Functional coupling of adenosine A_{2a} receptor to inhibition of the mitogen-activated protein kinase cascade in Chinese hamster ovary cells

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Activation of G_s-coupled receptors enhances the increase in cyclic AMP mediated by adenylate cyclases. As it has been shown that cyclic AMP inhibits the epidermal growth factor-activated mitogen-activated protein kinase (MAPK) signalling pathway, stimulation of G_s-coupled receptors may lead to the inhibition of MAPK activation. To investigate the effect of a G_s-coupled receptor on the MAPK cascade, we cloned the adenosine (Ado) A_{2a} receptor from a guinea-pig leucocyte cDNA library, and established Chinese hamster ovary (CHO) cells stably expressing the receptor (CHOAdoA2R). The [³H]5'-N-ethylcarbamoyladenosine (NECA) binding characteristics ($K_d = 91.0 \pm 5.4$ nM, $B_{max} = 707 \pm 11$ fmol/mg of protein, $n = 3$) and NECA-induced cyclic AMP production indicate that the cloned Ado A_{2a} receptor was functionally expressed in the cells. In CHO cells, thrombin induced intracellular Ca²⁺ increase and

MAPK activation through the intrinsic G-coupled receptor. In CHOAdoA2R cells, NECA partially inhibited thrombin-elicited MAPK activation. When combining NECA-treatment with 1,2-bis-(*o*-aminophenoxy)ethane-*N,N,N',N'*-tetra-acetic acid acetoxymethyl ester (BAPTA-AM) loading, a nearly complete inhibition of the MAPK activation occurred. Forskolin also partially inhibited the MAPK activation and synergized with BAPTA-AM, suggesting that partial inhibition of MAPK activation by NECA results from cyclic AMP production via Ado A_{2a} receptor activation. The same synergism of MAPK inhibition between wortmannin and BAPTA-AM was observed, but not between wortmannin and NECA. These results suggest that cyclic AMP production through Ado A_{2a} receptor inhibits thrombin-elicited MAPK activation by a Ca²⁺-independent/wortmannin-sensitive pathway in CHO cells.

INTRODUCTION

Mitogen-activated protein kinases (MAPKs), also known as extracellular signal-regulated kinases (ERKs), are a family of protein-serine/threonine kinases that become activated in response to various extracellular stimuli such as various growth factors [1,2]. MAPKs can also be activated by agonists acting through G-protein-coupled receptors. For example, in neutrophils, chemoattractants such as *N*-formyl-methionyl-leucyl-phenylalanine [3], platelet-activating factor (PAF) [4], and the activated complement component C5a [5], whose receptors are coupled to G_i/G_q-protein(s), activate MAPKs. MAPKs are activated by phosphorylation of both threonine and tyrosine residues [6] by MAPK kinase [7] also known as MEK [8]. MEK itself is activated by upstream kinases. Two activators of MEK are known: Raf-1, which phosphorylates MEK in response to growth factor stimulation [9,10], and MEK kinase [11], for which an upstream activator has not yet been found.

In Rat1 fibroblasts, cyclic AMP inhibits the epidermal growth factor-activated MAPK signalling pathway by inhibiting Raf-1 kinase activity [12,13]. Raf-1 is activated by Ras [14,15] and Ca²⁺-dependent protein kinase C (PKC) [16]. However, it is not clear whether cyclic AMP also inhibits MAPK activation through G_i/G_q-coupled receptors. Moreover, since artificial cell-permeable cyclic AMP analogues were used in the previous reports, it is debatable whether cyclic AMP actually inhibits MAPK activation *in vivo*. In general, occupancy of G_s-coupled

receptors enhances the increase in cyclic AMP mediated by adenylate cyclases. If cyclic AMP actually inhibits MAPK activation *in vivo*, ligand binding to G_s-coupled receptors should inhibit MAPK activation. In an attempt to confirm the above notion, we isolated a cDNA encoding adenosine (Ado) A_{2a} receptor, a family of G_s-coupled receptors, from a guinea-pig leucocyte cDNA library and established Chinese hamster ovary (CHO) cells stably expressing the receptor (CHOAdoA2R).

