Cyclic AMP and Protein Kinase A Stimulate Cdc42: Role of A_2 Adenosine Receptors in Human Mast Cells

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ABSTRACT

The functional activity of Cdc42 is known to be regulated by proteins that control its GDP/GTP-bound state. However, there is still limited information on how Cdc42 is controlled by G-protein-coupled receptors. Adenosine receptors belong to the G-protein-coupled receptor family of cell surface receptors. Human HMC-1 mast cells express the high-affinity A_{2A} and the low-affinity A_{2B} subtypes of adenosine receptors known to increase intracellular cAMP levels. We found that both subtypes of A_2 adenosine receptors activate Cdc42 in HMC-1 cells. Furthermore, stimulation of adenylate cyclase with forskolin, or loading of HMC-1 with the cell-permeable cAMP analog 8-Br-cAMP, activated Cdc42. Stimulation of Cdc42 by cAMP was also observed in CHO-K1 and COS-7 cells. Protein kinase A (PKA)-mediated phosphorylation is likely involved in cAMP-dependent Cdc42 activation, because transient expression of the PKA catalytic subunit in COS-7 cells activated Cdc42. Inhibition of protein phosphatases 1 and 2A with calyculin A potentiated the effects of 5′-*N*-ethylcarboxamidoadenosine and 8-Br-cAMP, whereas the selective PKA inhibitor H-89 reversed the activation of Cdc42. We demonstrated that Cdc42 is a poor substrate for PKA phosphorylation in vitro and in intact cells. Our data suggest that PKA does not phosphorylate Cdc42 directly. Instead, the proteins that modulate the GDP/GTP-bound state of Cdc42 may be the primary targets of PKA phosphorylation.

The endogenous nucleoside adenosine modulates various cellular functions acting via specific receptors that belong to the serpentine G-protein-coupled receptor (GPCR) family. There is ample evidence that adenosine regulates mast cell secretion. Stimulation of mast cell secretion is likely the mechanism that explains the adenosine-provoked broncho-constriction in asthmatic patients (Church and Holgate, 1986; Feoktistov et al., 1998). The spectrum of bioactive compounds, released from mast cells, ranges from preformed mediators to newly synthesized mediators, including cytokines. We have previously shown that the human mast cell line HMC-1 expresses A_{2A} and A_{2B} receptors, but only A_{2B} receptors induce production of interleukin 8. Both receptors stimulate adenylate cyclase via G_s-protein, but only A_{2B} receptors also stimulate phospholipase C via G_q-proteins (Feoktistov and Biaggioni, 1995).

Recently we demonstrated that at least two mitogen-acti-

vated protein kinase (MAPK)-signaling pathways were involved in A_{2B}-mediated interleukin 8 secretion, because the selective MEK1 inhibitor PD 98059 and the p38 MAPK inhibitors SB 202190 and SB 203580 blocked this process. Adenosine, acting via A_{2B} receptors, stimulates Ras, the upstream regulator of the extracellular signal-regulated kinase (ERK) pathway (Feoktistov et al., 1999). The p38 MAPK pathway appears to be linked to another upstream regulator, the Ras-related small G-protein of the Rho family Cdc42 (Bagrodia et al., 1995; Zhang et al., 1995). Mammalian Cdc42 has been implicated in the regulation of diverse functions, including actin rearrangements, inflammatory and stress responses, mitogenesis, differentiation, cell growth, cell cycle progression, apoptosis, prostaglandin biosynthesis, myocyte hypertrophy, and gene expression (for review, see Benard et al., 1999b; Johnson, 1999). In the rat RBL mast cells, Cdc42 was shown to regulate $Fc_\epsilon RI$-dependent degranulation and serotonin secretion (Guillemot et al., 1997). It is not known, however, whether adenosine receptors regulate Cdc42 activity.

ABBREVIATIONS: GPCR, G-protein coupled receptor; ERK, extracellular signal regulated kinase; GEF, guanine nucleotide exchange factor; GST, glutathione *S*-transferase; MAPK, mitogen-activated protein kinase; NECA, 5′-N-ethylcarboxamidoadenosine; PAK, p21-activated kinase; PBD, p21-binding domain; PKA, protein kinase A; TCA, trichloroacetic acid; FBS, fetal bovine serum; CGS 21680, 4-[(N-ethyl-5′-carbamoyladenos-2-yl)-aminoethyl]-phenylpropionic acid; ZM241385, 4-(2-[7-amino-2-)2-furyl(triazolo{2,3-a}-[1,3,5]triazin-5-ylamino]ethyl)phenol; HMC, human mast cells; HEL, human erythroleukemia; NP-40, Nonidet P-40; PAGE, polyacrylamide gel electrophoresis; DMSO, dimethyl sulfoxide; PMA, phorbol 12-myristate 13-acetate.

The discovery of guanine nucleotide exchange factors (GEFs), GTPase-activating proteins, and GDP dissociation inhibitors has improved our understanding of how small G-proteins are regulated. It is possible that subunits of activated heterotrimeric proteins coupled to GPCR can directly bind to GEFs, as recently demonstrated for $G_{\alpha12}/G_{\alpha13}$ and PDZ-RhoGEF (Fukuhara et al., 1999). In addition, GPCR can regulate small G-proteins through other pathways triggered by heterotrimeric G-proteins, including tyrosine kinases, protein kinase C, and cAMP. Of interest, in recent reports, cAMP was shown to bind directly to, and activate, the GEF for the small G-protein Rap1 (de Rooij et al., 1998; Kawasaki et al., 1998). However, the role of cAMP in the regulation of Cdc42 activity is not known. In this study we present evidence indicating that adenosine activates Cdc42 in human mast cells via both A_{2A} and A_{2B} receptors, by a mechanism that includes cAMP and a protein kinase A (PKA)-dependent component.

Materials and Methods

Cell Culture and Reagents. Human mast cells (HMC-1) were a generous gift from Dr. J. H. Butterfield (Mayo Clinic, Rochester, MN). HMC-1 cells were maintained in suspension culture at a density between 3 and 9×10^5 cells/ml by dilution with Iscove's medium supplemented with 10% (v/v) fetal bovine serum (FBS), 2 mM glutamine, antibiotics, and 1.2 mM α-thioglycerol. Human erythroleukemia (HEL) cells were obtained from the American Type Culture Collection (TIB 180; Rockville, MD) and maintained in suspension culture at a density between 3 and 9×10^5 cells/ml by dilution with RPMI 1640 medium supplemented with 10% (v/v) FBS, 10% (v/v) newborn calf serum, antibiotics, and 2 mM glutamine. Monkey kidney simian virus 40-transformed COS 7 cells were obtained from the American Type Culture Collection (CRL-1651) and maintained in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) FBS and antibiotics. Chinese hamster ovary CHO-K1 cells were obtained from the American Type Culture Collection (CRL-9618) and maintained in Ham's F12 medium supplemented with 10% (v/v) FBS and antibiotics. All cells were kept under humidified atmosphere of air/CO_2 (19:1) at 37°C.

