A₃ Adenosine Receptor Activation Inhibits Cell Proliferation via Phosphatidylinositol 3-Kinase/Akt-dependent Inhibition of the Extracellular Signal-regulated Kinase 1/2 Phosphorylation in A375 Human Melanoma Cells*

Received for publication, December 7, 2004, and in revised form, March 8, 2005. Published, JBC Papers in Press, March 17, 2005, DOI 10.1074/jbc.M413772200

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Adenosine exerts its effects through four subtypes of G-protein-coupled receptors: A₁, A₂A, A₂B, and A₃. Stimulation of the human A₃ receptor has been suggested to influence cell death and proliferation. The phosphatidylinositol-3-OH kinase (PI3K)/Akt and the Raf/mitogen-activated protein kinase (MAPK/ERK) kinase (MEK)/mitogen-activated protein kinase (MAPK) pathways have central roles in the regulation of cell survival and proliferation. Due to their importance, the cross-talk between these two pathways has been investigated. Here, we show that the A₃ adenosine receptor agonist CI-IB-MECA stimulates PI3K-dependent phosphorylation of Akt leading to the reduction of basal levels of ERK1/2 phosphorylation, which in turn inhibits cell proliferation. The response to CI-IB-MECA was not blocked by A₁, A₂A, or A₂B receptor antagonists, although it was abolished by A₃ receptor antagonists. Furthermore, the response to CI-IB-MECA was generated at the cell surface, since the inhibition of A₃ receptor expression, by using small interfering RNA, abolished agonist effects. Using A375 cells, we show that A₃ adenosine receptor stimulation results in PI3K-dependent phosphorylation of Akt, leading to the reduction of basal levels of ERK1/2 phosphorylation, which in turn inhibits cell proliferation.

Recent studies have hypothesized a role of adenosine in promoting the development and growth of tumor masses (1, 2). An increasing amount of work suggests a contradictory role of adenosine in the viability of the normal and cancer cells (1–3). General opinion is that adenosine’s antiproliferative effect is due to the stimulation of the four adenosine receptor subtypes named A₁, A₂A, A₂B, and A₃, which are coupled to opposite signal transduction pathways (4–6). In particular, it has been demonstrated that A₃ adenosine receptor agonists (the natural ligand adenosine and synthetic analogues) protect cells from apoptosis and interfere with cell proliferation (7–12). One of the different mechanisms through which A₃ adenosine receptors are able to inhibit cell proliferation was found to involve inhibition of telomerase activity and a cell cycle arrest in the G₀/G₁ phase, leading to a cytostatic effect (7, 13, 14). Furthermore, it has been demonstrated that the antigrowth signal exerted by A₃ receptors blocks cells into Gi-late cell cycle phase (9). Prompted by the antiproliferative effect of A₃ adenosine receptor stimulation, adenosine derivatives have been examined in vivo for their ability to suppress tumor growth with success demonstrated in different experimental tumor models in mice (15, 16).

The molecular pathway sustaining the antiproliferative action of A₃ receptor has been defined. The activation of this Gi-coupled receptor increases Ca²⁺ intracellular levels and decreases cAMP concentration (17, 18), and recently, it has been demonstrated that it can activate the phosphatidylinositol 3-kinase (PI3K)/Akt pathway (8). On the other hand, it has been reported that A₃ stimulation correlates with the protein kinase A, Akt, c-myc, and cyclin D down-regulation (19).

The other branch of the adenosine signaling cascade has been demonstrated with studies performed in Chinese hamster ovary cells transfected with the human A₃ adenosine receptor (CHO-A₃) (20–23) and in microglia cells (24). These studies show that A₃ receptor signaling in CHO cells leads to stimulation of ERK1/2 phosphorylation and activity (20, 21). In particular, A₃ receptor signaling to ERK1/2 depends on βγ release from pertussis toxin-sensitive G proteins, PI3K, Ras, and Rad-18 binding domain; ANOVA, analysis of variance; PLC, phospholipase C; GTPγS, guanosine 5′-3-O-(thiotriphosphate).
MEK (21). Functional A3 receptors activating ERK1/2 have been also described in microglia cells (24).

Together, the Ras-MEK-ERK1/2 and the PI3K-Akt routes form the two major branches of intracellular adenosine A3 receptor signaling. It should be noted that PI3K might be upstream/downstream of Ras, thus regulating ERK1/2. As for A3 receptors, it has been demonstrated that Ras, in CHO-A3 cells, is activated downstream of PI3K (21).

In the present study, we focused on the regulation of both Akt and ERK1/2 by A3 receptors, to gain more insight into adenosine signal transduction. We studied the dynamics between these two routes by use of specific inhibitors. We present a molecular mechanism able to explain the antiproliferative activity of a selective antagonist of A3 adenosine receptor, CI-IB-MECA, by using human melanoma cell line A375 (25). A375 cells were chosen because the expression and the active functional role of all adenosine receptor subtypes has been firmly established and, furthermore, the singular role of the A3 receptor in cell survival and proliferation has been evaluated (9). Thus, we have inquired into the ability of the A3 adenosine receptor to modulate the MEK/ERK1/2 and the PI3K/Akt pathways, focusing our study on the cross-talk signaling that has been demonstrated to be present in several cellular systems (26–29).

Using A375 cells, we show that A3 adenosine receptor stimulation results in PI3K-dependent phosphorylation of Akt, leading to the reduction of basal levels of ERK1/2 phosphorylation levels, which in turn inhibits cell proliferation.