Here, we report evidence that the thrombin receptor, which is coupled to a G-protein, mediates activation of MAPK, through at least two distinct pathways, one Ca²⁺-dependent and the other Ca²⁺-independent, in the CHO cells. Cyclic AMP production through the Ado A_{2a} receptor inhibited the thrombin-induced MAPK cascade by a Ca²⁺-independent pathway. We further demonstrate that wortmannin also inhibits MAPK activation, on a target directed to the Ca²⁺-independent pathway.

MATERIALS AND METHODS

Oligonucleotide primers and PCR amplification

Taq DNA polymerase and all other PCR reagents were purchased from Pharmacia. Two oligonucleotides corresponding to the third and the sixth transmembrane region of human Ado A_{2a} receptor cDNA sequences [17] (forward: 5'-ATTGCCATCCG-CATCCCGCTCCGG-3', reverse: 5'-CCCCACAATGATGGC-

Abbreviations used: Ado, adenosine; BAPTA-AM, 1,2-bis-(*o*-aminophenoxy)ethane-*N,N,N',N'*-tetra-acetic acid acetoxymethyl ester; CHO, Chinese hamster ovary; MBP, myelin basic protein; MAPK, mitogen-activated protein kinase; NECA, 5'-*N*-ethylcarbamoyladenosine; PAF, platelet-activating factor; PI 3-kinase, phosphatidylinositol 3-kinase; PKC, protein kinase C; PLA₂, phospholipase A₂; R-PIA, *N*⁶-[(*R*)-1-methyl-2-phenylethyl]adenosine.

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The nucleotide sequence data for guinea-pig mRNA for adenosine A_{2a} receptor will appear in the GenBank DNA database under the accession number D63674.

CAGTGACTT-3') were synthesized. Template cDNAs were synthesized using mRNAs derived from HL-60 cells. One μg of the cDNA templates, 100 pmol of each primer, 20 pmol of each dNTP and 5 units of *Taq* DNA polymerase were used in a 100 μl -volume reaction. The PCR temperature cycle was set as follows: for the first cycle, denaturation at 94 °C for 5 min, annealing at 60 °C for 2 min, extension at 72 °C for 1 min, and for the next 30 cycles, denaturation at 94 °C for 1 min, annealing at 60 °C for 2 min, extension at 72 °C for 1 min. An amplified DNA fragment (411 bp, termed hAR) was subcloned into pBluescript SK⁻ (Stratagene) for sequencing analysis and for preparing ³²P-labelled probes for the plaque hybridization.

Cloning of guinea-pig Ado A_{2a} receptor cDNA

About 3×10^5 plaques from the guinea-pig leucocyte cDNA library [λ ZAPII (Stratagene)] were screened with hAR fragment ³²P-labelled by random priming. Clones were analysed by restriction digestion and sequence analysis using the dideoxy chain termination method on double-stranded template [18,19].

Expression of guinea-pig Ado A_{2a} receptor in CHO cells

A full-length cDNA fragment of the guinea-pig Ado A_{2a} receptor was excised and subcloned into expression vector pcDNA1NEO carrying a neomycin-resistant gene. The resultant plasmid was transfected into CHO-K1 cells by electroporation, and the transformants were selected in culture medium [Ham's F-12 (Nissui) containing 10% (v/v) fetal-calf serum, 100 units/ml penicillin, 100 $\mu\text{g}/\text{ml}$ streptomycin] supplemented with 1 mg/ml geneticin. Geneticin-resistant clones were isolated by limiting dilution and the receptor expression was confirmed by Northern-blot analysis. The clone most highly expressing Ado A_{2a} receptor mRNA (termed CHOAdoA2R) was maintained in culture medium supplemented with 0.2 mg/ml geneticin.

Membrane preparation

Membrane preparation was carried out as described [20]. Cells at subconfluence were scraped into ice-cold homogenization buffer (25 mM Hepes/NaOH, pH 7.4, 0.25 M sucrose, 10 mM MgCl₂) and homogenized. The 1000 g supernatant of the homogenate was centrifuged at 100000 g for 1 h at 4 °C, and the pellet was resuspended in homogenization buffer at 5–10 mg/ml protein and stored at –80 °C until use.

