Nikon Axiovert 35 microscope. Fluorescence intensities in images were analyzed using the MCID M4 (Imaging Research, Ontario, Canada) image analysis software. Four to ten cells were counted in each experiment.

PKC ζ Activity Assay. CHO A₃ cells were seeded out in 10-cm plastic dishes, grown overnight, serum deprived for 18 h, stimulated as described above, and lysed in lysis buffer (20 mM Tris, HCl, pH 7.5, 0.25 M sucrose, 1.2 mM EGTA, 20 mM β -mercaptoethanol, 5 μ g/ml leupeptin, 5 μ g/ml aprotinin, 1 mM Na₃VO₄, 1 mM Na₄P₂O₇, 1 mM NaF, 1% Triton X-100, and 0.5% Nonidet P-40). Cellular lysates were cleared by centrifugation and the protein concentration of the supernatant was determined. About 1 mg of protein was used

for immunoprecipitation with 1 μ l/100 μ g protein of rabbit anti-PKC ζ and protein A/G Sepharose beads. Samples were rotated overnight at 4°C, washed three times in lysis buffer and once in 50 mM Tris/HCl, pH 7.5, 1 mM NaHCO₃, and 5 mM MgCl₂. For the kinase reaction, kinase buffer (125 mM Tris/HCl, pH 7.5, 1.25 mM NaHCO₃, 6.25 mM MgCl₂, 100 μ M CaCl₂, 100 μ M NaF, 10 mM Na₃VO₄, 10 mM Na₄P₂O₇, 50 μ M phosphatidyl serine, and PKC ϵ -peptide substrate) was added and the reaction was started by addition of ATP (final concentration, 625 nM) and [³²P]ATP (5 μ Ci/sample). Addition of 20 μ l of 1% phosphoric acid stopped the reaction after 15 min at 30°C; 30 μ l of the kinase assay was dropped onto a phosphocellulose filter, washed intensively in 1% phosphoric acid, and counted in a Wallac 1209 Rackbeta scintillation counter (PerkinElmer Wallac, Gaithersburg, MD).

Transfection of CHO Cells. Two hundred thousand CHO A₃ cells were grown overnight in six-well plates and transfected with 0.5 μ g HA-ERK1/2 and 0.5 μ g AU5-PKB in combination with either 0.05 μ g RasS17N, 0.25 μ g β ARK-ct, or respective amounts of empty vector using the Fugene transfection kit according to the manufacturer's instructions. Transfected cells were split the next day into 3.5-cm Petri dishes and serum-starved (0.5% FCS, v/v) overnight. Stimulation and lysis as described above.

Ras-Activity Assay. The GST-fused Ras-binding domain of Raf (GST-Ras RBD) was expressed in *Escherichia coli* and a crude bacterial lysate was prepared in phosphate-buffered saline, 0.5 M dithiothreitol, 20 μ g/ml aprotinin, 5 μ g/ml leupeptin, and 1 mM Na₃VO₃. For the pull-down assay, the crude bacterial lysate was added to the lysis buffer and CHO A₃ cells were lysed in the presence of the minimal Ras-binding domain of Raf and incubated rotating at 4°C for 1 h. The cell lysate was then cleared by centrifugation and the supernatant was incubated rotating at 4°C for 1 h with glutathione-Sepharose 4B beads. The precipitate was washed in lysis buffer, denatured in Laemmli buffer, and analyzed by immunoblotting using a mouse monoclonal anti-pan Ras and a goat anti-mouse horseradish peroxidase coupled secondary antibody.

Data Analysis and Statistics. Quantification of immunoblots was done by densitometry using the Scion image software (Scion, Frederick, MD). We used Prism2 software (GraphPad, San Diego, CA) for nonlinear regression for the dose-response curves (fixed Hill slope = 1).

Results

The effects of the unselective adenosine receptor agonist NECA in CHO A₃ cells on the phosphorylation of diverse proteins were exclusively mediated by the recombinant adenosine A₃ receptor, as described previously for ERK1/2 phosphorylation (Schulte and Fredholm, 2000). Untransfected CHO cells did not respond to NECA (up to 10 μ M) when measuring cAMP (increase or decrease), ERK1/2 (Schulte and Fredholm, 2000), PKB or CREB phosphorylation, even if they respond to 2 U/ml thrombin (Fig. 1). Thus, we feel justified using NECA instead of other more selective adenosine A₃ receptor agonists because the participation of endogenously expressed adenosine A_{2B} receptors could effectively be excluded.

The high potency and efficacy of NECA to induce ERK1/2 phosphorylation in CHO A₃ cells, as reported earlier (Schulte and Fredholm, 2000), suggested, but did not prove, an increase in the enzymatic activity of ERK1/2 as well. Here, a nonradioactive kinase activity assay used in parallel with the detection of ERK1/2 phosphorylation indeed showed a similar concentration-response relationship (Fig. 1). The EC₅₀ of the ERK1/2 activity was similar [EC₅₀ = 28.25 nM (95% CI, 7.467 to 106.9)] to the one we described earlier for the

ERK1/2 phosphorylation [EC₅₀ = 65.4 nM (95% CI, 35.96 to 119.1)] (Schulte and Fredholm, 2000).

Involvement of $\beta\gamma$ Subunits Released from Pertussis Toxin-Sensitive G Protein, but No Involvement of Calcium or Protein Kinase C. Despite the fact that A₃ receptors have been reported to couple to G_{q/11} in stably transfected CHO cells (Palmer et al., 1995), overnight treatment with PTX (200 ng/ml) abolished the NECA-induced increase in ERK1/2 phosphorylation (results not shown) as shown