**EXPERIMENTAL PROCEDURES**

**Chemicals and Reagents**

A375 cells were obtained from American Type Culture Collection (ATCC). Tissue culture media and growth supplements were obtained from Cambrex (Bergamo, Italy). Anti-ACE2bMEAPK and anti-ERK1/2 (pAb) were from Promega (Milan, Italy). Anti-adenosine A3 receptor (pAb) was from Aviva Antibody Corp. (DBA; Milano, Italy). Phospho-Raf (Ser259) and (Ser326) on Raf is an inhibitory phosphorylation site, phospho-Akt (Ser 473), phospho-PI3K/Akt (Ser 473/477), and MEK1/2 antibodies were from Cell Signaling Technology (Celbio; Milano, Italy). Anti-adenosine A3 receptor (pAb) was from Santa Cruz Biotechnology (DBA; Milano, Italy). Unless otherwise noted, all other chemicals were purchased from Sigma.

**Cell Culture**

A375 cells were grown adherently and maintained in Dulbecco's modified Eagle's medium, containing 10% fetal calf serum, penicillin (100 units/ml), streptomycin (100 μg/ml), l-glutamine (2 mM) at 37 °C in 5% CO2, 95% air. Cells were passaged two or three times weekly at a ratio between 1:5 and 1:10.

**Trypan Blue Exclusion**

Cells were collected and stained with 0.4% of trypan blue for 5 min at room temperature before being examined under the microscope. The number of viable cells was determined by trypan blue exclusion. The dead cells that stained blue were scored positive and counted against the total number of cells to determine the percentage of cell death.

**MTT Assay**

The number of living cells was determined by evaluating the mitochondrial dehydrogenase activity by using MTT that is converted into a formazan product in living cells. 105 cells were plated in 24-multwell plates; 500 μl of complete medium were added to each well with different concentrations of CI-IB-MECA. The cells were then incubated for 24 h. At the end of the incubation period, 50 μl of MTT solution (5 mg/ml) were added to each well. The plates were incubated for 2 h at 37 °C, and then 550 μl of an acid protein solution (0.1 N HCl in isopropanol alcohol) were added to each well to dissolve the formazan. The optical density of each well was read on a spectrophotometer at 570 nm. For each experiment, four individual wells of each drug concentration were prepared. Each experiment was repeated three times.

**ATPLite Assay**

The intracellular ATP concentration was determined with a luminescent ATP detection kit (ATPLite-M; PerkinElmer Life Sciences) according to the manufacturer's directions. Light units generated by ATP in each sample were normalized to control (solution with known ATP concentration) and expressed as the absolute ATP levels.

**[3H]Thymidine Incorporation**

**Cell Proliferation Test**—Cells were seeded in fresh medium with 1 μCi/ml [3H]thymidine in Dulbecco's modified Eagle's medium containing 10% fetal calf serum, penicillin (100 units/ml), streptomycin (100 μg/ml), l-glutamine (2 mM) and simultaneously treated with adenosine analogues. After different times of labeling, cells were trypsinized, dispersed in four wells of a 96-well plate, and filtered through Whatman GF/C glass fiber filters using a Micro-Mate 196 cell harvester (PerkinElmer Life Sciences). The filter-bound radioactivity was counted on Top Count Microplate Scintillation Counter (efficiency 57%) with Micro-Skint 20.

**JAM Test**—This assay measures cell death by quantifying the amount of fragmented DNA. Target cells were labeled with 1 μCi/ml [3H]thymidine for 20 h in Dulbecco's modified Eagle's medium containing 10% fetal calf serum, penicillin (100 units/ml), streptomycin (100 μg/ml), l-glutamine (2 mM). The cells were then washed and treated with new unlabeled medium containing adenosine analogues for 24 h. At the end of the incubation period, the cells were trypsinized and dispersed in four wells of a 96-well plate, filtered through Whatman GF/C glass fiber filters using a Micro-Mate 196 cell harvester (PerkinElmer Life Sciences). The filter-bound radioactivity was counted on Top Count Microplate Scintillation Counter (efficiency 57%) with Micro-Skint 20.

The amount of apoptotic and necrotic cells, measured as the loss of radioactivity associated with the loss of fragmented and degraded DNA, was detected by filtration and subsequent washing with a Micro-Mate 196 cell harvester followed by quantification with a Top Count Microplate Scintillation Counter.

The percentage of cell death is expressed as 100 × (dpm(U) – dpm(T)) / dpm(U), where dpm(U) represents the radioactivity of untreated cells and dpm(T) is the radioactivity of treated cells (9).

**Analysis of Propidium Iodide Incorporation and Phosphatidylserine Exposure by Flow Cytometry**

A375 cells (1.5 × 105) were seeded into 6-mm plates and cultured for 16 h before the addition of 10 μM CI-IB-MECA. At various incubation times (0, 3, 8, 16, and 24 h), attached and floating cells were harvested and resuspended with cold PBS for immediate treatment and analysis of propidium iodide incorporation and annexin-V-fluorescein labeling, according to the manufacturer’s instructions ( Annexin-V-FLUOS staining kit; Roche Applied Science). Triplicate samples for each experimental condition were analyzed using FL2 and FL1 channels, respectively, of a FACScan flow cytometer (BD Biosciences).

**Morphological Analysis**

To recover all seeded cells, the adherent culture fraction was trypsinized and mixed with the supernatant fraction. Then the cell suspension was spun to a slide by CytoSpin 3 cytocentrifuge (Shandon) at 250 rpm for 10 min. As previously described (30), cells were fixed in 4% paraformaldehyde for 10 min and permeabilized in PBS solution containing 0.1% Triton X-100, and the DNA was stained with 4',6-diamidino-2-phenylindole. Slides were mounted in DABCO glycerol-PBS and observed on a Zeiss Axiohot fluorescent microscope.