[³H]5'-N-ethylcarbamoyl-adenosine (NECA) binding assay

The [2,8-³H]NECA (DuPont NEN, 1.11 TBq/mmol) binding assays were performed as described previously [21] by incubation of the membranes (0.6–0.7 mg of protein/ml) at 25 °C in the binding assay buffer (25 mM Hepes/NaOH, pH 7.4, 10 mM MgCl₂, 0.1% BSA) including [2,8-³H]NECA (15–240 nM). After the mixture had stood at 25 °C for 2 h, reactions were terminated by addition of the ice-cold binding assay buffer (2 ml). Free and bound [2,8-³H]NECA were separated by rapid filtration through GF/C filters (Whatman), which were washed three times with 3 ml each of the ice-cold binding assay buffer. When necessary, the non-specific binding was determined in the presence of a 300-fold excess of unlabelled NECA.

Cyclic AMP production

Cells (5×10^3) were seeded on a 96-well plate, incubated for 48 h, washed with Ham's F-12 medium, and preincubated with Ham's F-12 medium containing 0.5 mM 3-isobutyl-1-methylxanthine

for 20 min. The cells were stimulated with various concentrations of NECA or *N*⁶-[(*R*)-1-methyl-2-phenylethyl]adenosine (*R*-PIA) for 20 min. The reaction was terminated by the addition of HCl (final concentration, 0.1 M). The plate was stored at –80 °C overnight. The supernatants were collected and their pH values adjusted to 7.0. Cyclic AMP levels were determined by using the BIOTRAK[®] cAMP enzyme immunoassay (EIA) system (Amersham) according to the manufacturer's instructions.

MAPK assay, Ca²⁺ measurement and arachidonic acid release

MAPK assays were conducted as described in [22]. After purification with Q-Sepharose beads, the sample was incubated with myelin basic protein (MBP) (1 mg/ml) in 25 μl of kinase assay buffer (20 mM Tris/HCl, pH 7.5, 2.5 mM MgCl₂, 250 μM MnCl₂, 25 μM protein kinase inhibitor and 50 μM ATP) containing 3.7 kBq of [γ -³²P]ATP for 25 min at 25 °C. A 10 μl aliquot was spotted on to P81 phosphocellulose paper (Whatman) and washed extensively with 0.5% phosphoric acid. The paper was dried, and the ³²P incorporation into MBP was measured by Cerenkov counting.

The intracellular calcium concentration was determined by loading cells with 3 μM fura-2-penta-acetoxymethyl ester (Dojindo, Kumamoto, Japan) and using a CAF-110 spectrofluorometer (JASCO) as described in [23].

Release of arachidonic acid was measured as described elsewhere [24,25]. Cells (5×10^4) were seeded on a 24-well plate, incubated for 24 h and labelled with [5,6,8,9,11,12,14,15-³H]arachidonic acid (DuPont NEN, 2.22 TBq/mmol) for another 24 h. After washing the cells twice with Hanks' balanced salt solution (pH 7.4) containing 0.1% BSA and preincubation with Ham's F-12 medium containing 0.1% BSA for 20 min, the reaction was initiated by addition of thrombin (final concentration, 0.1 unit/ml) and various ligands. After 20 min, the radioactivity released into the medium was determined.

RESULTS AND DISCUSSION

Isolation and expression of guinea-pig Ado A_{2a} receptor

From pharmacological aspects, the presence of Ado A_{2a} receptor on neutrophils has been indicated [26–28]. To investigate the effect of Ado A_{2a} receptor on the MAPK cascade, we isolated an Ado A_{2a} receptor cDNA from a guinea-pig leucocyte cDNA library using hAR fragment as a probe. The cloned cDNA encoded a 409-amino-acid sequence which has two amino acid residue substitutions from that reported for the guinea-pig Ado A_{2a} receptor from the brain: Ser²⁶⁸ → Tyr and Met²⁶⁹ → Leu [29]. The relative abundance of mRNAs for the Ado A_{2a} receptor in various tissues was investigated by Northern-blot analysis using the coding region of the cloned cDNA as a probe. As previously reported, the Ado A_{2a} receptor cDNA probe mainly hybridized with a 3.0 kb mRNA in brain and spleen. It is noteworthy that Ado A_{2a} receptor mRNA was abundantly expressed in neutrophils as well as these tissues (results not shown).