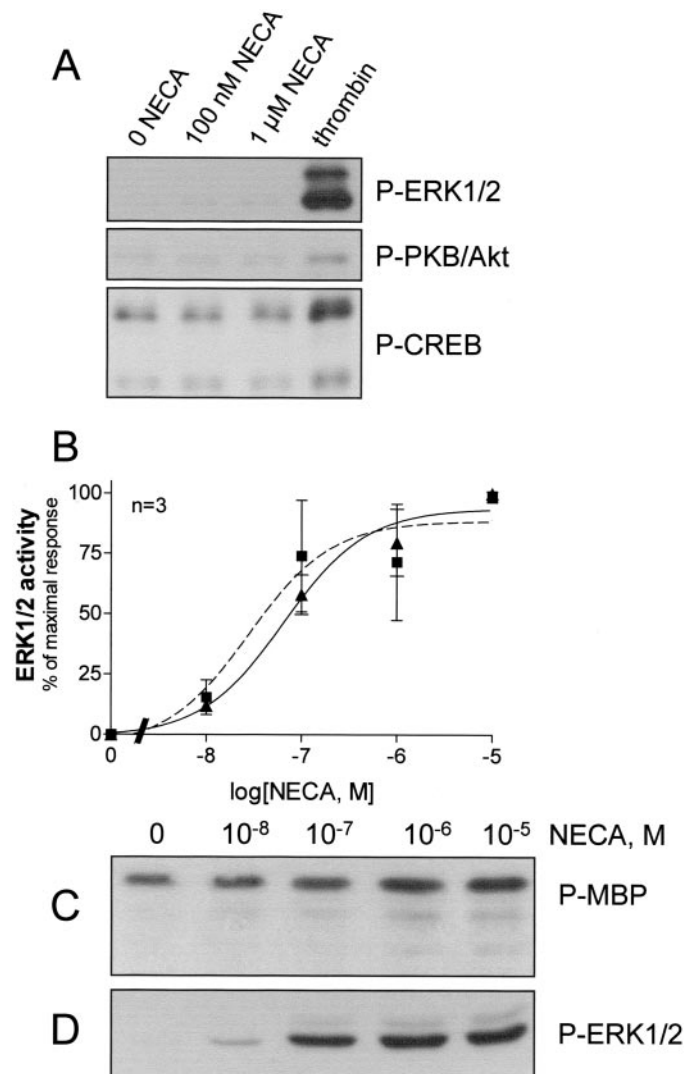


Fig. 1. A, NECA has no effects in untransfected CHO cells. CHO cells were stimulated with NECA or thrombin (2 U/ml) for 5 min and the cells lysates were analyzed for ERK1/2, PKB/Akt, and CREB phosphorylation. This experiment was repeated twice (P-CREB, P-PKB) or more than five times (P-ERK1/2). Quantification of phospho-CREB in the experiment shown gave a value of 5.2 (arbitrary units) in lane 1, 4.3 in lane 2 (100 nM NECA), 5.2 in lane 3 (1 μ M NECA), and 16.8 in lane 4 (thrombin). B, activation by NECA of the human adenosine A₃ receptor expressed in CHO cells induced a concentration-dependent ERK1/2 phosphorylation and an increase in the enzymatic activity. Enzymatic activity was measured by the detection of phosphorylated substrate (MBP) with a phosphospecific anti MBP antibody after an in vitro kinase assay. Phospho-MBP in unstimulated samples results from basal phosphorylation of the MBP preparation. ERK1/2-phosphorylation was determined using a P-ERK1/2 antibody. For further details, see text. The graph in B summarizes three independent experiments (P-MBP, \blacktriangle —; P-ERK1/2, \blacksquare —). Error bars show S.D. C, results from one representative experiment in the kinase assay. D, a representative Western blot using the phospho-ERK1/2 antibody.

previously by Graham et al. (2001). This provides evidence that the relevant G protein in these cells is $G_{i/o}$. Furthermore, with overexpression of the $\beta\gamma$ -sequestering peptide β ARK-ct together with a HA-tagged ERK1, it was possible to blunt the NECA-induced HA-ERK1 phosphorylation (Fig. 2), strongly indicating the involvement of $\beta\gamma$. The release of $\beta\gamma$ -subunits from activated $G_{i/o}$ proteins by adenosine receptor agonists is known to activate PLC β and to induce a rise in intracellular free Ca^{2+} ($[Ca^{2+}]_i$) (Ali et al., 1990). Indeed, this was confirmed in the CHO A_3 cells using calcium-imaging experiments. Overnight pretreatment with pertussis toxin blocked the NECA-induced changes in $[Ca^{2+}]_i$ completely (Fig. 3A, insert). Furthermore, loading of CHO A_3 cells with BAPTA-AM, a chelator of $[Ca^{2+}]_i$, abolished both the NECA- and the UTP-induced mobilization of $[Ca^{2+}]_i$ (Fig. 3A). Whereas 10 μ M of BAPTA-AM was sufficient to completely suppress the UTP- and NECA-induced changes in $[Ca^{2+}]_i$, this concentration of the Ca^{2+} -chelator had no effect on the NECA-induced ERK1/2 phosphorylation (Fig. 3B).

In analogy with adenosine A_1 receptors expressed in CHO cells, it is likely that the A_3 receptor also activates PKC (Dickenson and Hill, 1997). Although this has not been directly shown, we decided to examine the possible involvement of PKC in mediating ERK1/2 phosphorylation. Pretreatment of CHO A_3 cells with 100 nM PDBu overnight, to

down-regulate protein kinase C (Chen, 1993), did not reduce the NECA-induced ERK1/2 phosphorylation (Fig. 3C). The down-regulation of PKC by phorbol esters, which affect only PKC isoforms (Chen, 1993) that contain the diacylglycerol/phorbol ester-binding domain C1 consisting of two Zn^{2+} finger motifs, such as the conventional and novel PKC isoforms (Mellor and Parker, 1998), did not decrease the NECA-induced ERK1/2 phosphorylation. This, in connection with the insensitivity to the $[Ca^{2+}]_i$ chelation, excludes the involvement of Ca^{2+} and diacylglycerol/phorbol ester-sensitive PKC isoforms such as those of the conventional and novel PKC groups. Phorbol esters do not affect PKC isoforms of the group of atypical PKC (aPKC), which do not contain the C1 domain.

The broad PKC inhibitor chelerythrine decreased A_3 receptor-mediated ERK1/2 phosphorylation in a dose-dependent manner (Schulte and Fredholm, 2002), but chelerythrine is a potent antagonist at adenosine A_1 and A_{2A} and probably also at adenosine A_3 receptors (Schulte and Fredholm, 2002). Compet-