**Flow Cytometry Analysis**

A375 adherent cells were trypsinized, mixed with floating cells, washed with PBS, and permeabilized in 70% (v/v) ethanol/PBS solution at 4 °C for at least 24 h. Cells were washed with PBS, and the DNA was stained with a PBS solution, containing 20 μg/ml of propidium iodide and 100 μg/ml of RNase, at room temperature for 30 min. Cells were analyzed with an EPICS XL flow cytometer (Beckman Coulter, Miami, FL), and the content of DNA was evaluated by the Cell-LISYS program (BD Biosciences). Cell distribution among cell cycle phases and the percentage of apoptotic cells were evaluated as previously described (30). Briefly, the cell cycle distribution is shown as the percentage of cells containing 2n (G0/G1 phases), 4n (G2 and M phases), 4n (> 2n) DNA amount (S phase) judged by propidium iodide staining. The apoptotic population is the percentage of cells with DNA content lower than 2n.

**Small Interfering RNA (siRNA) Design**

To generate a small interfering RNA that targets A3 receptor mRNA (siRNA3), eight oligonucleotides consisting of ribonucleosides, except
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FIG. 1. Effect of A3 receptor stimulation on human A375 melanoma cell growth. A, antiproliferative activity measured by [3H]thymidine incorporation assay. A375 cells were treated with CI-IB-MECA at the indicated concentrations. [3H]Thymidine incorporation is reported as a percentage of drug-vehicle-treated cells. The ordinate shows means of four different [3H]thymidine incorporation quantifications with S.E. (vertical bar). B, cytotoxic activity measured by an MTT test. A375 cells were treated with CI-IB-MECA at the indicated concentrations. The cell growth is expressed as a percentage of the OD measured on untreated cells (control) assumed as 100% of cell viability. The ordinate shows means of four different OD quantifications with S.E. (vertical bar). C, JAM test. The dose-response curve of A375 cell sensitivity to CI-IB-MECA is reported. A375 cells were treated with CI-IB-MECA at the indicated doses for 24 h, and cell death was quantified by the JAM test. The percentage of cell death (loss of [3H]thymidine-labeled DNA) is reported on the ordinate with S.E. (vertical bar). Values represent means ± S.E. of four separate DNA loss quantifications in the same experiment. 100% indicates 100% loss of radioactivity incorporated by untreated cells. D, viability and apoptosis of A375 cells after treatment with 10 μM CI-IB-MECA; density curves show propidium iodide (PI) nuclear permeability versus annexin V cell surface expression. E, DNA content analysis of A375 cells by flow cytometry. The curves show relative cell number (vertical axis) versus propidium iodide fluorescence (DNA content). DMSO panel, A375 cells treated for 24 h with CI-IB-MECA vehicle (Me2SO). CI-IB-MECA panel, A375 cells treated for 24 h with 10 μM CI-IB-MECA.

Western Blotting

After serum deprivation (growth medium without serum) overnight, A375 cells were treated with adenosine analogues for different times (2–24 h). Cells were harvested and washed with ice-cold PBS containing 1 mM sodium orthovanadate, 104 mM (2-aminoethyl)benzene sulfonyl fluoride, 0.08 mM aprotinin, 2 mM leupeptin, 4 mM benzatin, 1.5 mM pepstatin A, 1.4 mM E-64. Cells were then lysed in Triton lysis buffer. The protein concentration was determined using a BCA protein assay kit (Pierce). Aliquots of total protein sample (50 μg) were analyzed using antibodies specific for phosphorylated Thr183/Tyr185 (1:500 dilution) or total ERK-1/ERK-2 MAPK (1:5000 dilution). Membranes were then stripped and reprobed with tubulin (1:250) to ensure equal protein loading.

Real Time RT-PCR Experiments

Total cytoplasmic RNA was extracted by the acid guanidinium thiocyanate phenol method (39). Quantitative real time RT-PCR assay (34) of A3 mRNA transcript was carried out using gene-specific double fluorescently labeled TaqMan MGB probe (minor groove binder) in an ABI Prism 7700 Sequence Detection System (Applied Biosystems, Warrington, Cheshire, UK) (35). The following primer and probe sequences were used for real time RT-PCR: A3 forward primer, 5′-ATG CCT TTG GCC ATT GTT G-3′; A3 reverse primer, 5′-ACA CUC AAC CAC TTC TAC AGC TGC CT-3′; A3 MGB probe, 5′-FAM-TCA GCC TGG GCA TC-3′ (where the fluorescent reporter FAM and the quencher TAMRA are 6-carboxyfluorescein and 6-carboxy -N,N,N′,N′-tetramethylrhodamine, respectively). For the real time RT-PCR of the reference gene, the endogenous control human β-actin was used, and the probe was fluorescence-labeled with VIC™ (Applied Biosystems, Monza, Italy).

Signaling Pathway

Cells were treated for 30 min with metabolic inhibitors or with drug vehicle (Me2SO) prior to being challenged with adenosine analogues.
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U0126 was used as an inhibitor of MEK-1 and MEK-2 to prevent ERK-1 and ERK-2 MAPK activation. LY294002 was used as an inhibitor of PI3K, U73122 as an inhibitor of PLC, and SH6 as an inhibitor of Akt (Akt-I; Vinci-Biochem).

Ras Activity Assay

Raf-1 Ras-binding domain (RBD) fragment (Raf-1 amino acid residues 1–149) fused to glutathione S-transferase was purchased from Upstate Biotechnology, Inc. Ras-GTP from various treated lysates was "pulled down" using the glutathione S-transferase fusion protein corresponding to the human Ras binding domain of Raf-1 bound to agarose. The activated Ras affinity precipitation assay was performed as described according to the manufacturer's protocol. Briefly, A375 melanoma cells were serum-starved for 24 h, treated with Cl-IB-MECA at different concentrations (0.1–10 μM) for different times (from 5 to 60 min), washed twice with cold PBS, and lysed with Mg2+/EGTA lysis/wash buffer. Then the lysate was incubated with 10 μg of Raf-1 RBD-conjugated agarose at 4 °C for 30 min. Raf-1 RBD-conjugated agarose specifically binds to and precipitates Ras-GTP from cell lysates. After washing the beads three times with Mg2+/EGTA lysis/wash buffer, they were suspended in 2× Laemmli sample buffer, subjected to SDS-PAGE and immunoblot analysis using 1 μg/ml anti-Ras monoclonal antibody as a primary antibody (Upstate Biotechnology), and visualized using the ECL Western blotting detection system.