4-[(N-ethyl-5′-carbamoyladenos-2-yl)-aminoethyl]-phenylpropionic acid (CGS 21680) and 5′-N-ethylcarboxamidoadenosine (NECA) were purchased from Research Biochemicals, Inc. (Natick, MA). 4-(2-[7-Amino-2-)2-furyl(triazolo{2,3-a]-[1,3,5]triazin-5-ylamino]ethyl)phenol (ZM241385), was purchased from Tocris Cookson, Inc. (Ballwin, MO). Forskolin and 8-Br-cAMP were obtained from Sigma Chemical Co. (St. Louis, MO). Bovine heart catalytic subunit of PKA, microcystin-LR, calyculin A, H-89, synthetic PKA inhibitor 6-22 amide, recombinant glutathione S-transferase (GST), GST-RhoA, and GST-Cdc42 were purchased from Calbiochem-Novabiochem Corp. (San Diego, CA).

Evaluation of Cdc42 Activation. The active GTP-bound form of Cdc42 was detected using the p21-binding domain of p21-activated kinase (PAK)-1 (PBD) according to a recently published technique (Benard et al., 1999a). The pGEX 4T3 PBD prokaryotic expression vector was kindly provided by Dr. Garry Bokoch (Scripps Research Institute, La Jolla, CA). GST-PBD expression in BL-21 strain of *Escherichia coli* was induced with 0.4 mM isopropyl β-d-thiogalactopyranoside, and the bacteria were sonicated on ice for six 1-min periods in phosphate-buffered saline containing 0.5 mM dithiothreitol, 0.1 μM aprotinin, 1 μM leupeptin, and 1 mM phenylmethylsulfonyl fluoride. Triton X-100 was added to a final concentration of 1%, and after gently stirring for 30 min at 4°C, glycerol was added to a final concentration of 10%. The lysate was aliquoted and stored at −80°C. The desired amount of crude GST-PBD (0.25 mg protein/40 μl glutathione-agarose/sample) was thawed and incubated with glutathione-agarose beads at room temperature for 30 min. The beads

were isolated by centrifugation and washed three times with lysis buffer containing 6 mM Na_2HPO_4, 4 mM NaH_2PO_4, 1% Nonidet P-40 (NP-40), 150 mM NaCl, 2 mM EDTA, 50 mM NaF, 0.1 mM Na_3VO_4, 4 μg/ml leupeptin, 2 mM benzamidine, and Complete Mini protease inhibitor cocktail (Boehringer Mannheim, Mannheim, Germany). HMC-1 cells were harvested and resuspended to a concentration of 10^7 cells/ml in a buffer, pH 7.4, containing 150 mM NaCl, 2.7 mM KCl, 0.37 mM NaH_2PO_4, 1 mM $MgSO_4$, 1 mM $CaCl_2$, 5 g/l D-glucose, 10 mM HEPES (HEPES)-NaOH, and 1 U/ml adenosine deaminase. After a 15-min preincubation at 37°C, 1-ml aliquots of cell suspension were incubated for various times at 37°C with the reagents indicated under *Results*. In experiments when CHO-K1 or COS-7 were used, all incubations with reagents were performed in 35-mm plates in the same buffer. Following each stimulation, 10^7 HMC-1 cells were collected by centrifugation for 15 s at 1000g and lysed by addition of 200 μl of 6 mM Na_2HPO_4, 4 mM NaH_2PO_4, 1% NP-40, 150 mM NaCl, 2 mM EDTA, 50 mM NaF, 0.1 mM Na_3VO_4, 4 μg/ml leupeptin, 2 mM benzamidine, and Complete Mini protease inhibitor cocktail. CHO-K1 or COS-7 were lysed directly in the plates. GST-PBD, precoupled to glutathione-agarose beads in the lysis buffer, was added, and lysates were incubated at 4°C for 30 min. The beads were then washed five times with the lysis buffer by centrifugation, and resuspended in 40 μl of the sample buffer (250 mM Tris-HCl, pH 6.8; 10% SDS, 10% glycerol, 5% β-mercaptoethanol, and 0.5% bromphenol blue). After boiling for 5 to 10 min, the supernatant was collected by centrifugation, and the protein samples (20 μl) were separated on a 12% SDS-polyacrylamide gel electrophoresis (PAGE) gel and subsequently transferred to a polyvinylidene fluoride membrane by Western blotting. Cdc42 was detected by incubating the membrane overnight at 4°C with rabbit polyclonal anti-human Cdc42 antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). Horseradish peroxidase-conjugated anti-rabbit IgG (Sigma) was used as a second antibody (2 h at room temperature). The bands on the membrane were visualized with an enhanced chemiluminescence method (Nesbitt and Horton, 1992).

Transfection of COS-7 Cells. The plasmids utilized in our transfection studies were from the following origins: pcDNAI-α_s-Q227L, a plasmid encoding a constitutively active mutant of the $G\alpha_s$-subunit, was kindly provided by Dr. Tatyana A. Voyno-Yasenetskaya (University of Illinois, Chicago, IL); pFC-PKA, a plasmid encoding the catalytic subunit of PKA, was purchased from Stratagene (La Jolla, CA).

The COS-7 cells were transfected using Fugene 6 transfection reagent (Boehringer Mannheim). One microgram of plasmid DNA was mixed with 100 μl of serum-free Dulbecco's modified Eagle's medium containing 6 μl of Fugene 6. After a 15-min incubation at room temperature, the transfection mixture was added to the cells growing on 35-mm plates at 40 to 60% confluency. The cells were then incubated for 48 h under a humidified atmosphere of air/CO_2 (19:1) at 37°C.