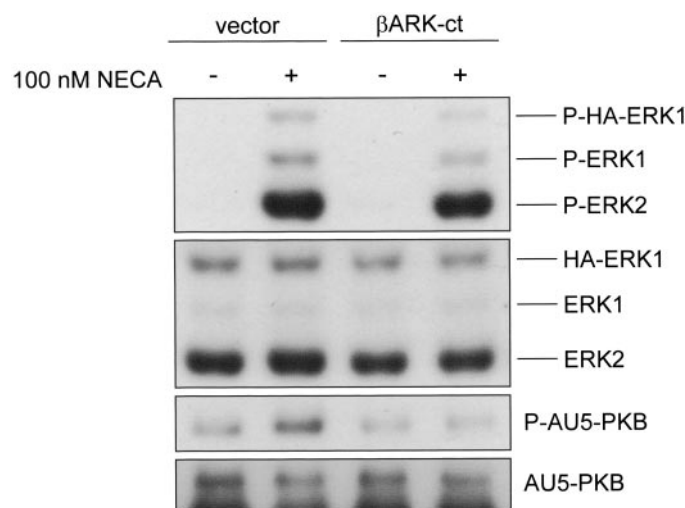


Fig. 2. Involvement of $\beta\gamma$ subunits in adenosine A_3 receptor-mediated phosphorylation of ERK1/2. CHO A_3 cells were cotransfected with a hemagglutinin-tagged HA-ERK 1, an AU5-tagged AU5-PKB, and either the $\beta\gamma$ subunit-sequestering peptide β ARK-ct or a corresponding empty vector. Cells were then stimulated with 100 nM NECA for 5 min. The AU5-PKB was immunoprecipitated and both the supernatant and the precipitate were analyzed by immunoblotting for P-ERK1/2 and P-AU5-PKB, respectively. The immunoblot shows the endogenously expressed ERK 1, ERK 2 and the transfected HA-ERK 1 in the supernatant, whereas only the transfected AU5-PKB is visible in the immunoprecipitate. To confirm equal expression levels of the tagged reporter constructs, the total ERK1/2 or AU5 in the supernatant or the immunoprecipitate, respectively, is also shown. The experiment was repeated once. Expression of β -ARK-ct reduced the NECA-induced HA-ERK1 phosphorylation to $54 \pm 10\%$ compared with vector-transfected cells (protein phosphorylation—P-HA-ERK1/2 and P-AU5-PKB—in vector-transfected cells before and after NECA stimulation was set to 0 and 100%, respectively; values give mean \pm S.E.M.). NECA-induced changes in phosphorylation of endogenously expressed ERK1/2 were only minor because of low transfection efficiency. Basal AU5-PKB phosphorylation was reduced to $-87 \pm 41\%$ in unstimulated β -ARK-ct transfected cells, whereas it was $-76 \pm 12\%$ after NECA stimulation (i.e., basal AU5-PKB phosphorylation was slightly reduced and the NECA-induced increase in P-AU5-PKB was blocked).

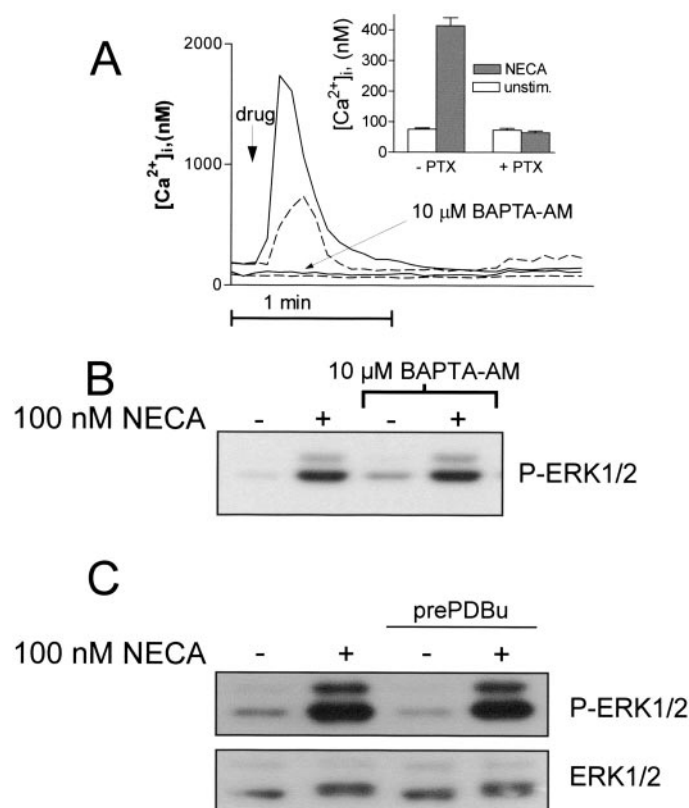


Fig. 3. Lack of involvement of calcium and phorbol ester-sensitive protein kinase C isoforms in the NECA-induced ERK1/2 phosphorylation in CHO A_3 cells. A, the adenosine analog NECA (10 μ M, —) and the nucleotide UTP (100 μ M, —) induced a strong increase in $[Ca^{2+}]_i$, which can be completely abolished by 20 min of pretreatment with the intracellular Ca^{2+} chelator BAPTA-AM. $[Ca^{2+}]_i$ was examined in Fura-2 AM-loaded cells by determining the ratio between intensity levels of fluorescence of Fura-2 monitored at 340 nm in the Ca^{2+} free and at 380 nm in the Ca^{2+} bound form. The data show a single cell that was representative of the NECA/UTP-responsive cell population during three separate experiments. Without BAPTA-AM, $\sim 30\%$ of the CHO A_3 cells responded to NECA, whereas after 20 min of treatment with 10 μ M BAPTA-AM, none of the cells showed an increase in $[Ca^{2+}]_i$. The bar graph (inset) shows the effect of overnight PTX treatment on the NECA-induced increase in $[Ca^{2+}]_i$ in CHO A_3 cells. B, the immunoblot shows the lack of effect of 10 μ M BAPTA-AM on the NECA-induced ERK1/2 phosphorylation in CHO A_3 cells ($n = 3$). C, the NECA effect on ERK1/2 phosphorylation is unaffected by overnight treatment with the phorbol ester PDBu.

itive radioligand binding experiments using [³H]NECA and CHO A₃ cell membranes revealed a K_D for chelerythrine at the human adenosine A₃ receptor near 10 μM (K.-N. Klotz, personal communication), which mirrors the effective concentration in inhibiting ERK1/2 phosphorylation by ~50% in CHO A₃ cells as reported previously (Schulte and Fredholm, 2002). Therefore, we interpret the reduction of adenosine A₃ receptor-mediated ERK1/2 phosphorylation with chelerythrine not mainly as an indication of a role of PKC but rather as an antagonistic effect at the receptor. Furthermore, the bisindolylmaleimide Ro 31-8220 did not affect NECA-induced ERK1/2 phosphorylation at concentrations <5 μM, whereas Ro 31-8220 at 5 μM abrogated the NECA-induced ERK1/2 phosphorylation (Fig. 4). However, despite a negligible affinity of Ro 31-8220 to the adenosine A₁ receptor (G. Schulte and B. B. Fredholm, unpublished observations), this substance has also been shown to suffer from a lack of specificity [i.e., it affects other non-PKC kinases (Alessi, 1997; Davies et al., 2000; Han et al., 2000)]. In CHO cells, we could detect the aPKCζ (Fig. 4B); however, using a radioactive kinase assay, we could not detect an increase in aPKCζ activity upon NECA (100 nM, 1 μM) stimulation (Fig. 4).

PI3K Is Activated by Adenosine A₃ Receptors and Is Necessary for ERK1/2 Phosphorylation. Many G_{i/o} coupled receptors are known to activate ERK1/2 in a PI3K-dependent manner (Gutkind, 1998; Marinissen and Gutkind, 2001). We first confirmed results from a recent report (Graham et al., 2001) that two inhibitors of the PI3K, wortmannin (10, 30, 100, 300 nM) and LY294002 (30, 100 μM), efficiently block the NECA-induced ERK1/2 phosphorylation in CHO A₃ cells (Fig. 5A). Thus, the adenosine A₃ receptor-mediated ERK1/2 phosphorylation is either dependent on a direct activation of PI3K or required a basal activity of PI3K. To provide additional support for the first possibility, we investigated the changes in PKB/Akt phosphorylation after NECA

treatment. PKB/Akt is a well-described downstream target of PI3K activity that was expected to be phosphorylated at serine 473 upon an increase in PI3K activity (Downward, 1998). Indeed, NECA stimulation for 5 min induced a dose-dependent increase in PKB/Akt phosphorylation with an EC₅₀ = 61 nM (95% CI, 31 to 117) (Fig. 5B) and this effect was also sensitive to the PI3K inhibitor wortmannin (Fig. 5A).