To elucidate the pharmacological properties of the cloned receptor, a stable transformant was constructed using CHO-K1 cells (termed CHOAdoA2R). The membranes prepared from CHOAdoA2R cells displayed a dose-dependent and saturable binding of [³H]NECA (Figure 1A). Scatchard analysis revealed that dissociation constant (*K*_d) and the maximal binding (*B*_{max}) were 91.0 ± 5.4 nM and 707 ± 11 fmol/mg of protein, respectively (mean $\pm$ S.D., *n* = 3, Figure 1B). The *K*_d value was comparable with that of rat brain receptor [30]. It is known that stimulation of Ado A_{2a} receptors activates adenylate cyclase to increase cyclic AMP levels [31]. We confirmed that Ado A_{2a} receptor

Figure 1 Characterization of the guinea-pig Ado A_{2a} receptor expressed in CHOAdoA2R cells

(A) Saturation isotherm of specific bindings of the CHOAdoA2R membrane fractions. Results are from one of three independent experiments. Each point is the mean of three assays $\pm$ S.D. (B) Scatchard analysis of [³H]NECA binding. K_d and B_{max} values are 85 nM and 707 fmol/mg of protein respectively. Results are from one of three independent experiments. Each point is the mean of three assays. (C) Comparative effect of adenosine analogues on cAMP production in CHOAdoA2R cells: CHOAdoA2R cells were incubated at 37 °C in the presence of 0.5 mM 3-isobutyl-1-methylxanthine (IBMX) with either NECA (●) or R-PIA (□) at various concentrations. After 20 min, the incubations were terminated by adding one-tenth the volume of 1 M HCl, followed by freezing at -80 °C. After thawing and centrifugation, the cyclic AMP contents of the supernatants were determined as described in the Materials and methods section. Results are from one of three independent experiments. Each point is the mean of three assays $\pm$ S.D.

agonists (NECA and R-PIA) triggered cyclic AMP production in CHOAdoA2R cells in dose-dependent manners (ED_{50} around 100 nM each) (Figure 1C). A ligand-stimulated adenylate cyclase activity exhibited the expected Ado A_{2a} receptor pharmacology, i.e. an effective order of NECA > R-PIA. Maximal accumulations of cyclic AMP by NECA (10 μ M) and R-PIA (10 μ M) were around 400 pmol/10⁶ cells and 200 pmol/10⁶ cells respectively. In the mock-transfected control cells, cyclic AMP production by NECA or R-PIA was below the detectable limits of the assays used in this study (results not shown). These results suggest that the cloned Ado A_{2a} receptor was functionally expressed in CHOAdoA2R cells.

Effect of NECA on thrombin-induced MAPK activation in CHOAdoA2R cells

As shown in Figures 2(A) and 2(B), in both CHOAdoA2R and mock-transfected control cells, MAPK was activated by thrombin (0.1 unit/ml) through its intrinsic G-coupled receptor, peaking at 5 min after stimulation and decreasing thereafter (4.19 ± 0.29 - and 4.23 ± 0.10 -fold increase respectively; mean $\pm$ S.D., $n = 3$). Pretreatment of CHOAdoA2R cells with NECA partially inhibited MAPK activation in response to

Figure 2 Effect of NECA on thrombin-stimulated MAPK activation in CHOAdoA2R (A) and mock-transfected control (B) cells, and dose-dependent inhibition of thrombin-induced MAPK activation by NECA in CHOAdoA2R cells (C)

(A) and (B) Quiescent CHOAdoA2R and mock-transfected control cells were treated for 20 min at 37 °C with (CHOAdoA2R, ●; Mock, ■) or without (CHOAdoA2R, △; Mock, ◇) 1 μ M NECA in the presence of 0.5 mM 3-isobutyl-1-methylxanthine (IBMX), followed by 0.1 unit/ml thrombin stimulation. The reactions were terminated at indicated times by freezing at -80 °C. Cell lysates were analysed for MAPK as described in the Materials and methods section. Each point and bar is the mean of triplicate assays $\pm$ S.D. Both results are from one of three independent experiments. ** $P < 0.01$; *** $P < 0.001$ versus non-treatment (Student's t test). (C) CHOAdoA2R cells were incubated with NECA at indicated concentrations for 20 min at 37 °C in the presence of 0.5 mM IBMX, and stimulated with 0.1 unit/ml thrombin for 5 min. Cell lysates were analysed for MAPK using a Biotrak[®] p42/p44 MAP kinase enzyme assay system (Amersham) according to the manufacturer's instructions. Each point and bar is the mean of three assays $\pm$ S.D. The basal activity (0% of control) corresponds to 18206 c.p.m., and 100% activation with thrombin was 194627 c.p.m. Results are from one of three independent experiments. ** $P < 0.01$ versus 100% activation (Student's t test).