Measurement of cAMP. Before each experiment, HMC-1 or HEL cells were harvested, washed by centrifugation (100g for 10 min) and resuspended in a buffer containing 150 mM NaCl, 2.7 mM KCl, 0.37 mM NaH_2PO_4, 1 mM $MgSO_4$, 1 mM $CaCl_2$, 5 g/l D-glucose, 10 mM HEPES-NaOH, pH 7.4, and 1 U/ml adenosine deaminase, to a concentration of 3×10^6 cells/ml. Cells were preincubated for 15 min at 37°C in the same buffer containing the cAMP phosphodiesterase inhibitor papaverine, 1 mM. Adenosine agonists and antagonists were added to cells as indicated. Cells were suspended in a total volume of 200 μl and mixed with a vortex, and the incubation was allowed to proceed for 2 min at 37°C. The reaction was stopped by the addition of 50 μl of 25% trichloroacetic acid (TCA) to cell suspensions. To determine cAMP concentrations in transfected COS-7 cells, 250 μl of 5% TCA were added to confluent cell monolayers growing on 35-mm plates. TCA-treated extracts were washed five times with 10 volumes of water-saturated ether. cAMP concentrations were determined by competition binding of tritium-labeled cAMP to a protein derived from bovine muscle, which has high specificity for

cAMP (cAMP assay kit, TRK.432; Amersham Corp., Arlington Heights, IL).

Measurement of PKA Activity. Confluent COS-7 cells were harvested by repetitive pipetting in 1 ml of phosphate-buffered saline per 35-mm well, followed by centrifugation at 100g for 10 min. The cells were lysed in 30 μl of ice-cold buffer, containing 20 mM 3-(N-morpholino)propanesulfonic acid, pH 6.6, 1 mM dithiothreitol, 0.05% (v/v) Triton X-100, 100 μM phenylmethylsulfonyl fluoride and Complete Mini protease inhibitor cocktail. PKA activity was assayed by a modification of the procedure proposed by Witt and Roskoski (1975). In brief, the final incubation mixture contained 20 mM 3-(N-morpholino)propanesulfonic acid, pH 6.6, 1 mM dithiothreitol, 1 mM EGTA, 10 mM $MgCl_2$, 50 μM [γ-^{32}P]ATP (0.5 μCi), 1 μM microcystin-LR, 0.5 mg/ml histone IIAS, and 0.05% (v/v) Triton X-100. The reaction was initiated by addition of 25 μl of cell lysate to 25 μl of the other components. The incubation was carried out at 30°C for 5 min and was terminated by the blotting of 25 μl of the mixture onto phosphocellulose P81 filter paper (2 × 2 cm; Whatman, Clifton, NJ). Filters were washed five times with 0.5% (v/v) phosphoric acid for 10 min and once with acetone. After drying in air, the radioactivity absorbed onto the phosphocellulose was measured by liquid scintillation counting. The activity of PKA was calculated as the difference between total protein kinase activity and activity in the presence of 1 μM PKA inhibitor 6-22 amide.

In Vitro Phosphorylation. Phosphorylation of the recombinant GST fusion proteins (1 μg) by the catalytic subunit of PKA (10 U, 20 ng) was carried out in 20 μl of 50 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 1 mM dithiothreitol, 20 μM [γ-^{32}P]ATP (10 μCi), 1 μM microcystin-LR, and Complete Mini protease inhibitor cocktail for 30 min at 30°C. In some experiments, the GST fusion proteins were precoupled to glutathione-agarose (Lang et al., 1996). The reaction was stopped either by boiling in SDS-PAGE sample buffer or by the addition of 1 ml of the cold phosphorylation buffer containing 25 μg glutathione-agarose beads. After incubation for 1 h at 4°C, the beads were washed three times with cold phosphorylation buffer, and proteins were eluted by boiling in SDS-PAGE sample buffer. The samples were then separated on a 4 to 12% gradient SDS-PAGE gel and processed for Western blotting and autoradiography.

^{32}P Labeling of Cells. COS-7 cells growing on 35-mm plates were washed twice with phosphate-free Dulbecco's modified Eagle's medium. The cells were then incubated in 4 ml of the same medium containing 1 mg/ml bovine serum albumin, 50 μM Na_3VO_4, and 2.5 mCi $^{32}P_i$ under a humidified atmosphere of air/CO_2 (19:1) at 37°C. After 4 h, an appropriate volume of stimulants or their vehicle was added to the medium, and cells were further incubated for 15 min. The reaction was stopped by removal of medium, and the cells were then lysed by addition of 200 μl of 6 mM Na_2HPO_4, 4 mM NaH_2PO_4, 1% NP-40, 150 mM NaCl, 2 mM EDTA, 50 mM NaF, 0.1 mM Na_3VO_4, 4 μg/ml leupeptin, 2 mM benzamidine, and Complete Mini protease inhibitor cocktail.

Results

Both A$_{2A}$- and A$_{2B}$-Subtypes of Adenosine Receptors Stimulate Cdc42. An increase in guanine nucleotide exchange on Cdc42 results in binding of this small G-protein to PAK and stimulation of its protein kinase activity. To determine whether adenosine induces the formation of the GTP-bound active form of Cdc42, we incubated cells with the stable adenosine analog NECA (10 μM) in the presence of 1 U/ml adenosine deaminase. Samples were collected at different time points and the extracted proteins were incubated with the recombinant GST-p21-binding domain of PAK (Benard et al., 1999a) coupled to glutathione-agarose. The absorbed proteins were then analyzed by immunoblotting with anti-Cdc42 antibody. As shown in Fig. 1A, the nonselective

A$_{2A}$/A$_{2B}$ agonist NECA induced maximal formation of active Cdc42 during the first minute of incubation. We then incubated cells for 1 min with increasing concentrations of NECA and observed a parallel rise in the active form of Cdc42 (Fig. 1B).

NECA stimulates both A$_{2A}$ and A$_{2B}$ adenosine receptors in HMC-1 cells (Feoktistov and Biaggioni, 1995). Therefore, we used the selective A$_{2A}$ agonist CGS 21680 and the selective A$_{2A}$ antagonist ZM241385 to differentiate between the effects of A$_{2A}$ and A$_{2B}$ receptors. To choose the correct concentration of agonist and antagonist to be used in these studies, we first determined their potency on A$_{2A}$ adenosine receptor-mediated cAMP accumulation in our cell model. As shown in Fig. 2A, the selective A$_{2A}$ agonist CGS 21680 produced a 4-fold increase in cAMP in the absence of ZM241385. Nonlinear regression analysis of this concentration-response curve revealed a pD$_2$ of 7.2 ± 0.15 for CGS 21680 (EC$_{50}$ of 61 ± 18 nM), consistent with A$_{2A}$ receptor activation (Klotz et al., 1998). Increasing concentrations of ZM241385, from 1 to 30 nM, produced rightward shifts in the concentration-response curve of CGS 21680. Schild analysis of this interaction yielded a slope of unity (inset in Fig. 2A), indicating that ZM241385 is a simple competitive antagonist of A$_{2A}$-mediated cAMP accumulation in HMC-1 cells. The intercept of this linear regression was used to estimate a pK$_B$ of 9.5, which is in close agreement with the previously reported affinity of ZM241385 at A$_{2A}$ adenosine receptors (Jacobson and Suzuki, 1996).