As mentioned above, the adenosine A₃ receptor-mediated HA-ERK1 phosphorylation could be blunted by overexpression of the βγ-sequestering peptide βARK-ct. The same was true for the adenosine A₃ receptor-mediated phosphorylation of AU5-PKB (Fig. 2).

Adenosine A₃ Receptors Do Not Activate Src, but Do Activate Ras. The cytoplasmic tyrosine kinase c-Src has been described as an important link between PI3K and ERK1/2 (Gutkind, 1998; Marinissen and Gutkind, 2001). Therefore, we investigated the involvement of c-Src using the

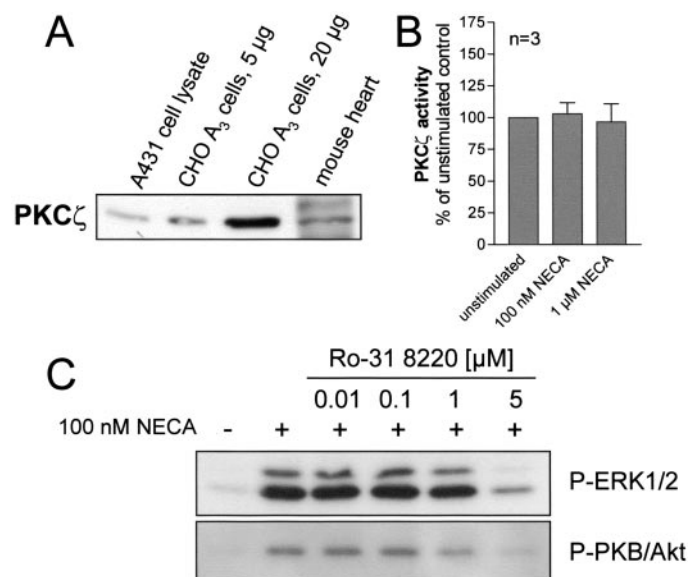


Fig. 4. The role of PKCζ in adenosine A₃ receptor signaling. Despite the fact that PKCζ is present in CHO A₃ cells, in A431 cells—the positive control supplied by the antibody producer—as well as in mouse heart lysate (A), we were not able to detect PKCζ activation in CHO A₃ cells upon NECA treatment (B), using a PKCζ activity assay as described under *Materials and Methods* ($n = 3$ in triplicates or quadruplicates). The effect of the bisindolylmaleimide Ro 31-8220, a PKC inhibitor, on ERK1/2 and PKB/Akt phosphorylation is shown in C ($n = 3$).

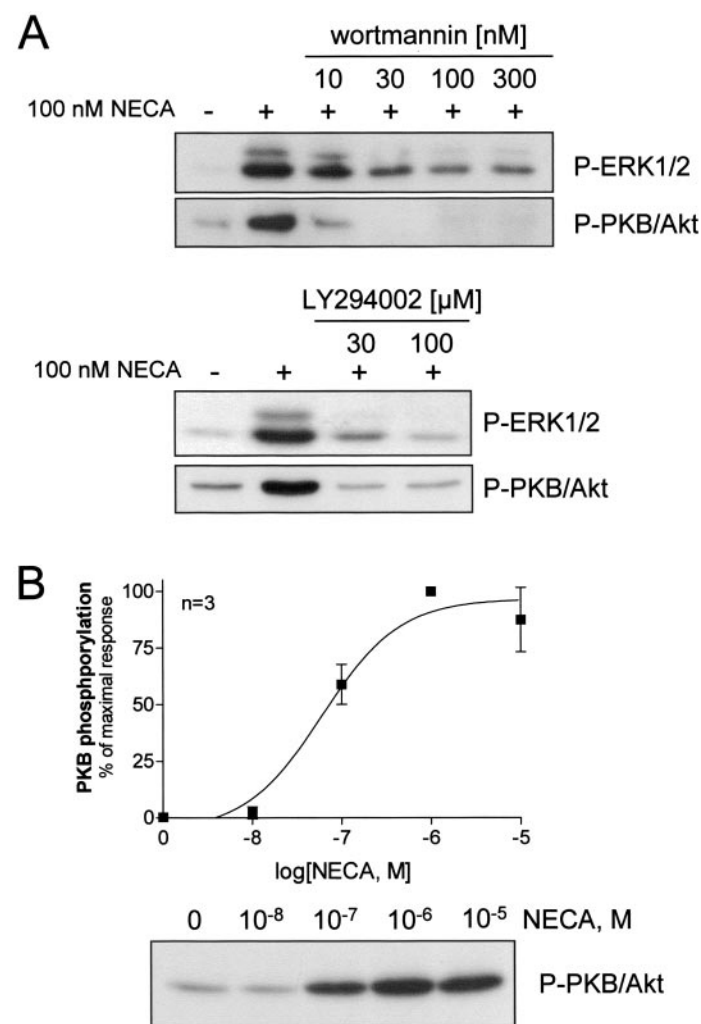


Fig. 5. Role of PI3K in adenosine A₃ receptor signaling. The phosphorylation of ERK1/2 and PKB/Akt by adenosine A₃ receptor activation is blocked by the PI3K inhibitors wortmannin and LY294002 (A). CHO A₃ cells were pretreated with wortmannin/LY294002 for 20 min before NECA stimulation and protein phosphorylation was examined ($n = 3$). B, dose-dependent increase in PKB/Akt phosphorylation at serine 473 [EC₅₀ = 61 nM (95% CI, 31 to 117)] after 5 min of stimulation with NECA in CHO A₃ cells. The graph summarizes three independent experiments (error bars give S.D.) from which one immunoblot is shown.

c-Src-family specific tyrphostin PP2 and an anti-phospho c-Src antibody. PP2 did not affect the adenosine A₃-receptor-mediated effects on ERK1/2 and PKB/Akt serine phosphorylation (Fig. 6A) any more than the inactive homolog PP3 did (Fig. 6B). Both substances affected ERK1/2 and PKB/Akt phosphorylation at higher micromolar concentrations (1 and 5 μ M PP2 or PP3). Phosphorylation of c-Src at tyrosine residue Y416, which is an autophosphorylation site in the kinase activation loop (Thomas and Brugge, 1997), is closely linked to c-Src kinase activation. In CHO A₃ cells, bFGF (5 ng/ml for 5 min) but not NECA stimulation increased Y416 phosphorylation, as detected by immunoblotting using an anti-phospho Y416 c-Src antibody (Fig. 6C). However, even high concentrations of NECA (up to 10 μ M, data not shown) failed to change the phosphorylation state of Y416 c-Src.