Densitometry Analysis

The intensity of each band in immunoblot assay was quantified using molecular analysis software (Bio-Rad). Mean densitometry data from independent experiments were normalized to control. The data were presented as the mean ± S.E. and analyzed by Student's t test.

Statistical Analysis

All values given throughout are expressed as means ± S.E. from three independent experiments except where indicated. Data sets were examined by analysis of variance (ANOVA) and Dunnett's test (when required). A p value less than 0.05 was considered statistically significant.

RESULTS

Cl-IB-MECA Inhibits Cell Proliferation and ERK1/2 Activation—In this study, we investigated the functionality of A3 adenosine receptors expressed in A375 human melanoma cells by using the selective adenosine analogue Cl-IB-MECA (25). A375 cells were treated in the presence of increasing concentrations (0.1–10 μM) of Cl-IB-MECA for 24 h, and [3H]thymidine incorporation was measured. As shown in Fig. 1A, Cl-IB-MECA induced a reduction in [3H]thymidine incorporation in a

FIG. 2. Time- and dose-dependent effects of Cl-IB-MECA on phosphorylation of ERK1/2. A, serum-starved A375 cells were stimulated with MeSO vehicle (Untreated) or 10 μM Cl-IB-MECA for the times indicated (n = 3). B, the immunoblot signals were quantified using Molecular Analyst/PC densitometry software (Bio-Rad). Densitometric analysis of ERK1/2 phosphorylated isoforms is reported. C, for dose-response experiments, A375 serum-deprived cells were stimulated for 30 min with either vehicle or various concentrations of Cl-IB-MECA before determination of ERK1/2 phosphorylation. The immunoblot shows one representative experiment. D, the immunoblot signals were quantified using Molecular Analyst/PC densitometry software (Bio-Rad). Densitometric analysis of ERK1/2 phosphorylated isoforms is reported. Data were normalized; the untreated control was set to 100%. The error bars give S.E. of three independent experiments. E, effect of increasing concentration of DPCPX, SCH 58261, MRE 2029F20, and MRE 3008F20 A1, A2A, A2B, and A3 adenosine receptor antagonists, respectively on 5 μM Cl-IB-MECA-mediated inhibition of cell proliferation. A375 proliferation was monitored by [3H]thymidine incorporation for 24 h. The proliferation rate at different antagonist concentrations (from 0 to 5 μM) is reported in the ordinate as percentage of [3H]thymidine incorporation of mock-untreated cells. Reported values represent the mean of four [3H]thymidine incorporation quantifications with S.E. values (vertical bar). 100% indicates the thymidine incorporation of A375 cells grown in the absence of Cl-IB-MECA. *, p < 0.05 with respect to untreated cells; analysis was by ANOVA followed by Dunnett's test. F, A375 cells were treated with Cl-IB-MECA (lane 1, control) or with 10 μM CI-IB-MECA (lanes 2–6) and exposed to the A3 receptor antagonist DPCPX (100 nM) (lane 3), A2A receptor antagonist SCH 58261 (100 nM) (lane 4), A2B receptor antagonist MRE 2029F20 (100 nM) (lane 5), or A3 receptor antagonist MRE 3008F20 (100 nM) (lane 6) for 1 h. Cellular extracts were prepared and subjected to immunoblot assay using an Anti-ACTIVE®MAPK antibody. The blot was then stripped and used to determine total ERK1/2 expression using an anti-ERK1/2 (pAb).

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dose-dependent manner, with an IC₅₀ = 1.2 ± 0.3 μM. Similar results were obtained with another A₃ adenosine receptor agonist, IB-MECA (data not shown). By using the MTT assay, we observed that CI-IB-MECA produced small but reproducible antiproliferative effects (Fig. 1B). To evaluate whether the reduced number of viable cells quantified by [³H]thymidine incorporation assay. The PLC inhibitor U73122 (A), the PI3K inhibitor LY 294002 (B), and the Akt inhibitor (C) were added at the indicated concentrations 30 min before the addition of CI-IB-MECA (1 and 5 μM). Cells were harvested after 3 h of treatment. Data were normalized; the unstimulated control was set to 100%. Error bars give S.E. of three independent experiments. * p < 0.01 versus CI-IB-MECA-treated cells in the absence of inhibitors (0). Analysis was by ANOVA followed by Dunnett’s test.

Fig. 3. A₃ adenosine receptor stimulation impairs cell proliferation via PLC-PI3K-Akt signaling pathway. [³H]Thymidine incorporation assay. The PLC inhibitor U73122 (A), the PI3K inhibitor LY 294002 (B), and the Akt inhibitor (C) were added at the indicated concentrations 30 min before the addition of CI-IB-MECA (1 and 5 μM). Cells were harvested after 3 h of treatment. Data were normalized; the unstimulated control was set to 100%. Error bars give S.E. of three independent experiments. * p < 0.01 versus CI-IB-MECA-treated cells in the absence of inhibitors (0). Analysis was by ANOVA followed by Dunnett’s test.