Because our experimental approach depended critically on the selectivity of ZM241385 as an A$_{2A}$ antagonist, and its lack of efficacy at A$_{2B}$ receptors, we believed it would be important to validate the selectivity of this compound in our model. For this purpose we tested the effects of ZM241385 on NECA-induced cAMP accumulation in HEL cells. These cells express only A$_{2B}$ adenosine receptors, and CGS 21680 has no effect on adenylate cyclase at concentrations up to 1 mM (Feoktistov and Biaggioni, 1993). As shown in Fig. 2B, ZM241385, at concentrations ranging from 1 to 30 nM, had no significant effect on the A$_{2B}$-mediated increase in cAMP produced by NECA.

We selected 1 μM CGS 21680 and 10 μM NECA, agonist concentrations producing submaximal activation of A$_{2A}$ and
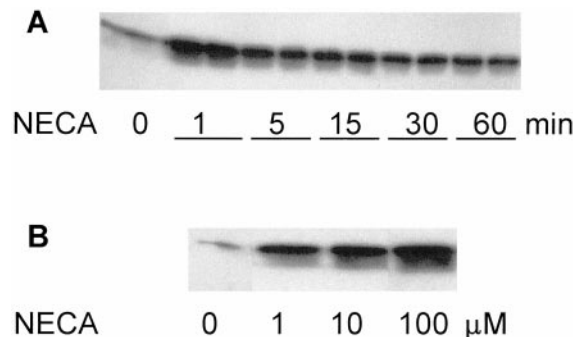

Fig. 1. Cdc42 activation in HMC-1 cells stimulated with the stable adenosine analog NECA. Activation of Cdc42 was determined by affinity precipitation with GST-PBD followed by Western blotting as described under *Materials and Methods*. A, time course of Cdc42 activation in HMC-1 cells stimulated with 10 μM NECA. Data are presented in duplicates. Results are representative of three independent experiments. B, effect of increasing concentrations of NECA on Cdc42 activation measured after 1 min of incubation with NECA. Results are representative of three independent experiments.

A_{2B}, respectively, to examine whether they would stimulate Cdc42. We found that Cdc42 was stimulated within the first minute of incubation with 1 μM CGS 21680 and that ZM241385 inhibited this effect in a concentration-dependent manner (Fig. 3A). This indicates that A_{2A} receptors stimulate Cdc42, and this effect can be blocked by a selective A_{2A} antagonist. In contrast, ZM241385, at concentrations ranging from 1 to 100 nM, did not block the stimulation of Cdc42 produced by 10 μM NECA (Fig. 3B), indicating that A_{2B} adenosine receptors also activate Cdc42. We then verified whether the effects of adenosine agonists and antagonists on Cdc42 would correlate with their effects on cAMP in HMC-1 cells. As shown in Fig. 3C, 1 μM CGS 21680 and 10 μM NECA increased cAMP in HMC-1 from 2.2 $\pm$ 0.16 to 8.5 $\pm$ 0.74 and 19.7 $\pm$ 0.95 pmol/10^6 cells, respectively. The selective A_{2A} antagonist ZM241385 (10 nM) inhibited the cAMP accumulation produced by CGS 21680 by 64%, but it was virtually ineffective when cells were stimulated with 10 μM NECA.

cAMP Stimulates Cdc42. Both A_{2A}- and A_{2B}-subtypes of adenosine receptors are expressed in HMC-1 cells and can regulate distinct intracellular pathways (Feoktistov and Biaggioni, 1995, 1998; Feoktistov et al., 1999). The only known pathway that they share is stimulation of adenylate cyclase via coupling to G_s-protein. Therefore, we investigated the possible role of cAMP in Cdc42 activation by using the stable cell-permeable cAMP analog 8-Br-cAMP. We used 10 μM 8-Br-cAMP, a concentration we have previously shown to produce maximal stimulation of PKA (Feoktistov et al., 1994). Figure 4A shows stimulation of Cdc42 by 8-Br-cAMP that was evident after 1 min of incubation. In contrast to the effect produced by NECA, which reached maximum in the first minute and then decreased (Fig. 1A), the stimulation of Cdc42 by 8-Br-cAMP remained relatively stable for a 60-min period. This can be explained by the lack of degradation of this cAMP analog, which is resistant to hydrolysis by phosphodiesterases. Direct stimulation of adenylate cyclase with 100 μM forskolin also activated Cdc42 with a time course similar to that observed with 8-Br-cAMP (Fig. 4B). Inhibition of protein dephosphorylation, by preincubation of HMC-1 cells with a cell-permeable PP1/PP2A protein phosphatase inhibitor calyculin A (50 nM) for 2 h, resulted in potentiation of the effects of NECA on Cdc42. As shown in Fig. 5, the time course of the NECA-induced Cdc42 stimulation also was affected in the presence of calyculin A, delaying the reversal phase of Cdc42 stimulation compared with control. Furthermore, pretreatment of HMC-1 cells for 40 min with 1 μM H-89, a selective antagonist of PKA, inhibited Cdc42 activation by NECA or CGS 21680 (Fig. 6). Taken together, these results demonstrate that increases in cAMP stimulate Cdc42

in human mast cells and strongly suggest that activation of PKA plays an important role in adenosine-mediated stimulation of Cdc42.