The PI3K class I_A and I_B subtypes contain a Ras binding domain, but the role of this binding domain is still obscure (Vanhaesebroeck and Waterfield, 1999). The involvement of the small GTPase Ras in the NECA-induced ERK1/2 phosphorylation could be confirmed by expressing the dominant negative mutant RasS17N in CHO A₃ cells, which eliminated the effects of NECA on ERK1/2 phosphorylation (Fig. 7). In addition, a pull-down assay using the minimal Ras-binding domain of Raf fused to a GST-flag, showed clearly that NECA stimulation led to an increase in the GTP bound form of Ras (Fig. 7). Ras activation could be inhibited by the PI3K inhibitors wortmannin and LY294002 (Fig. 7), indicating that Ras is activated downstream of PI3K. Furthermore, when co-transfecting CHO A₃ cells with AU5-PKB, HA-ERK1, and RasS17N, only NECA-induced phosphorylation of HA-ERK1 was reduced, not AU5-PKB phosphorylation (Fig. 7), confirming the position of PI3K upstream of Ras.

Adenosine A₃ Receptor Stimulation Activates MEK. Ras prototypically activates the MAPK kinase kinase Raf-1, which in turn phosphorylates and activates the MAPK kinase MEK (Marinissen and Gutkind, 2001). Indeed, MEK was dose-dependently phosphorylated upon NECA stimulation with a potency comparable with that of NECA-induced ERK1/2 phosphorylation [EC_{50} = 46.14 nM (95% CI, 34.22 to 62.20); Fig. 8A]. This has the importance that in this particular phosphorylation cascade, there is no major amplification (because if that had been the case, the EC_{50} for MEK phosphorylation should have been higher than that for ERK 1/2 phosphorylation). Furthermore, the MEK inhibitor PD98059 (1–50 μ M) completely inhibited the NECA-mediated ERK1/2 phosphorylation, confirming the involvement of MEK in ERK1/2 phosphorylation (Fig. 8B).

Discussion

Previously, we have described the pharmacological profile of the adenosine A₃ receptor-mediated ERK1/2 phosphorylation (Schulte and Fredholm, 2000). Here we demonstrate what was previously only surmised, that the enzymatic activity of ERK1/2 also increased after NECA stimulation of CHO A₃ cells in a dose-dependent manner. Furthermore, the adenosine A₃ receptor agonist increased enzyme activity and phosphorylation state with a similar potency. Therefore, we could in the remaining studies rely on an assay for ERK1/2 phosphorylation and draw conclusions about ERK1/2 activation.

The major part of our study was designed to clarify the

intermediate steps between receptor activation and ERK1/2 activation. A schematic description is provided in Fig. 9. As expected, adenosine A₃ receptor-mediated ERK1/2 phosphorylation required PTX-sensitive G proteins (Graham et al., 2001). Such an involvement is also required for adenosine A₃ receptor-mediated inhibition of adenylyl cyclase and activation of calcium mobilization (Fredholm, Berts unpublished data). Furthermore, we show that the release of $\beta\gamma$ -subunits is of importance by using the $\beta\gamma$ -sequestering peptide β ARK-ct, which inhibited both the NECA-induced ERK1/2 and PKB phosphorylation.

It is generally believed that $\beta\gamma$ subunits released from G_{i/o} proteins can increase PLC β activity, resulting in a mobilization of intracellular Ca²⁺. This was confirmed by the finding that NECA-induced changes in IP₃ increases were PTX-sensitive, as shown previously in HEK293 cells expressing the human adenosine A₃ receptor (Linden et al., 1999). However, our results exclude the involvement of Ca²⁺ in the activation of ERK1/2, because elimination of the NECA-induced Ca²⁺ transient by mean of Ca²⁺ chelation with BAPTA-AM had no effect on ERK 1/2 phosphorylation. Activation of G_{i/o} proteins and PLC β can also activate PKC. We therefore investigated the role of PKC in the NECA-induced ERK1/2 phosphorylation. As described under *Results*, our study, using different ways to inhibit PKC, tends to exclude PKCs of either the classic, novel, or atypical isoforms. The atypical PKC ζ , which is present in CHO cells and was shown to have an important role in GPCR signaling to MAPK (Cussac et al., 1999; Takeda et al., 1999), is not activated by adenosine A₃ receptors in CHO cells. The broad PKC inhibitor chelerythrine, an isoflavone derivative, reduced adenosine A₃ receptor-mediated ERK1/2 phosphorylation but has been shown to be a rather potent antagonist at adenosine A₁ and A_{2A} receptors (Schulte and Fredholm, 2002) and even at A₃ receptors (K_D = 10 μ M, not shown). To dissect the role of PKC ζ , we also used the bisindolylmaleimide Ro 31-8220. This compound, however, did not affect the adenosine A₃ receptor-mediated ERK1/2 phosphorylation at concentrations \leq 1 μ M, despite the fact that the reported IC_{50} (in vitro) for aPKC is in the nanomolar range (Wilkinson et al., 1993). Inhibitory effects did appear with Ro 31-8220 \geq 5 μ M. However, several previous reports show that at such concentrations, it inhibits many different kinases (Alessi, 1997; Davies et al., 2000; Han et al., 2000). The most parsimonious explanation for the effects of Ro 31-8220 on the adenosine receptor-mediated phosphorylation of ERK1/2 and PKB, therefore is that it is unrelated to inhibition of a PKC subform. Given that we could not detect any NECA stimulation in the PKC ζ kinase activity assay, we consider an involvement of aPKC ζ in this signaling pathway highly unlikely.