thrombin (Figure 2A). On the other hand, in mock-transfected control cells, the inhibition was not observed (Figure 2B). Thus, this inhibitory effect was due to the activation of Ado A_{2a} receptor. As shown Figure 2(C), NECA inhibited thrombin-induced MAPK activation in a dose-dependent manner. Half-maximal inhibition was observed around 10 nM. A maximal inhibition of thrombin-induced MAPK activation by NECA was observed about 1 μ M (62.9 ± 8.1 % inhibition; mean $\pm$ S.D., $n = 3$, Figure 2C).

Inhibition of thrombin-induced MAPK activation by various inhibitors

Various inhibitors of MAPK cascade activation, such as 1,2-bis-(*o*-aminophenoxy)ethane-*N,N,N',N'*-tetra-acetic acid acetoxymethyl ester (BAPTA-AM) [4], forskolin [12,32] and wortmannin [4,25], have been reported. BAPTA-AM acts on Ca²⁺-dependent pathways by chelating Ca²⁺, and the effect of forskolin is due to the accumulation of cyclic AMP. To investigate the signal-transduction mechanism of thrombin-induced MAPK activation in CHO cells, the effects of these inhibitors on MAPK activation

Figure 3 Dose-dependent inhibition of thrombin-induced MAPK activation by BAPTA-AM (A), forskolin (B) or wortmannin (C)

After incubation with these inhibitors at indicated concentrations for 60 min (BAPTA-AM) or 20 min (forskolin or wortmannin) at 37 °C, quiescent CHOAdoA2R cells were stimulated with thrombin (0.1 unit/ml) for 5 min. Cell lysates were analysed for MAPK using a BIOTRAK® p42/p44 MAP kinase enzyme assay system (Amersham) according to the manufacturer's instructions. Each point and bar is the mean of three assays $\pm$ S.D. The basal activity (0% of control) and 100% activation with thrombin was the same as in Figure 2. All results are from one of three independent experiments. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ versus 100% activation (Student's t test).

were tested. As shown in Figure 3, all these inhibitors inhibited thrombin-induced MAPK activation in dose-dependent manners. Half-maximal inhibitions by BAPTA-AM, forskolin and

Table 1 Inhibition of thrombin-induced MAPK activation in CHOAdoA2R cells after various treatments

After pretreatment by various inhibitors at 1 μM (NECA) or 10 μM (BAPTA-AM, forskolin or wortmannin) for 20 min (NECA, forskolin or wortmannin) or 60 min (BAPTA-AM) at 37 °C, quiescent CHOAdoA2R cells were stimulated with thrombin (0.1 unit/ml) for 5 min. MAPK activities in the cell lysate were determined as described in the Materials and methods section. The basal activity of non-stimulated cells (2070 ± 121 c.p.m.) was taken as 0%. Each datum is the mean $\pm$ S.D. ($n = 3$). This experiment was performed three times with essentially identical results.

Treatment	Activity (c.p.m.)	% of control
None	8723 ± 426	100
NECA	4820 ± 316^a	41.3
BAPTA	5027 ± 586^a	44.4
Forskolin	5150 ± 370^a	46.3
Wortmannin	5357 ± 437^a	49.4
NECA + BAPTA	3037 ± 172^b	14.5
Forskolin + BAPTA	3023 ± 266^c	14.3
Wortmannin + BAPTA	3483 ± 325^d	21.2
Wortmannin + NECA	5190 ± 273^e	46.9

^a $P < 0.01$ versus none, Student's t test.

^b $P < 0.01$ versus NECA alone, Student's t test.

^c $P < 0.01$ versus forskolin alone, Student's t test.

^d $P < 0.01$ versus wortmannin alone, Student's t test.

^e Not significant versus NECA alone, Student's t test.

wortmannin were observed around 20 nM, 40 nM and 50 nM respectively, and had a maximal inhibitory effect on MAPK activation around 10 μM ($56.1 \pm 2.0\%$ by BAPTA-AM, $63.4 \pm 2.2\%$ by forskolin and $59.3 \pm 4.7\%$ by wortmannin; mean $\pm$ S.D., $n = 3$, Figures 3A, 3B and 3C).