Role of PKA in Stimulation of Cdc42 by cAMP. PKA has been considered for a long time to be the essential mediator of the wide range of physiological effects initiated by increased intracellular cAMP levels. However, there is also accumulating evidence that cAMP can regulate effector molecules independently of PKA, as shown for some ion channels (Zufall et al., 1997). More recently, cAMP was implicated in binding to the GEFs that directly activate the small G-pro-
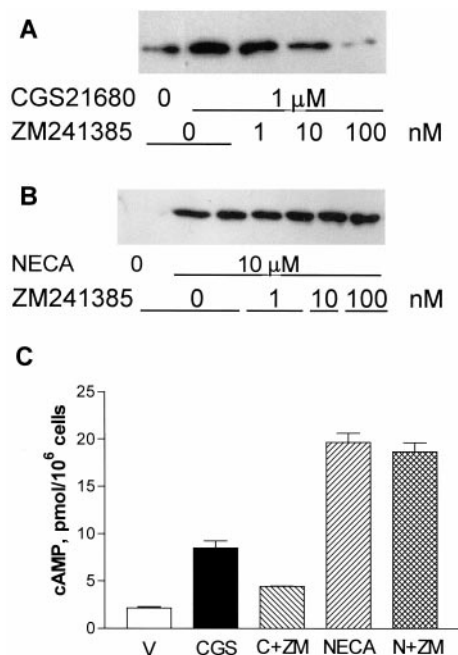
Fig. 3. Effects of the selective A_{2A} adenosine receptor antagonist ZM241385 on A_{2A}- and A_{2B}-mediated Cdc42 activation and cAMP accumulation in HMC-1 cells. Activation of Cdc42 was determined by affinity precipitation with GST-PBD followed by Western blotting as described under *Materials and Methods*. A, effect of increasing concentrations of the selective A_{2A} adenosine receptor antagonist ZM241385 on Cdc42 activated after 1 min of incubation with the selective A_{2A} adenosine receptor agonist CGS 21680 (1 μM). Results are representative of three independent experiments. B, effect of increasing concentrations of the selective A_{2A} adenosine receptors antagonist ZM241385 on Cdc42 activated after 1 min of incubation with 10 μM NECA. Results are representative of three independent experiments. Data for Cdc42 activation by NECA in the absence and in the presence of 1 nM ZM241385 are presented in duplicates. C, intracellular concentrations of cAMP in HMC-1 cells in the presence of DMSO as vehicle (V), after stimulation with 1 μM CGS 21680 in the absence (CGS) and in the presence of 10 nM ZM241385 (C+ZM), or after stimulation with 10 μM NECA in the absence (NECA) and in the presence of 10 nM ZM241385 (N+ZM). Results are presented as means $\pm$ S.E.M. of four experiments.
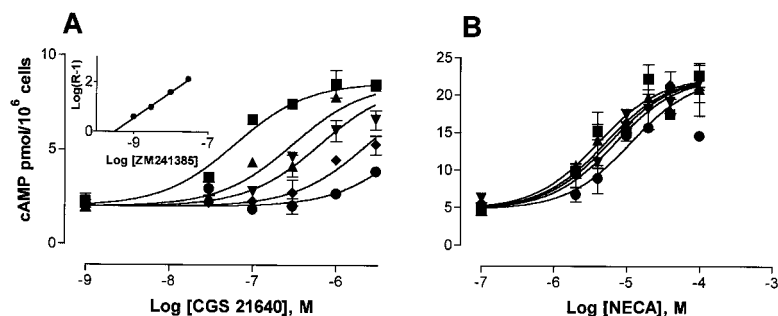
Fig. 2. Effects of ZM241385 on A_{2A}- and A_{2B}-mediated cAMP accumulation. ■, vehicle; ▲, 1 nM ZM241385; ▼, 3 nM ZM241385; ◆, 10 nM ZM241385; ●, 30 nM ZM241385. A, antagonistic effects of ZM241385 on A_{2A}-mediated cAMP accumulation in HMC-1 cells induced by CGS 21680. Concentration-response curves were repeated in the absence and in the presence of increasing concentrations of ZM241385, which produced a progressive shift to the right. Values are means $\pm$ S.E.M. of three experiments. Inset, Schild analysis of the data revealed a slope of unity, indicating simple competitive antagonism at A_{2A} adenosine receptors. B, effects of ZM241385 on A_{2B}-mediated cAMP accumulation in HEL cells induced by NECA. Values are means $\pm$ S.E.M. of three experiments.

tein Rap1 (de Rooij et al., 1998; Kawasaki et al., 1998). Therefore, we thought it important to verify whether PKA can stimulate Cdc42 in transfection studies. Unfortunately, we found it very difficult to efficiently transfect HMC-1 cells. We tried various transfection techniques, but only 1 to 3% of HMC-1 cells could be transfected with the pSV-β-galactosidase as determined by incubation with 5-bromo-4-chloro-3-indolyl-D-galactoside (data not shown).

In a search for a better cell model for PKA transfection studies, we chose CHO-K1 and COS-7 cells. Both cell lines have been shown to efficiently express proteins in transfection experiments. As shown in Fig. 7A, incubation of CHO-K1 and COS-7 cells with 10 μM 8-Br-cAMP for 15 min produced stimulation of Cdc42 in both cell lines. Preincubation of COS-7 cells with 50 nM calyculin A for 2 h potentiated Cdc42 stimulation by 8-Br-cAMP (Fig. 7B). Stimulation of β-adrenoceptors with 10 μM isoproterenol for 1 min in the presence of 1 mM papaverine increased intracellular cAMP concentrations in COS-7 from 4 $\pm$ 2.4 to 39.4 $\pm$ 2.4 pmol/10^6 cells, $n = 3$, and stimulated Cdc42 (data not shown). These results indicate that the phenomenon of cAMP-dependent Cdc42

stimulation is not limited to human mast cells only and may be shared by different cell types.

To study the role of PKA in regulation of Cdc42 activity, we transiently expressed the catalytic subunit of PKA in COS-7 cells. Cells transiently expressing the constitutively active α-subunit of G_s served as a positive control to mimic receptor-mediated activation of adenylate cyclase. Transfection of COS-7 with an empty expression vector (mock transfection) was used as a negative control. We verified that the average number of viable cells and the protein concentration in their lysates was the same for all confluent COS-7 cells 48 h after transfection. We also verified the expression of the catalytic subunit of PKA by measuring PKA activity in cell lysates. Protein kinase activity was assayed in the absence and in the presence of 10 μM 8-Br-cAMP or 1 μM PKA inhibitor 6-22 amide. The specific PKA activity was calculated as the difference between total protein kinase activity and activity in the presence of the PKA inhibitor (Fig. 8A). Mock-transfected COS-7 cells exhibited very low basal PKA activity (0.4 $\pm$ 0.4 pmol P_i/min/10^6 cells). PKA activity increased to 4.8 $\pm$ 0.7 pmol P_i/min/10^6 cells in the presence of the cAMP analog. The cells transfected with the catalytic subunit of PKA displayed a very high level of PKA activity (30.1 $\pm$ 0.4 pmol P_i/min/10^6 cells). As expected for a catalytic subunit, this
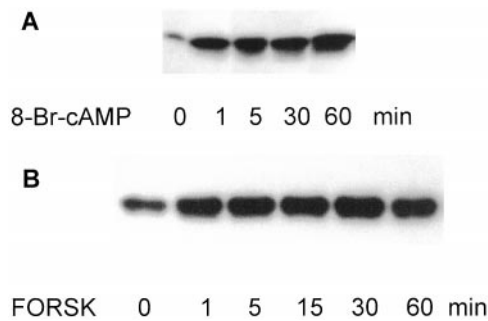
Fig. 4. Increase in intracellular cAMP levels activates Cdc42 in HMC-1 cells. Activation of Cdc42 was determined by affinity precipitation with GST-PBD followed by Western blotting as described under *Materials and Methods*. A, time course of Cdc42 activation in HMC-1 cells incubated with the cell-permeable cAMP analog 8-Br-cAMP (10 μM). Results are representative of three independent experiments. B, time course of Cdc42 activation in HMC-1 cells incubated with 100 μM forskolin (FORSK). Results are representative of three independent experiments.