By contrast, activation of PI3K by $\beta\gamma$ -subunits released from activated G_{i/o} proteins (Lopez-Illasaca et al., 1997) was apparently involved in the NECA-induced ERK1/2 phosphorylation. Graham et al. (2001) recently described inhibition of adenosine A₃ receptor-mediated ERK1/2 phosphorylation in CHO A₃ cells by wortmannin. In addition, Gao et al. (2001) reported that adenosine A₃ receptors activate the PI3K pathway and trigger the phosphorylation of PKB/Akt in rat basophilic leukemia cells. We show that this occurs also in CHO cells transfected with human adenosine A₃ receptors. The increase in PKB/Akt phosphorylation indicated that NECA led to an increase in PI3K activity in CHO A₃ cells and that

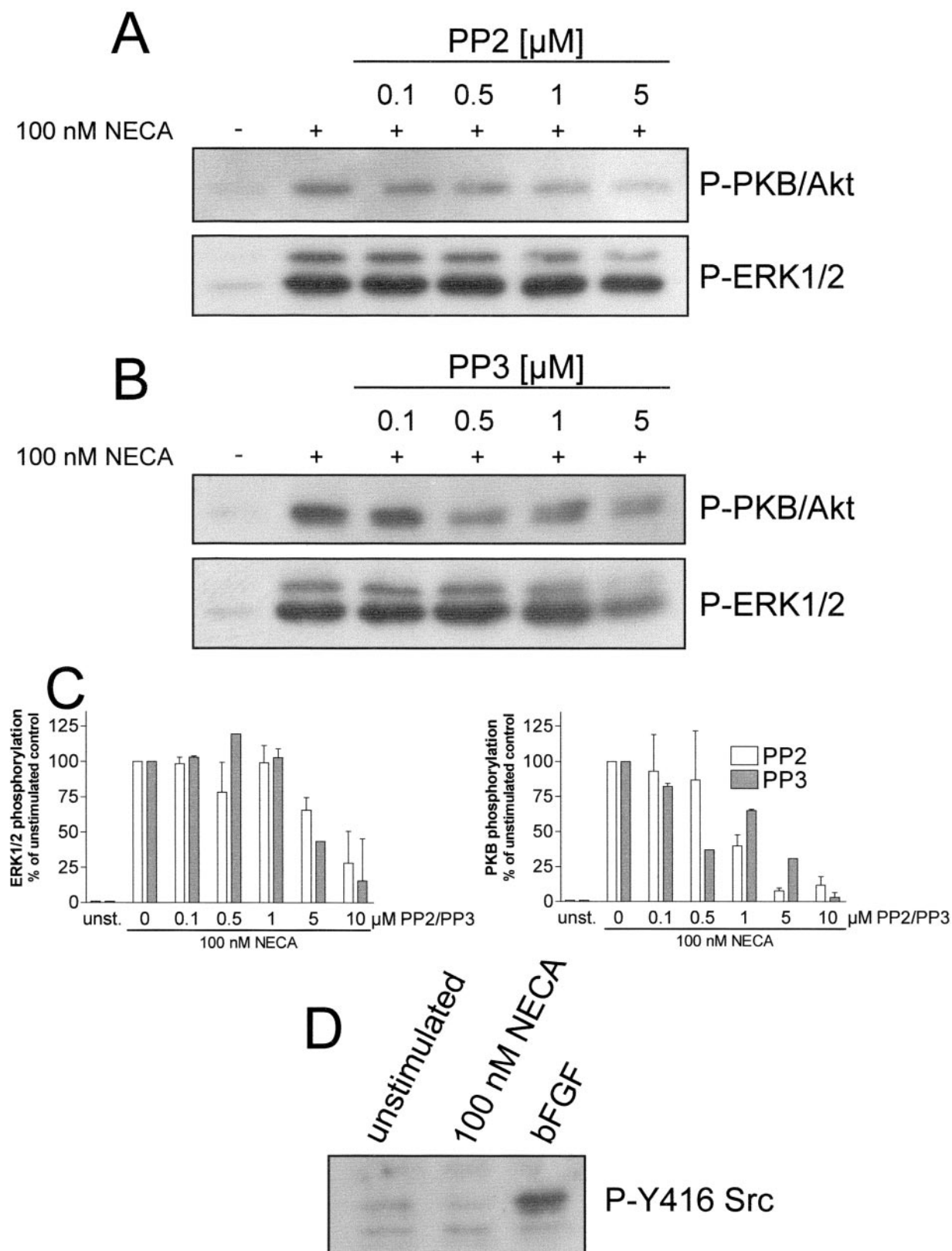


Fig. 6. Lack of involvement of c-Src-like tyrosine kinases in adenosine A₃ receptor-mediated ERK1/2 phosphorylation. A, CHO A₃ cells were treated with the c-Src-kinase family specific tyrophostin PP2 for 20 min before NECA stimulation ($n = 4$). To underline the specificity of PP2, B shows the effects of PP3, an inactive homolog to PP2, on ERK1/2 and PKB/Akt phosphorylation. To compare the effects of PP2 and PP3 on the NECA-induced ERK1/2 and PKB phosphorylation, we summarized one to five experiments for each data point in the bar graph shown in C. Results were normalized: protein phosphorylation in unstimulated cells was set to 0% and in NECA-stimulated cells to 100%. Error bars show S.D. D, one representative experiment (of five) showing that NECA (100 nM) did not affect phosphorylation of c-Src. As positive control for the phosphorylation of c-Src CHO, A₃ cells were stimulated with bFGF (5 ng/ml). c-Src phosphorylation was examined in cell lysates prepared 5 min after addition of agonist.

the adenosine A₃ receptor-mediated ERK1/2 phosphorylation did not depend only on a basal PI3K activity. The PI3K and PKB/Akt signaling pathway has been shown to be involved in

pro-survival signaling, insulin responses, and various inflammatory effects (Downward, 1998). Together with our results, this may represent a strong intracellular link between adenosine A₃ receptor signaling and its involvement in inflammatory processes (Salvatore et al., 2000). Further studies, however, are necessary to confirm this association in a more physiological context.

Thus, adenosine A₃ receptor activation led to PI3K activation dependent on the $\beta\gamma$ release from G_{i/o}. This is important both for ERK1/2 activation and PKB/Akt phosphorylation. PKB/Akt, however, cannot be located upstream of ERK1/2, because the two processes are differentially affected by overexpression of RasS17N.

When we investigated the effect of the c-Src kinase family-specific tyrphostin PP2 and its inactive homolog PP3 on adenosine A₃ receptor signaling, we found that those two substances did not differ in their effect on NECA-induced ERK1/2 and PKB/Akt phosphorylation. Together with the fact that PP2 has an IC₅₀ at Src-like kinases in the nanomolar range, we conclude that the inhibitory effects of PP2 at micromolar concentrations may be ascribed to unspecific side effects rather than to inhibition of Src-like kinases. In addition, we showed that stimulation of adenosine A₃ receptors did not increase the phosphorylation at the autophosphorylation site Y416 of c-Src. The anti-phospho Y416 c-Src antibody is expected to recognize all members of this kinase family. The Src-like tyrosine kinase inhibitor PP2 had no