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CI-IB-MECA Inhibits Cell Proliferation and ERK1/2 Activation via Adenosine A₃ Receptors—The family of adenosine receptors consists of four subtypes of G protein-coupled receptors, designated A₁, A₂A, A₂B, and A₃. We have previously demonstrated that all four adenosine receptors are expressed in human melanoma A375 cells (36, 37). To evaluate the functional role of adenosine receptor subtypes on A375 melanoma cell proliferation and on ERK1/2 activation, we tested the effect of CI-IB-MECA in combination with DPCPX (an A₁ receptor antagonist), SCH 58261 (a selective A₂A receptor antagonist), MRE 209F20 (a selective A₂B receptor antagonist), and MRE 3008F20 (a selective A₃ receptor antagonist) (36–39). MRE 3008F20 reduced the CI-IB-MECA effect on cell proliferation in a dose-dependent manner, but concentrations of DPCPX, SCH 58261, and MRE 209F20 sufficient to block A₁, A₂A, or A₂B receptors, respectively, failed to block the CI-IB-MECA effect on cell proliferation (Fig. 2E). Furthermore, although the A₁, A₂A, and A₂B receptor antagonists were not able to prevent ERK1/2 inhibition induced by CI-IB-MECA, the selective A₃ receptor antagonist MRE 3008F20 (0.1 μM) abrogated the CI-IB-MECA-induced inhibition of ERK1/2 activation (Fig. 2F).
Therefore, we hypothesized that the A3 adenosine receptor subtype may be responsible for CI-IB-MECA-mediated inhibition of cell proliferation and of ERK1/2 activation in A375 melanoma cells.

**A3 Adenosine Receptor Signals through a Pathway Including PLC-PI3K-Akt**—We next sought to examine the signaling pathway by which A3 adenosine receptor activation inhibits A375 melanoma cell proliferation and ERK1/2 activation.

Many G<sub>α</sub>-coupled receptors are known to activate ERK1/2 in a PI3K-dependent manner (20, 40, 41). In particular, A<sub>3</sub> receptor activation has been shown to activate PLC and PI3K (20, 24). We have investigated the involvement of PLC-PI3K-Akt cascade on the CI-IB-MECA-induced impairment of cell proliferation. We studied the effect of U73122 (a membrane-permeable amino-steroid inhibiting PLC-dependent pathways) on CI-IB-MECA-dependent inhibition of melanoma cell proliferation. Pretreatment of cells with 0.25, 0.5, and 1 μM U73122 abrogated, in a dose-dependent manner, CI-IB-MECA effect on cell proliferation (Fig. 3A), suggesting a critical role for PLC in A<sub>3</sub> receptor-dependent inhibition of cell proliferation.

Furthermore, A375 cells were pretreated with increasing concentrations of LY294002 (PI3K inhibitor; 2.5, 10, and 20 μM) and Akt inhibitor (Akt-I; 1, 5, and 10 μM) for 30 min before adding CI-IB-MECA (1 and 5 μM). Fig. 3, B and C, shows that LY294002 and Akt-I, respectively, significantly reversed the inhibitory effect of CI-IB-MECA on cell proliferation.

These results indicate that the antiproliferative effect of CI-IB-MECA is mediated by a PLC-PI3K-Akt signaling pathway. To verify that PLC-PI3K-Akt signal was the molecular pathway sustained by A<sub>3</sub> receptor stimulation, we also investigated the effect of U73122, LY294002, and the Akt inhibitor on ERK1/2 phosphorylation levels under CI-IB-MECA treatment.

Pretreatment of A375 cells with 0.5 μM U73122 for 30 min impaired CI-IB-MECA inhibition of ERK1/2 phosphorylation (Fig. 4A). Fig. 4B shows the data obtained after densitometry analysis of the phospho-ERK-2 and phospho-ERK-1 protein levels.

Pretreatment of A375 cells with 10 μM LY294002 for 30 min impaired CI-IB-MECA inhibition of ERK1/2 phosphorylation (Fig. 4C). Fig. 4D shows the data obtained after densitometry analysis of the phospho-ERK-2 and phospho-ERK-1 protein levels. Similar results were obtained after the pretreatment of A375 cells with 5 μM Akt inhibitor (Fig. 4, E and F). These data suggest that the A<sub>3</sub> adenosine receptor signals through a pathway including PLC-PI3K-Akt.

To provide additional support for this possibility, we investigated the changes in Akt phosphorylation level in A375 cells after CI-IB-MECA treatment. 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 (42). A375 cells were starved for 24 h to reduce basal P-Akt levels and then were treated with CI-IB-MECA (10 μM). Fig. 5A shows the kinetics of Akt phosphorylation promoted by CI-IB-MECA. A<sub>3</sub> receptor stimulation induced Akt phosphorylation in a time-dependent manner. The maximum induction of Akt phosphorylation was at 5 min after the treatment (Fig. 5B). Furthermore, CI-IB-MECA stimulation for 30 min induced a dose-dependent increase in Akt phosphorylation with an