Fig. 6. Effect of the PKA inhibitor H-89 on activation of Cdc42 by NECA or by CGS 21680 (CGS). HMC-1 cells were preincubated at 37°C for 40 min with 1 μM H-89 (+) or with its vehicle (−) before 1 min of stimulation with 10 μM NECA or 1 μM CGS 21680. Activation of Cdc42 was determined by affinity precipitation with GST-PBD followed by Western blotting as described under *Materials and Methods*. Results are representative of three independent experiments.
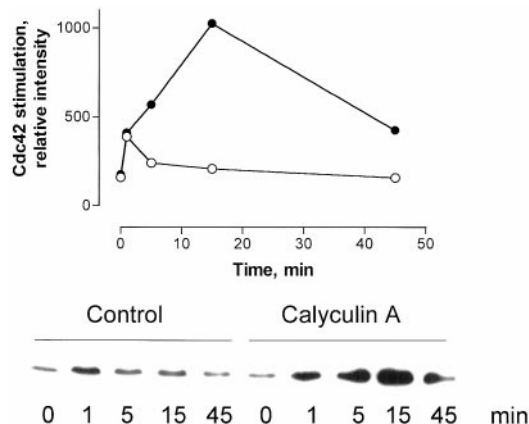
Fig. 5. Effect of calyculin A on activation of Cdc42 by NECA in HMC-1 cells. HMC-1 cells were preincubated at 37°C for 2 h with 50 nM calyculin A (closed symbols) or with its vehicle (open symbols) before stimulation with 10 μM NECA. Activation of Cdc42 was determined by affinity precipitation with GST-PBD followed by Western blotting and evaluated by increase in intensity of immunoreactive bands, using SigmaScan/Image software (Jandel Scientific, San Rafael, CA). Results are representative of three independent experiments.
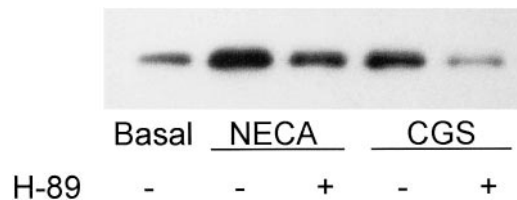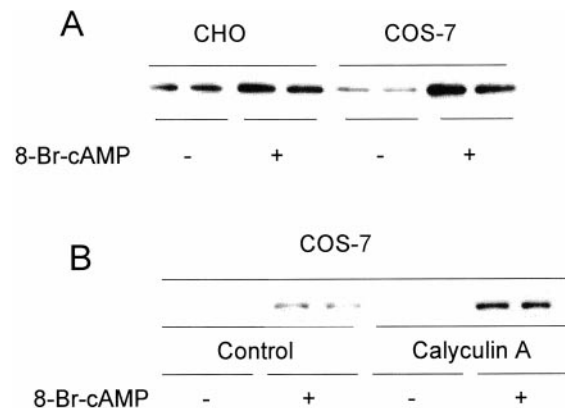
Fig. 7. 8-Br-cAMP activates Cdc42 in CHO-K1 and COS-7 cells. Activation of Cdc42 was determined by affinity precipitation with GST-PBD followed by Western blotting as described under *Materials and Methods*. A, CHO-K1 and COS-7 cells were incubated with the cell-permeable cAMP analog 8-Br-cAMP (10 μM) (+) or with its vehicle (−) for 15 min. B, COS-7 cells were preincubated at 37°C for 2 h with 50 nM calyculin A or with its vehicle (control) before stimulation with 10 μM 8-Br-cAMP (+). The film from these experiments was underexposed, compared with A, to emphasize the potentiation by calyculin A. Data are presented in duplicates. Results are representative of three independent experiments.

PKA activity was not sensitive to stimulation by cAMP. If anything, PKA activity was slightly decreased to 26.9 ± 1.1 pmol P_i/min/10^6 cells in the presence of 8-Br-cAMP. In cells expressing the constitutively active α-subunit of G_s, cAMP-stimulated PKA activity was the same (5.0 ± 0.6 pmol P_i/min/10^6 cells) as in mock-transfected cells. However, the basal PKA activity was higher in these cells (1.9 ± 0.8 pmol

P_i/min/10^6 cells) than in mock-transfected cells, possibly because of elevated cAMP concentrations still present in the cell lysates. Indeed, cells expressing the constitutively active α-subunit of G_s had 5 times higher intracellular cAMP concentrations compared with mock-transfected cells or cells expressing the catalytic subunit of PKA (Fig. 8B). As shown in Fig. 8C, expression of the catalytic subunit of PKA stimulated Cdc42 activity in COS-7 cells to levels similar to those produced by elevating intracellular cAMP concentrations, either by expression of the constitutively active α-subunit of G_s or by 8-Br-cAMP. These data confirm the concept that an increase in PKA activity can also stimulate Cdc42 in the absence of a rise in intracellular cAMP levels.

Cdc42 as a Substrate for PKA Phosphorylation. It has been reported that PKA phosphorylates the Cdc42-related small G-protein RhoA, thus regulating its active state (Lang et al., 1996). To investigate the possibility that Cdc42 may also be a target of PKA-mediated phosphorylation, a recombinant human GST-Cdc42 fusion protein absorbed onto glutathione-agarose was phosphorylated in vitro in the presence of the catalytic subunit of PKA. Beads were then washed extensively and boiled for 2 min in sample buffer, and proteins were subjected to SDS-PAGE. A recombinant human GST-RhoA was used as a positive control and GST alone was used as a negative control. The phosphoimage presented in Fig. 9A shows that both GST-Cdc42 and GST-RhoA were phosphorylated by PKA.