Two distinct pathways of thrombin-induced MAPK activation in CHO cells

Since the above inhibitors, including NECA, could not inhibit thrombin-induced MAPK activation completely (55–65% inhibition), it can be proposed that there are multiple pathways in thrombin-induced MAPK activation in CHO cells. To confirm this hypothesis, these inhibitors were used in combination to inhibit MAPK activation (Table 1). As described above, BAPTA-AM, forskolin or wortmannin at 10 μM partially inhibited MAPK activation when used separately. However, when combining BAPTA-AM loading with NECA or forskolin, a nearly complete inhibition occurred, indicating that NECA and forskolin act on Ca^{2+} -independent pathways. Since cyclic AMP production was stimulated by NECA (Figure 1C) and forskolin in CHOAdoA2R cells, cyclic AMP production by NECA might lead to the inhibition of thrombin-induced MAPK activation.

Activation of MAPK involves an array of signals that ultimately converge on MEK. At least two mechanisms are defined for activation of MEK [1,2,24,33]; Ras-dependent activation of Raf-1 that is an immediate upstream activator of MEK [5,32,34–37] and PKC α -Raf-1 axis [16,38,39]. Since NECA pretreatment did not affect thrombin-stimulated intracellular Ca^{2+} increases in CHOAdoA2R cells (Figure 4A), NECA did not appear to change conventional PKC activity. On the other hand, BAPTA-AM loading completely inhibited thrombin-stimulated Ca^{2+} increase (Figure 4A). BAPTA-AM loading partially inhibited thrombin-stimulated MAPK activity and strongly synergized with NECA or forskolin to inhibit MAPK activation, indicating the presence of two distinct pathways of thrombin-induced MAPK activation; one that is Ca^{2+} -dependent and the

Figure 4 Effect of intracellular Ca²⁺ increase on MAPK activation

(A) Effect of NECA and BAPTA-AM on thrombin-elicited Ca²⁺ increase. Representative traces of intracellular Ca²⁺ increase by 0.1 unit/ml thrombin in fura-2-loaded CHOAdoA2R cells. (1) Non-treatment, (2) pretreatment with NECA (1 μM, 20 min at 37 °C) in the presence of 0.5 mM 3-isobutyl-1-methylxanthine (IBMX), and (3) loading with BAPTA-AM (10 μM, 60 min at 37 °C). Results are from one of three independent experiments. (B) Effect of A23187 on the MAPK activity. CHOAdoA2R cells were incubated with A23187 at indicated concentrations for 20 min at 37 °C. Cell lysates were analysed for MAPK using a BIOTRAK® p42/p44 MAP kinase enzyme assay system (Amersham). Each point and bar is the mean of three assays ± S.D. Results are from one of three independent experiments. ****P* < 0.001 versus non-treatment (Student's *t* test).

other that is Ca²⁺-independent/cyclic AMP-sensitive. Indeed, pretreatment of CHOAdoA2R cells with A23187, a Ca²⁺ ionophore, activated MAPK in a dose-dependent manner (Figure 4B), suggesting the presence of a Ca²⁺-dependent MAPK activation pathway in CHO cells. Although, Cook and McCormick [13] reported that cyclic AMP inhibited the interaction between Ras and Raf-1, the real target molecule(s) of cyclic AMP-dependent inhibition of MAPK by NECA remains unclear.

Recently, Ferby et al. [4] and Sakanaka et al. [25] found that PAF- and somatostatin-stimulated MAPK activations are mediated by Ca²⁺-dependent and -independent pathways. They reported that the Ca²⁺-independent pathway was completely inhibited by wortmannin. To confirm the relationship between the NECA-sensitive MAPK cascade and the wortmannin-sensitive one in CHOAdoA2R cells, wortmannin was used in combination with other inhibitors. In CHOAdoA2R cells, wortmannin (10 μM) partially inhibited thrombin-induced MAPK activation (Table 1). When combining BAPTA-AM loading with wortmannin pretreatment, a nearly complete inhibition occurred.