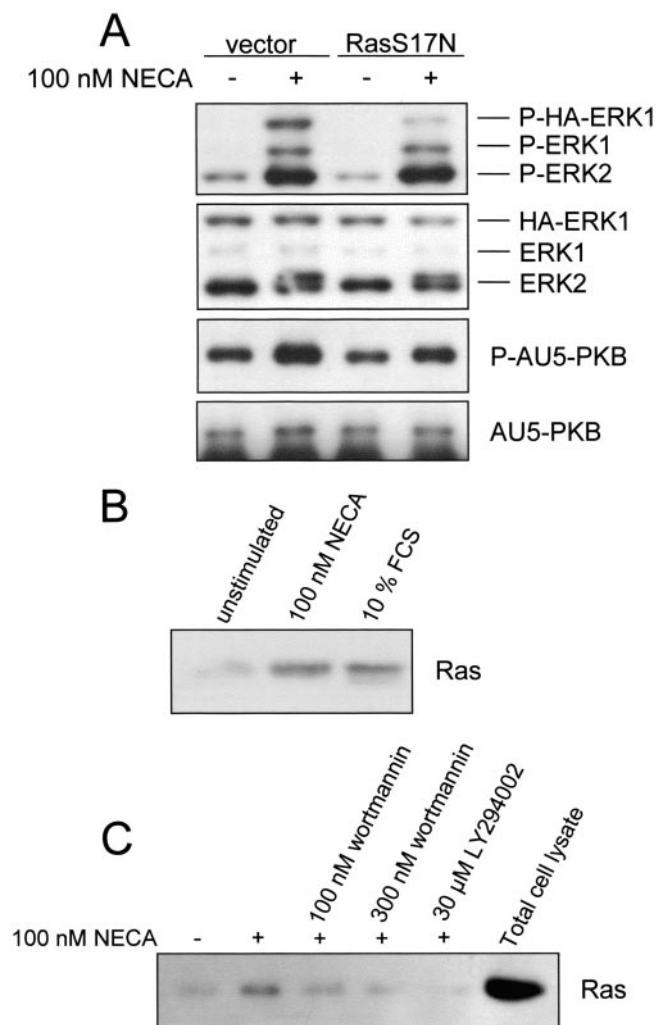


Fig. 7. Activation of Ras is required for adenosine A₃ receptor-mediated ERK1/2 phosphorylation. A shows the effect of a dominant negative mutant of Ras, RasS17N, on the adenosine A₃ receptor-mediated HA-ERK1/2 and AU5-PKB/Akt phosphorylation. CHO A₃ cells were cotransfected with mammalian expression vectors coding for HA-ERK 1, AU5-PKB, and either RasS17N or empty vector. Cells were stimulated, immunoprecipitated with an anti-AU5 antibody, and both the supernatant and the precipitate were analyzed for HA-ERK1/2 and AU5-PKB phosphorylation, respectively. The immunoblot shows the endogenously expressed ERK 1, ERK 2, and the transfected HA-ERK 1 in the supernatant, whereas only the transfected AU5-PKB is visible in the immunoprecipitate. To confirm equal expression levels of the reporter constructs, immunoblots were run in parallel, detecting total ERK1/2 and AU5-PKB in the supernatant and the precipitate, respectively. In RasS17N-transfected and NECA-stimulated CHO A₃ cells, HA-ERK1 and AU5-PKB phosphorylation was 13 ± 18 and $54 \pm 10\%$, respectively, compared with NECA stimulated vector-transfected cells (set to 100%). The fact that dominant-negative RasS17N did not affect phosphorylation of the endogenously expressed ERK1/2 is most likely attributable to low transfection efficiency. B shows the increase in active GTP-bound Ras after NECA and fetal calf serum (10%, FCS) stimulation for 5 min. Active Ras was precipitated using the minimal Ras-binding domain of Raf fused to a GST tag and glutathione Sepharose. The immunoblot shows the amount of precipitated Ras detected with a mouse monoclonal pan-Ras antibody after stimulation with 100 nM NECA or 10% FCS for 5 min ($n = 3$). C, the activation of Ras by stimulation of adenosine A₃ receptors is blocked by the PI3K inhibitors wortmannin and LY294002. To confirm the correct molecular weight of the precipitated Ras, results obtained with a total cell lysate are also shown.

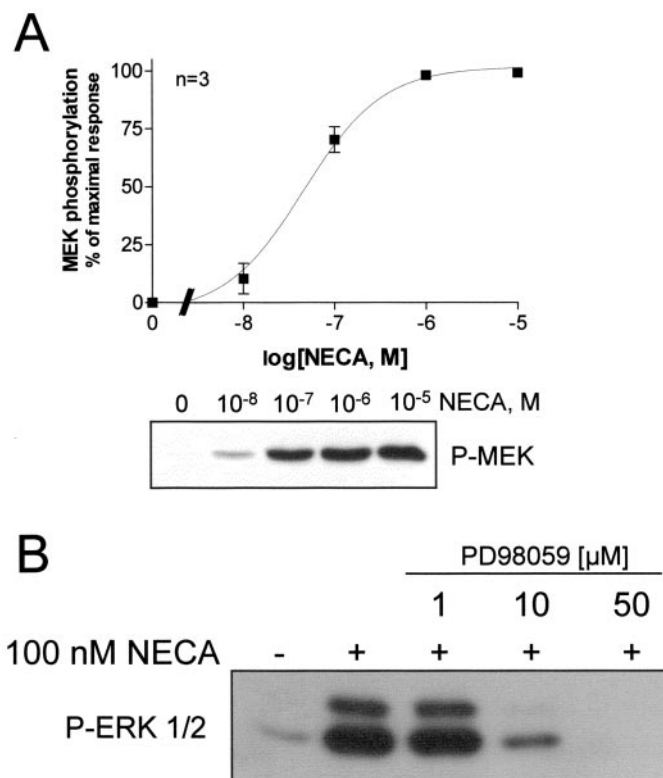


Fig. 8. Adenosine A₃ receptor stimulation increases MEK phosphorylation and MEK activity is required for ERK phosphorylation. A, MEK, the upstream kinase of ERK1/2, was phosphorylated in a dose-dependent manner by NECA. Immunoblotting using the anti-phospho MEK1/2 antibody is described in the text. Values are presented as mean and S.D. from three separate experiments. The inset shows a representative blot. B, immunoblot showing that the MEK1 inhibitor PD98059 inhibited the NECA-induced ERK1/2 phosphorylation in CHO A₃ cells in a concentration-dependent manner ($n = 3$).

effect on ERK1/2 and PKB/Akt phosphorylation in nanomolar concentrations, thus confirming the lack of importance of Src-like kinases.

The present results do not, however, rule out the possibility that transactivation of a receptor tyrosine kinase is involved in the cascade of events from the adenosine A₃ receptor to ERK1/2. Recently Graham et al. (2001) described a genistein-sensitive pathway leading from the adenosine A₃ receptor to ERK1/2. The concentration of genistein used was so high that receptor tyrosine kinases might be blocked. However, our recent results (Schulte and Fredholm, 2002) show that even at lower concentrations this inhibitor directly binds to and inhibits adenosine receptors. Hence, there is no data directly implicating receptor transactivation in the adenosine A₃ receptor signaling to ERK1/2. Furthermore, a recent report (Andreev et al., 2001) has elegantly demonstrated that G protein-coupled receptor transactivation of epidermal growth factor receptors and their activation of the MAP kinase cascade are differently regulated. Thus, it seems most parsimonious to assume that the adenosine A₃ receptor

activates a PI3K enzyme directly via $\beta\gamma$ subunits. Either PI3K γ class I_B or PI3K β class I_A subtype enzymes could be involved (see Marinissen and Gutkind, 2001, and references therein).