We used two different approaches to evaluate the stoichiometry of the phosphorylation reaction. First, we coupled recombinant GST fusion proteins or GST to glutathione-agarose (Lang et al., 1996). After incubation with the catalytic subunit of PKA in the presence of [^{32}P]ATP, the agarose beads were collected by centrifugation and washed, and radioactivity was assayed in a liquid scintillation counter. The radioactivity determined in GST samples was considered nonspecific and was subtracted from the corresponding values measured for GST-Cdc42 and GST-RhoA. The molar ratio of phosphate transferred to proteins was 8- to 10-fold lower for Cdc42 (0.005 and 0.006 pmol/pmol Cdc42) compared with RhoA (0.05 pmol/pmol RhoA). We then decided to exclude the possibility of potential interference of glutathione-
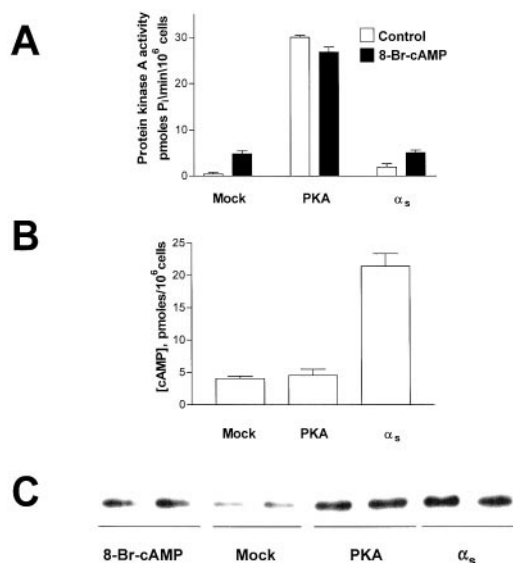

Fig. 8. Expression of PKA catalytic subunit activates Cdc42 in COS-7 cells. The COS-7 cells were transiently transfected with 1 μg of pFC-PKA, a plasmid encoding the catalytic subunit of PKA, with pcDNAI-α_s-Q227L, a plasmid encoding the constitutively active mutant of the Gα_s-subunit (α_s), or with an empty expression vector (Mock). A, PKA activity in COS-7 cells determined in the absence (clear bars) or in the presence of 10 μM 8-Br-cAMP (filled bars) 48 h after transfections. Values are presented as means $\pm$ S.E.M. of three experiments. B, intracellular cAMP concentrations in COS-7 cells 48 h after transfections. Values are presented as means $\pm$ S.E.M. of three experiments. C, activation of Cdc42 in COS-7 cells 48 h after transfection. Mock-transfected cells were incubated with 10 μM 8-Br-cAMP or with DMSO as vehicle (Mock) for 15 min. Cells expressing PKA or α_s were incubated with DMSO only. Activation of Cdc42 was determined by affinity precipitation with GST-PBD followed by Western blotting as described under *Materials and Methods*. Data are presented in duplicates. Results are representative of three independent transfections.
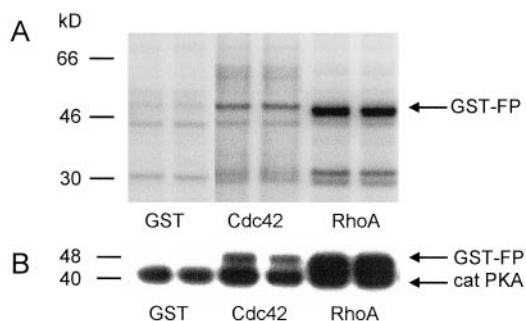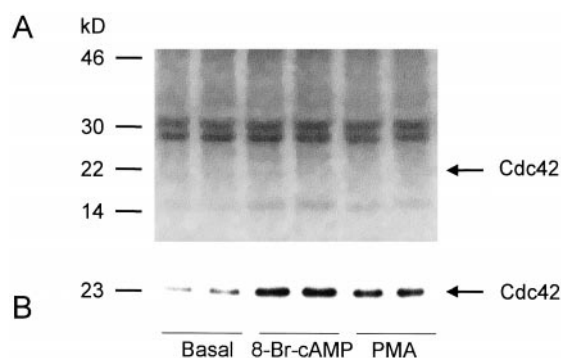

Fig. 9. In vitro phosphorylation of Cdc42 by PKA. Purified GST, GST-Cdc42, and GST-RhoA (1 μg) were incubated with 20 ng of PKA catalytic subunit (10 U) and [^{32}P]ATP for 30 min at 30°C. A, phosphorylated GST fusion proteins coupled to glutathione-agarose were separated by SDS-PAGE and analyzed using a phosphoimager. B, phosphorylated GST fusion proteins mixed with autophosphorylated PKA catalytic subunit were separated by SDS-PAGE and processed for autoradiography. Data are presented in duplicates. Results are representative of two independent experiments. Arrows indicate positions on the gels of GST-Cdc42 and GST-RhoA fusion proteins (GST-FP) and the catalytic subunits of PKA (cat PKA).

Fig. 10. Stimulation of Cdc42 with activators of PKA and PKC in COS-7 cells labeled with $^{32}P_i$. Metabolic labeling of COS-7 cells was accomplished by incubation with $^{32}P_i$ in phosphate-free medium for 4 h. The cells were then incubated in the absence or in the presence of 10 μM 8-Br-cAMP or 10 nM PMA for an additional 15 min. Cdc42 was precipitated from cell lysates with GST-PBD, separated by SDS-PAGE, and processed for Western blotting (B) and phosphoimaging (A). Data are presented in duplicates. Results are representative of two independent experiments. Arrows indicate positions of Cdc42 on the gels.

agarose with phosphorylation of bound proteins by PKA. Therefore, we initially incubated recombinant GST fusion proteins or GST with the catalytic subunit of PKA in the presence of [^{32}P]ATP and then precipitated with glutathione-agarose. The molar ratio of phosphate transferred to fusion proteins was 0.012 pmol/1 pmol Cdc42 and between 0.072 and 0.076 pmol/1 pmol RhoA.

This low level of phosphate incorporation into Cdc42 is further illustrated in Fig. 9B, which shows an autoradiograph of the reaction mixture separated by SDS-PAGE before glutathione-agarose precipitation. It is known that the catalytic subunit of PKA has only three potential sites for autophosphorylation (Francis and Corbin, 1994). The molecular ratio of the catalytic subunit of PKA to Cdc42 in the reaction mixture was 1:60. However, a 40-kDa band, corresponding to the catalytic subunit of PKA, produced a much stronger radioactive signal than the 48-kDa band corresponding to GST-Cdc42.