![Fig. 4. Cl-IB-MECA impairs ERK1/2 activation via the PLC-Pi3K-Akt signaling pathway: Western blot assay. 0.5 μM PLC inhibitor U73122 (A), 10 μM PI3K inhibitor LY294002 (C), and 5 μM Akt inhibitor (E) were added 30 min before the addition of CI-IB-MECA (5 and 10 μM). Cells were harvested after 1 h of treatment. A375 cells were treated without (lane 1) or with CI-IB-MECA 5 μM (lanes 2 and 5) and 10 μM (lanes 3 and 6) for 1 h. U73122, LY294002, and Akt inhibitor are shown in lanes 4–6 of A, C, and E, respectively. Cellular extracts were prepared and subjected to immunoblot assay using an anti-ACTIVE®MAPK antibody. The blot was then stripped and used to determine total ERK expression using an anti-ERK1/2 (pAb). A, C, and E, lane 1, Me2SO vehicle. B, D, and F, the immunoblot signals were quantified using Molecular Analyst/PC densitometry software (Bio-Rad). Densitometric analysis of ERK1/2 phosphorylated isoforms is reported. Data were normalized; the unstimulated control (lane 0) was set to 100% as well as the cells treated with the inhibitors alone. The error bars give S.E. of three independent experiments. *, p < 0.01 versus Me2SO-treated cells. Analysis was by ANOVA followed by Dunnett’s test.](http://www.jbc.org/content/jbc/195/21/19521.full)
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**Fig. 5.** Time- and dose-dependent effects of CI-IB-MECA on Akt phosphorylation in A375 cells. A, serum-starved A375 cells were incubated at 37 °C with Me2SO (DMSO; lane 1) or 10 μM CI-IB-MECA for 2.5, 5, 10, 15, 30, and 60 min (lanes 2–7, respectively). B, the immunoblot signals were quantified using Molecular Analyst/PC densitometry software (Bio-Rad). Densitometric analysis of Akt phosphorylated isomers is reported. The unstimulated control (lane 1) was set to 100%. *, p < 0.05 with respect to unstimulated control; analysis was by ANOVA followed by Dunnett’s test. C, serum-starved A375 cells were incubated for 30 min at 37 °C with Me2SO vehicle (lane 1) or 1, 10, 100, 1000, and 10000 nM CI-IB-MECA (lanes 2–6, respectively) before determination of Akt phosphorylation. D, densitometric analysis of the Akt phosphorylated isomer is reported. The mean values of three independent experiments (one of which is shown in C) were normalized to the result obtained in cells in the absence of CI-IB-MECA. Plots are mean ± S.E. values (n = 3). E, CI-IB-MECA-stimulated Akt phosphorylation in A375 cells is mediated by the A3 adenosine receptor. Serum-starved A375 cells were incubated at 37 °C with Me2SO vehicle (lane 6, control), 0.1 μM (lane 1), and 1 μM (lane 2) CI-IB-MECA. MRE 3008F20 1 μM was added alone (lane 5) or in the presence of 0.1 and 1 μM CI-IB-MECA (lanes 3 and 4, respectively) before determination of Akt phosphorylation. F, densitometric analysis of Akt phosphorylated isomer is reported. *, p < 0.05 with respect to untreated cells; analysis was by ANOVA followed by Dunnett’s test. Tubulin shows equal loading.

EC₅₀ of 151 ± 19 nM (Fig. 5, C and D). The addition of the selective antagonist of the human A₃ receptor, MRE 3008F20 (1 μM) blocked CI-IB-MECA (0.1 and 1 μM)-induced increase of Akt phosphorylation (Fig. 5, E and F).

To verify whether any cross-talk exists between PI3K/Akt and Raf/MEK/ERK pathways in human A375 melanoma cells after A₃ receptor stimulation, we investigated Raf-1 inactivation levels by Western blot. For this purpose, we utilized an antibody able to recognize an inhibitory phosphorylation site on Ser²⁵⁹ of Raf. CI-IB-MECA induced Raf inactive levels in a dose- and time-dependent manner. Raf phosphorylation was increased by CI-IB-MECA with an EC₅₀ value of 33 ± 2 nM (Fig. 6, A and B). The kinetics of Raf phosphorylation was comparable with the time course of Akt and ERK1/2 phosphoryration, with a maximum level at 5 min (Fig. 6, C and D). The addition of the selective antagonist of the human A₃ receptor, MRE 3008F20 (1–5 μM) blocked CI-IB-MECA inhibition of Raf activity (Fig. 6, E and F). Pretreatment of A375 cells with 10 μM LY294002 for 30 min impaired CI-IB-MECA inhibition of Raf (Fig. 6, G and H). Similar results were obtained after the pretreatment of A375 cells with 5 μM of Akt inhibitor (Fig. 6, G and H). These results indicate that PI3K and Akt are involved in CI-IB-MECA-induced Raf Ser²⁵⁹ phosphorylation.

**A3 Receptor Gene Silencing Attenuates the Akt/MAPK Signal Transduction Pathway**—To confirm the role of A₃ receptor stimulation in the Akt/MAPK signaling pathway in vitro, we tried to knock down A₃ receptor expression using siRNA, leading to a transient knockdown of the A₃ receptor gene. We designed four siRNAs from the human A₃ receptor gene sequence. Although there was a difference in silencing ability, all of the siRNAs were able to suppress endogenous A₃ receptor protein expression in human A375 cells (Fig. 7). Therefore, siRNA were useful to investigate the A₃ receptor stimulatory effect in A375 cells.

A375 cells were mock-transfected or transfected with small interfering RNAs that target A₃ receptor mRNA (siRNA₃) for degradation. To evaluate transfection efficiency, A375 cells were also transfected with a siRNA control labeled with fluoresein. By flow cytometry, we observed a transfection efficiency of 86 ± 5% (Fig. 7A). After transfection, the cells were cultured in complete medium, and at 24, 48, and 72 h, total RNA was isolated for real time RT-PCR analysis of A₃ receptor mRNA and for Western blot analysis of A₃ receptor protein. As expected, A₃ receptor mRNA levels were significantly reduced in cells transfected with siRNA₃ (Fig. 7B). Furthermore, A₃ receptor protein expression was strongly reduced in siRNA₃-treated cells (Fig. 7, C and D). A rescue experiment, extending the analysis of A₃ receptor protein expression at 96 and 120 h post-siRNA₃ transfection, was performed. Fig. 7C shows that A₃ receptor protein began to recover in A375 cells 96 h after siRNA₃ treatment. By 120 h, the protein had returned to base-line values.