We also examined intermediary steps between PI3K and ERK1/2. The present results provide good evidence that PI3K-dependent activation of Ras is of critical importance in adenosine A₃ receptor-mediated ERK1/2 phosphorylation. The way GPCRs signal via PI3K to the small G protein Ras is still an issue of debate (Vanhaesebroeck et al., 1997; Vanhaesebroeck and Waterfield, 1999): it has been proposed, for example, that Ras is located directly upstream of PI3K, and that c-Src is coupling PI3K to Ras. Both of these possibilities are ruled out in the present case. On the other hand, we do not know if there are steps between PI3K and Ras activation.

Finally, the sensitivity to PD98059, and the dose-dependent phosphorylation of MEK confirmed that ERK1/2 phosphorylation depended on MEK activity and that no other MEK-independent pathway led to ERK1/2 phosphorylation in CHO A₃ cells. The MEK kinase involved has not been

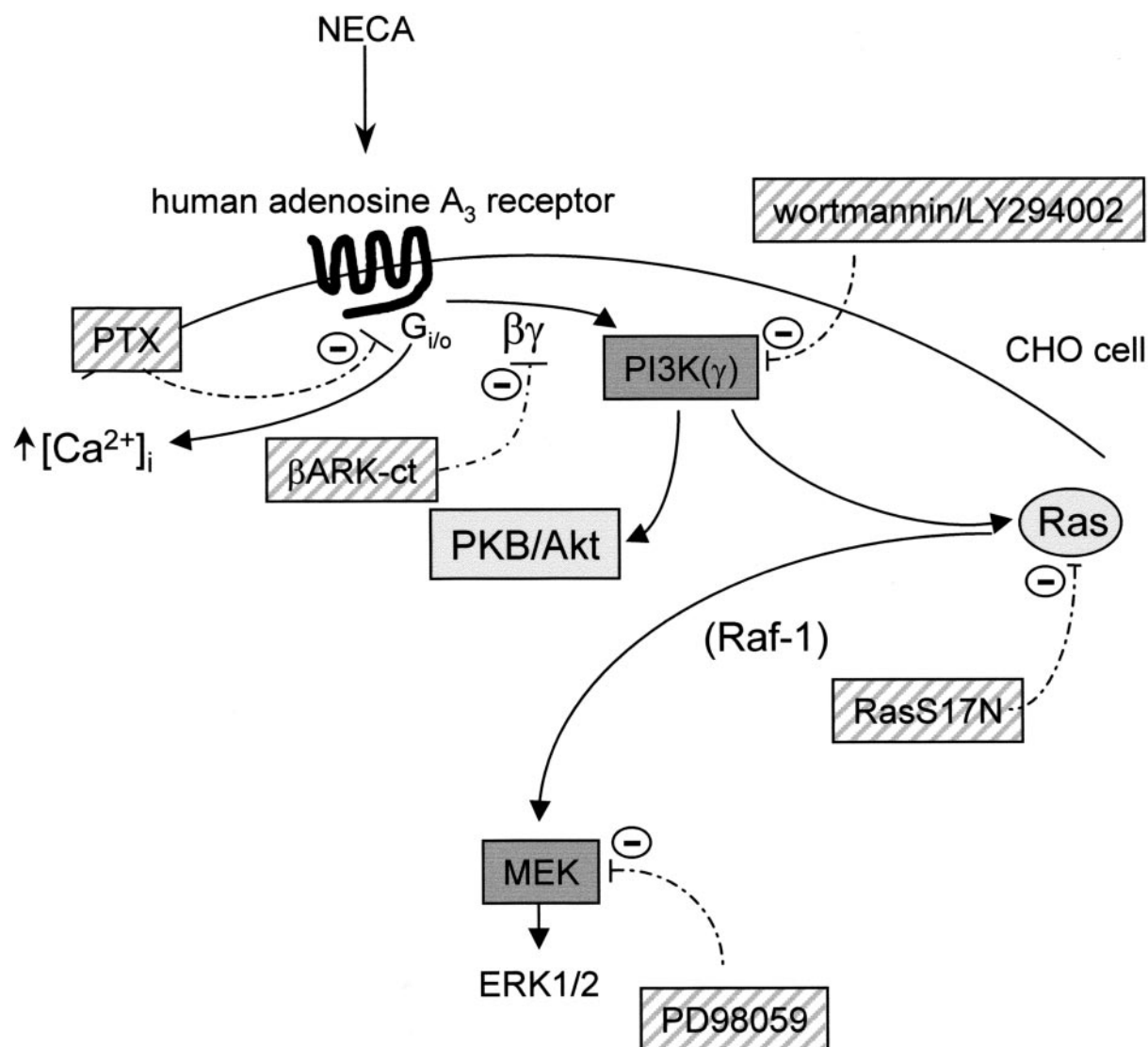


Fig. 9. Schematic representation of the pathways from NECA stimulation of CHO cells expressing the human adenosine A₃ receptor to ERK1/2. Striped boxes indicate drugs or molecular biological tools used in this study. Information in parentheses was not directly addressed by this study. For further details, see *Discussion*.

identified but it is known that Raf1 is present in CHO cells and is activated by Ras, and it is a well-characterized MEK kinase (Avruch et al., 1994; Marinissen and Gutkind, 2001).

Activation of human adenosine A₃ receptors expressed in CHO cells leads to a rapid and strong stimulation of ERK1/2 phosphorylation and activity. Furthermore, adenosine analogs are at least as potent in activating this signaling cascade as in affecting other parallel signaling pathways. The present results suggest one possible reason for this: that the signaling pathway is rather direct, involving G_{i/o}, PI3K, Ras, Raf-1, and MEK. Although it is possible that the signaling pathways may be different in other cellular backgrounds (Dumont et al., 2001), it seems likely that the biological events regulated by adenosine A₃ receptors under physiological and pathophysiological conditions may depend not only on changes in cAMP and Ca²⁺, but also on mitogenic signaling via ERK1/2 or PKB/Akt.

Acknowledgments

We thank Alf Berts for assistance with the experiments on adenosine receptor-mediated changes in [Ca²⁺]_i and the effect of BAPTA-AM thereon. We thank Karl-Norbert Klotz for sharing unpublished information with us. We thank M. Freissmuth, U. Rapp, W. F. Simonds, J. Toppmaier, and A. Wittinghofer for kindly providing the expression vectors used in this study. J. Zierath kindly provided the anti-phospho PKB/Akt and anti-phospho MEK antibodies. A. Chibalin and P. Gerwins are acknowledged for their support with the PKCζ and ERK1/2 activity assays, respectively.

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