The lack of efficient phosphorylation of Cdc42 was also confirmed in studies using COS-7 cells preloaded with ^{32}P. Activation of Cdc42 as a result of its direct phosphorylation would show as ^{32}P incorporation into the active form of Cdc42. In this study we incubated cells with 10 μM 8-Br-cAMP to stimulate PKA, or with 10 nM phorbol 12-myristate 13-acetate (PMA) to stimulate protein kinase C, or with dimethyl sulfoxide (DMSO) as their vehicle, for 15 min. The active form of Cdc42 was precipitated in cell lysates with GST-PBD coupled to glutathione-agarose, subjected to SDS-PAGE, and processed for phosphoimaging and Western blotting. As shown in Fig. 10A, no detectable ^{32}P radioactivity was associated with the 23-kDa region of the phosphoimage, whereas Western blotting reveals an increase in active forms of 23-kDa Cdc42 in cells stimulated by 8-Br-cAMP or PMA (Fig. 10B). Phosphoimaging of this gel revealed two bands of ^{32}P labeling in 30-kDa region of the gel. However, these bands were irrelevant to activation of Cdc42 because they were present in all samples, including nonstimulated cells. Interestingly, we also observed ^{32}P labeling of a 15-kDa band that parallels the activation of Cdc42. It seems unlikely that this band represents a product of the proteolytic hydrolysis of Cdc42, because we did not detect phosphorylation of the full-length Cdc42 molecule on the same gel. The nature of this low-molecular mass band is not known, but it was co-precipitated with Cdc42 activated by either cAMP or phorbol ester. These results agree with our in vitro data that Cdc42 is a poor substrate for phosphorylation and suggest that cAMP-dependent stimulation may involve phosphorylation of some other proteins regulating the active state of Cdc42.

Discussion

There is accumulating evidence that adenosine receptors control cell growth (Sexl et al., 1997; Grant et al., 1999), cell differentiation (Abbracchio, 1996; Neary and Burnstock, 1996), apoptosis (Chow et al., 1997; Barbieri et al., 1998), and gene expression (Chae and Kim, 1997; Heese et al., 1997), all events commonly associated with activation of protein kinase cascades that serve as information relays connecting cell-surface receptors to specific transcription factors. These protein kinase cascades include three separate groups of MAPKs: ERK, Jun N-terminal kinase, and p38 MAPK. We recently demonstrated that A$_2$ adenosine receptors stimulate all three groups of MAPKs in HMC-1 human mast cells. We also showed that only the A$_{2B}$-subtype of adenosine receptors stimulated the small G-protein Ras, the upstream regulator of the ERK pathway. The effect appeared to be cAMP independent, because stimulation of A$_{2A}$ adenosine receptors, believed to be linked only to adenylate cyclase in these cells, did not activate Ras (Feoktistov et al., 1999). Our data, in part, were confirmed by another group of investigators, who demonstrated that expression of dominant negative mutant Ras(N17) in HEK-293 cells inhibited A$_{2B}$-mediated stimulation of ERK (Gao et al., 1999).

The upstream regulators of p38 MAPK and Jun N-terminal kinase pathways include, among others, Cdc42, a small G-protein of the Rho family (Bagrodia et al., 1995; Zhang et al., 1995). Because we observed adenosine-dependent activation of those pathways in human mast cells (Feoktistov et al., 1999), in this study we tested the hypothesis that adenosine receptors would also activate Cdc42. Our data present the first evidence that stimulation of adenosine receptors activates Cdc42. In contrast to Ras, which was activated only via A$_{2B}$ adenosine receptors (Feoktistov et al., 1999), Cdc42 appeared to be regulated by both subtypes of A$_2$ receptors. Whereas only A$_{2B}$ adenosine receptors are coupled to a heterotrimeric G-protein of the G$_q$ family in HMC-1 cells, both A$_{2A}$ and A$_{2B}$ receptors are coupled to G$_s$-protein and, upon activation, they increase intracellular cAMP levels (Feoktistov and Biaggioni, 1995). Therefore, we hypothesized that cAMP can activate Cdc42 in HMC-1 cells. Indeed, our results show that both stimulation of adenylate cyclase and incubation of HMC-1 cells with the cell-permeable cAMP analog 8-Br-cAMP increased the level of the GTP-bound form of Cdc42. Furthermore, stimulation of Cdc42 by cAMP appears to be a common mechanism shared by different cell types; our data demonstrate that cAMP-dependent stimulation of Cdc42 was also observed in CHO-K1 and COS-7 cells.

The functional activity of small G-proteins of the Ras superfamily is regulated by proteins that modulate their GDP/GTP-bound state. Activation of small G-proteins is mediated by GEFs, which promote the exchange of GDP for GTP. Inactivation of small G-proteins is accelerated by GTPase-activating proteins. Other regulatory proteins, GDP dissociation inhibitors, maintain unstimulated small G-proteins in a cytosolic GDP-bound state. All these regulatory proteins are possible sites of modulation by GPCR via various tyrosine kinases, protein kinase C, and cAMP. For example, cAMP binds to a GEF for Rap1 and thereby activates this small G-protein by a PKA-independent mechanism (de Rooij et al., 1998; Kawasaki et al., 1998). cAMP can also modulate the activity of small G-proteins directly by PKA-mediated phosphorylation. This mechanism has been linked to inhibition of RhoA by cAMP (Lang et al., 1996; Laudanna et al., 1997; Dong et al., 1998).

Several lines of evidence indicate that cAMP stimulates Cdc42 by a PKA-dependent mechanism. First, inhibition of protein phosphatases 1 and 2A with calyculin A potentiated the effects of NECA and 8-Br-cAMP. Second, activation of Cdc42 via A$_{2A}$ and A$_{2B}$ receptors was attenuated in the presence of the selective PKA inhibitor H-89. Third, expression of the catalytic subunit of PKA stimulated Cdc42. Taken together, these results support our hypothesis that adenosine can stimulate Cdc42 by a cAMP/PKA-dependent mechanism.

The data presented here is the first evidence of cross-talk

between cAMP and Cdc42. It is unknown how widespread this phenomenon is, but we found it not only in HMC-1 but also in CHO-K1 and COS-7 cells. It has been proposed that PKA can directly modulate activity of some small G-proteins. Activation of Rap1 and inhibition of RhoA were ascribed to their phosphorylation by PKA (Lang et al., 1996; Vossler et al., 1997). However, we found that Cdc42 is a poor substrate for PKA phosphorylation in vitro. We also showed that Cdc42 activated by 8-Br-cAMP was not labeled with radioactivity in COS-7 cells preloaded with $^{32}P_i$. We suggest that PKA does not phosphorylate Cdc42 directly. Instead, the proteins that modulate the GDP/GTP-bound state of Cdc42 may be the primary targets of PKA phosphorylation. There are multiple potential regulators of Cdc42 in mammalian cells (for review, see Johnson, 1999). It remains to be determined which of them can be regulated by PKA phosphorylation.

It appears that activation of Cdc42 by GPCR can be mediated through different signaling pathways. It has been shown that protein kinase C can activate Cdc42 in human leukocytes. Furthermore, the formyl-Met-Leu-Phe-stimulated activation of Cdc42 can be blocked by tyrosine kinase inhibitors (Benard et al., 1999a). Our finding, that cAMP can activate Cdc42, extends the spectrum of possible pathways involved in transducing a signal from a GPCR to Cdc42 and reveals a link between Cdc42-mediated signaling and adenosine A_2 receptors.

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