Neither mock transfection nor transfection with an siRNA targeted to an irrelevant mRNA inhibited A₃ receptor mRNA or protein expression. To confirm the specificity of the siRNA₃-mediated silencing of the A₃ receptor, we investigated the expression of A₂₅ receptor protein in siRNA₃₅-treated cells (Fig. 7C). Fig. 7C demonstrates that treatment of A375 cells with siRNA₃ reduced the expression of A₃ protein but had no effect on the expression of the A₂₅ receptor. Therefore, at 72 h from the siRNA₃₅ transfection, A375 cells were exposed to increasing concentrations of the A₃ adenosine receptor agonist CI-IB-MECA (1–10 μM) for different times. Cell proliferation was evaluated by [³H]thymidine incorporation assay, and total protein was harvested for Western blot analysis. As control, A375 cells were exposed to random siRNA. We found that the inhibition of A₃ receptor expression is sufficient to block CI-IB-MECA-induced inhibition of cell proliferation and of ERK1/2 phosphorylation levels (Fig. 7, E and F, respectively). These results clearly show the connection between A₃ receptor stimulation and MAPK signaling in melanoma cells.
Adenosine A3 Receptors Do Not Activate Ras in A375 Melanoma Cells—To determine whether A3 receptor stimulation could modulate activity of Ras, we performed a Ras activation assay using cell lysates of serum-starved A375 melanoma cells as described under “Experimental Procedures.” The main component of the assay was a recombinant protein derived from Raf that contains a domain capable of binding only to GTP-bound Ras (Raf-1 Ras binding domain). Active GTP-bound Ras was pulled down from cell lysates with the glutathione S-transferase-Raf-RBD coupled to glutathione-agarose, and the fraction of activated Ras was determined by immunoblotting with a Ras antibody. The active form of Ras (Ras-GTP) was increased in response to 10% fetal calf serum and to GTP-

Adenosine A3 Receptor Stimulation Inhibits MEK1/2—We performed immunoblot analysis for MEK1/2 to assess their involvement in the signal transduction of A3 receptor stimulation for inhibition of melanoma cell proliferation and of ERK1/2 phosphorylation levels. We found that MEK1/2 phosphorylation was inhibited by A3 adenosine receptor stimulation.
The inhibition of MEK1/2 phosphorylation via Cl-IB-MECA was abrogated by the A3 receptor antagonist MRE 3008F20 (1 μM) and by the Akt inhibitor (5 μM) (Fig. 9).

**DISCUSSION**

Recently, the importance of small autacoids, primarily thought to be neurotransmitters, in mediating a variety of biological activities in the skin (9, 43–45) has been demonstrated. In particular, several studies have indicated that adenosine, via stimulation of its receptors, is involved in cell proliferation and cell death. In agreement with these studies, we also found that A3 receptor inhibits human melanoma A375 cell line proliferation.

Because it has been reported that the activity of Akt or MAPK or both is elevated in many cancer cells and is known to play critically important roles in cellular proliferation (46–49), we tested the hypothesis that A3 receptor stimulation regulates cell growth signaling via the ERK1/2 and/or Akt pathway in melanoma cells.

We found that serum-deprived A375 melanoma cells had no basal Akt phosphorylation, whereas Cl-IB-MECA treatment resulted in the phosphorylation of Akt at the Ser573 site. Furthermore, Akt phosphorylation matched the phosphorylation of Raf at an inhibitory site (Ser259). Surprisingly, serum-deprived A375 cells showed high basal levels of ERK1/2 phosphorylation (Fig. 2). The high levels of ERK1/2 phosphorylation were significantly inhibited in response to 5 and 10 μM Cl-IB-MECA (Fig. 9). The inhibition of MEK1/2 phosphorylation via CI-IB-MECA was abrogated by the A3 receptor antagonist MRE 3008F20 (1 μM), by the PI3K inhibitor LY294002 (10 μM), and by the Akt inhibitor (5 μM) (Fig. 9).
Adenosine A3 receptor stimulation decreases MEK1/2 phosphorylation via AKT and PI3K. A, serum-starved A375 cells were incubated at 37 °C with MeSO vehicle (lane 1) or 5 μM (lane 2) or 10 μM (lane 3) CI-IB-MECA for 30 min. MRE 3008F20 1 μM was added alone (lane 4) or in the presence of 5 and 10 μM CI-IB-MECA (lanes 5 and 6, respectively) before determination of MEK1/2 phosphorylation. LY294002 and Akt inhibitor are shown in lanes 7–9 and 10–12, respectively. Cellular extracts were prepared and subjected to immunoblot assay using a P-MEK1/2 antibody. The blot was then stripped and used quantitatively. The immunoblot signals were quantified using Molecular Analyst/PC densitometry software (Bio-Rad). Densitometric analysis of the MEK1 phosphorylated isoform is reported. Data were normalized; the unstimulated control (lane 1) was set to 100%. The error bars give S.E. of three independent experiments. *p < 0.05 with respect to untreated cells; analysis was by ANOVA followed by Dunnett’s test.

phosphorylation in unstimulated A375 cells may reflect a neuronspecific (melanocyte precursor cells derive from the neural crest) characteristic, since ERK1/2 is not usually phosphorylated after long periods of serum deprivation in cells of muscular and adipose origin (50, 51). Interestingly, CI-IB-MECA stimulation resulted in a time- and dose-dependent reduction in ERK1/2 phosphorylation (Fig. 2). It is suggested that this mechanism may be peculiar for melanoma cells, having a misregulation of proliferative pathways, since A3 receptors increased ERK1/2 phosphorylation in CHO-A3 cells in a dose-dependent manner (20) and induced a biphasic effect on the phosphorylation levels of ERK1/2 on microglia cells (24). Further studies in other different cell systems will enhance our understanding of the role of A3 receptors in the modulation of mitogenic signaling.