Table 2 Inhibition of thrombin-induced arachidonic acid release in CHOAdoA2R cells after various treatments

Pretreatments of the cells were the same as described in Table 1. Then cells were stimulated with thrombin (0.1 unit/ml) for 20 min. Radioactivity released into medium was determined as described in the Materials and methods section. The basal activity of non-stimulated cells (656 ± 134 d.p.m.) was taken as 0%. Each datum is the mean ± S.D. (*n* = 3). This experiment was performed three times with essentially identical results.

Treatment	Radiolabel released (d.p.m.)	Percentage of control
None	8618 ± 1170	100
NECA	5168 ± 208 ^a	56.7
BAPTA	4704 ± 272 ^a	50.8
Wortmannin	5749 ± 1629 ^a	63.9
NECA + BAPTA	2949 ± 686 ^b	28.8
Wortmannin + BAPTA	3153 ± 185 ^c	31.4

^a *P* < 0.01 versus none, Student's *t* test.

^b *P* < 0.01 versus NECA alone, Student's *t* test.

^c *P* < 0.01 versus wortmannin alone, Student's *t* test.

However, the combinational use of wortmannin and NECA did not inhibit MAPK activation further, indicating that wortmannin, as well as NECA, acts on a Ca²⁺-independent pathway of thrombin-induced MAPK activation. Recently, it has been reported that thrombin activates phosphatidylinositol 3-kinase (PI 3-kinase) through G-protein βγ-subunits [40]. Moreover, Koch et al. [41] suggested that Ras activation was mediated by βγ-subunits of G_i-protein and leads to MAPK activation. Although further experiments will be required to elucidate the signalling molecules for Ras-dependent MAPK activation pathway, our results and the above observation suggest the possible involvement of a wortmannin-sensitive molecule such as PI 3-kinase in the Ca²⁺-independent pathway of thrombin-elicited MAPK activation.

Inhibitors of MAPK activation reduce thrombin-induced arachidonic acid release

Cytosolic phospholipase A₂ (PLA₂) or other types of PLA₂ mediate the rapid production of arachidonic acid in response to a number of agonists, such as PAF, thrombin, ATP, phorbol ester or Ca²⁺ ionophore [42]. Lin et al. [43] have shown that MAPKs are involved in the agonist-induced release of arachidonic acid by phosphorylation of cytosolic PLA₂. Thus, we investigated whether inhibition of MAPK activation affects thrombin-elicited arachidonic acid release in CHOAdoA2R cells. We examined the effect of NECA, BAPTA-AM or wortmannin on the thrombin-induced PLA₂ activation by monitoring the release of [³H]arachidonic acid and its metabolites into the culture medium. In CHOAdoA2R cells, the ratio of thrombin (0.1 unit/ml)-elicited net release against total [³H]arachidonic acid incorporation was 4.2%. Thrombin-induced arachidonic acid release was partially inhibited by these inhibitors (Table 2). As in the case of MAPK, combination of NECA or wortmannin pretreatment and BAPTA-AM loading reduced arachidonic acid release more prominently. The similar behaviour of the MAPK activation and arachidonic acid release shows the possibility that the activation of Ado A_{2a} receptor regulates arachidonic acid release by the inhibition of MAPK. The inhibitory effect of NECA on thrombin-induced arachidonic acid release was, however, weaker than that on MAPK activation. This suggests the presence of an MAPK-independent mechanism of PLA₂ ac-

tivation. Kramer et al. [44] observed that, in human platelets, proline-directed kinases other than MAPK phosphorylate and activate cPLA₂. These kinases might be also involved in MAPK-independent cPLA₂ activation in CHO cells.

In summary, we investigated the effect of Ado A_{2a} receptor activation on the MAPK cascade using CHO-K1 cells expressing the cloned receptor. The results of this study provide evidence that Ado-stimulated cyclic AMP production regulates MAPK activity. The remarkable synergism observed with intracellular cyclic AMP production and Ca²⁺-chelating suggests that thrombin activates MAPK through at least two distinct pathways, a Ca²⁺-dependent and a Ca²⁺-independent/cyclic AMP-sensitive one. In contrast to the previous reports on the inhibition of Raf-1 kinase by cyclic AMP [13], our data show clearly that NECA-induced cyclic AMP has a target molecule(s) common to the wortmannin-sensitive pathway. As this report shows that activation of Ado A_{2a} receptor inhibits arachidonic acid release and the Ado A_{2a} receptor is highly expressed in guinea-pig neutrophils, adenosine might play anti-inflammatory roles *in vivo*.

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