There are two possible explanations for the CI-IB-MECA-induced inhibition of ERK1/2 phosphorylation, either inhibition of ERK1/2 kinase or induction of a ERK1/2 phosphatase. The possibility that an ERK1/2 kinase is inhibited has been examined by Rommel et al. (26). Recently, it was reported that the phosphorylation of Raf by Akt inhibits the activation of the ERK1/2 signaling pathway, suggesting the presence of cross-talk between the two signaling pathways (52). Furthermore, it has been shown that the Raf-MEK-ERK pathway can be inhibited by Akt in differentiated myotubes but not in their undifferentiated myoblast precursors. The authors suggested that regulation of a Raf/Akt interaction, underlying the ERK1/2 inhibition, might be mediated by stage-specific modification of these proteins or by stage-specific accessory proteins. This regulation might be intact in cells of neuronal origin also. To this end, we examined whether any cross-talk exists between ERK1/2 and Akt pathways in A375 melanoma cells. The classical MAPK cascade leads from the Ras kinases to the MAPK kinase MEK1/2. There is evidence that Akt is able to phosphorylate Raf, thereby efficiently abrogating Raf activity on downstream substrates (27, 28, 52). We studied the effects of A3 receptor stimulation on the proliferation of melanoma cells in the presence of specific inhibitors of the PI3K and Akt signal transduction pathways. We could effectively block the CI-IB-MECA-induced reduction of ERK1/2 phosphorylation with an inhibitor of PI3K (Fig. 4). Indeed, application of CI-IB-MECA in combination with PI3K inhibition resulted in a clear increase of ERK1/2 phosphorylation when compared with P-ERK1/2 in the presence of CI-IB-MECA alone. These data suggest that the Ras-Raf-MEK-ERK pathway is normally activated by A3 receptor stimulation, as is the PI3K-Akt route. It is clear that these apparently separate routes should actually interact.

In order to investigate the functionality of A3 receptors expressed in melanoma cells, we used the selective adenosine analogue CI-IB-MECA. It is not clear whether the growth-inhibitory action of micromolar concentrations of the A3 receptor agonist CI-IB-MECA is due to its role as an extracellular ligand for cell surface receptors or whether it acts intracellularly as a second messenger. In particular, this agonist may, in high concentrations, activate AKT receptors, which, however, when compared with A3 receptors, are expressed at a low level in A375 cells (Bmax = 23 ± 7 and Bmax = 291 ± 50 fmol mg−1 of protein for A1 and A3, respectively) (36). Thus, the effects we report here on melanoma cell proliferation and on ERK1/2 phosphorylation induced by CI-IB-MECA using high (10−6 M) concentrations of CI-IB-MECA are almost certainly due to A3 receptor stimulation. In particular, the effects of CI-IB-MECA on cell proliferation and on ERK1/2 phosphorylation are not mediated by A1, A2A, or A2B receptors. In support of this conclusion, DPCPX, SCH 58261, and MRE 20929F20, adenosine receptor antagonists highly selective for A1, A2A, and A2B receptors, respectively, did not block the inhibitory effect of A3 receptor stimulation on cell proliferation and on P-ERK1/2 modulation. On the contrary, the effects on cell proliferation and on P-ERK1/2 modulation were inhibited by the A3 receptor antagonist, MRE 3008F20. Furthermore, the CI-IB-MECA-induced effects on cell proliferation and ERK1/2 phosphorylation in human A375 melanoma cells were abolished in cells in which A3 receptor protein was knocked down by si-RNA treatment when compared with wild-type cells. These findings, together with the specificity of the agonist used, make us confident that the effects are due to the A3 receptor subtype.

A3 receptor stimulation inhibits the proliferation of melanoma cells partly by a PLC-sensitive mechanism. Pretreatment of cells with a PLC-γ inhibitor strongly abrogated the CI-IB-MECA effect on cell proliferation and on ERK1/2 phosphorylation, suggesting a critical role for PLC-γ in A3 receptor signaling. Furthermore, pretreatment of A375 cells with a PI3K inhibitor and an Akt inhibitor impaired CI-IB-MECA-induced inhibition of cell proliferation and the effects of A3 receptor stimulation on Raf, MEK1/2, and ERK1/2 phosphorylation. These data suggest that the A3 adenosine receptor signals through a pathway including PI3K-Akt. On the contrary, Ras was not activated, at least when measured with the pull-down assay. These results confirm the hypothesis of this study; in A375 cells, A3 receptors decrease MEK1/2-ERK1/2 phosphorylation and cell proliferation via the inhibition of Raf by a PI3K-Akt pathway without affecting Ras.

Our results indicate that CI-IB-MECA acts extracellularly as a first messenger for cell surface receptors. In A375 cells, the inhibition of PLC-PI3K-Akt signaling is able to block the effect of A3 receptor stimulation on cell proliferation, suggesting that in the melanoma cell system, an inhibitory connection between...
PLC-P13K-Akt and ERK1/2 is present.

Finally, we have described the molecular mechanism sustained by CI-IB-MECA interfering with cell proliferation. CI-IB-MECA via A3 adenosine receptor binding activates a PLC-P13K-Akt signaling that in turn reduces P-ERK1/2 levels necessary for cell proliferation. As a consequence, cells accumulated in G2/M cell cycle phases, and a low level of DNA incorporation was observed.

Further definition of the pathways leading to ERK1/2 inactivation and translation into an in vitro model are required to clarify whether adenosine signaling in vivo has characteristics similar to those observed in this in vitro model. In this scenario, the regulation of ERK1/2 by A3 receptor and PI3K would represent an important aspect of adenosine signaling.
A3 Adenosine Receptor Activation Inhibits Cell Proliferation via Phosphatidylinositol 3-Kinase/Akt-dependent Inhibition of the Extracellular Signal-regulated Kinase 1/2 Phosphorylation in A375 Human Melanoma Cells
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J. Biol. Chem. 2005, 280:19516-19526.
doi: 10.1074/jbc.M413772200 originally published online March 17, 2005

Access the most updated version of this article at doi: 10.1074/jbc.M413772200

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