Original Paper

Cellular Physiology and Biochemistry

Cell Physiol Biochem 2010;26:125-134

Accepted: June 22, 2010

Higher Concentrations of Extracellular ATP Suppress Proliferation of Caco-2 Human Colonic Cancer Cells via an Unknown Receptor Involving PKC Inhibition

Takahiro Yaguchi[1], Masaru Saito[1], Yoshiyuki Yasuda[1], Takeshi Kanno[1], Takashi Nakano[2] and Tomoyuki Nishizaki[1]

[1]Division of Bioinformation, Department of Physiology, Hyogo College of Medicine, Nishinomiya, [2]Department of Thoracic Oncology, Hyogo College of Medicine, Nishinomiya

Key Words

ATP • Protein kinase C • Mitogen-activated protein kinase • Caco-2 cell • Proliferation

Abstract

Background/Aims: Adenosine 5'-triphosphate (ATP) mediates a variety of signal transductions via ATP receptors such as P2X and P2Y receptors. The present study aimed at understanding the mechanism underlying extracellular ATP-induced suppression of Caco-2 human colonic cancer cell proliferation. Methods: Caco-2 cells were cultured. To examine cell viability and cell cycling, 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay, fluorescent cytochemistry, terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay, and flow cytometry were carried out. To see mRNA expression of ATP receptors, reverse transcription-polymerase chain reaction (RT-PCR) was performed. To examine PKC activity and mitogen-activated protein (MAP) kinase activity, in situ PKC assay and Western blotting using an anti-extracellular signal-regulated kinase 1 (ERK1)-antibody and an anti-phospho-ERK antibody were carried out. Results: Extracellular ATP or the unhydrolyzed ATP analogue 5'-adenylyimido-diphosphate (AMP-PNP) reduced Caco-2 cell viability in a concentration (10 μM-10 mM)-dependent manner at 48-h treatment, and the effect was not affected by caspase inhibitors. Caco-2 cells were little reactive to propidium iodide and Hoechst 33342 or little positive to TUNEL after 48-h treatment with ATP (1 mM). In the flow cytometry, 48-h treatment with ATP (1 mM) arrested cell cycling at the S phase in Caco-2 cells. P_2 purinoceptor agonists reduced Caco-2 cell viability with the order of potency: 2-methylthio ATP>UTP>β,γ-methylene ATP, and the ATP effect was partially inhibited by suramin, a non-selective inhibitor of P_2 purinoceptors. The PKC inhibitor GF109203X or the MAP kinase kinase inhibitor PD98059 reduced Caco-2 cell viability to an extent similar to that achieved by ATP (1 mM), and no further reduction was obtained with co-treatment with ATP. ATP and its ATP analogues such as AMP-PNP and ATPγS, at higher concentrations (1-10 mM), inhibited PKC activation in Caco-2 cells in a fashion that mimics the effect of GF109203X, but PD98059 exhibited no effect on PKC activation. The inhibitory effect of ATP on PKC activation was not found with SK-N-SH cells, a human neuroblastoma cell line, but the cells expressed all the mRNAs for P2X and P2Y receptors that Caco-2 cells did. ATP (10 mM) or GF109203X

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Prof. Tomoyuki Nishizaki
Division of Bioinformation, Department of Physiology
Hyogo College of Medicine
1-1 Mukogawa-cho, Nishinomiya 663-8501 (Japan)
Tel. +81-798-45-6397, Fax +81-798-45-6649, E-Mail tomoyuki@hyo-med.ac.jp

inhibited activation of ERK, a MAP kinase, in Caco-2 cells. Conclusion: Extracellular ATP, at higher concentrations, suppresses Caco-2 cell proliferation at the S phase of cell cycling by inhibiting PKC, possibly as mediated via an unknown ATP receptor, followed by MAP kinase.

Introduction

ATP, an energy source, is ubiquitously present in a wide range of organs and tissues. ATP serves as a transmitter and exerts its diverse actions via ATP receptors (P_2 purinoceptors). P_2 purinoceptors are classified into the major two classes of the receptors; the ligand-gated ion channels, P2X receptors, and the G-protein-linked receptors, P2Y receptors [1, 2]. ATP, synaptically co-released together with other transmitters, modulates inhibitory and facilitatory synaptic transmissions [3-6]. ATP released from astrocytes, alternatively, might be a mediator of neuron-astrocyte communications or astrocyte-astrocyte communications [7, 8]. Moreover, evidence has pointed to the implication of ATP/P_2 purinoceptors in pain [9].

Much attention has focused upon P2 purinoceptors in cell proliferation or apoptotic cell death. $P2X_2$ receptors have a sequence homology to RP-2, an apoptosis-related gene [10]. Extracellular ATP inhibits cell growth in prostate cancer cells via P2X receptors [11] or in esophageal cancer cells [12], endometrial cancer cells [13], ovarian cancer cells [14], and colorectal cancer cells [15, 16] via P2Y receptors, where intracellular Ca^{2+} rise following P2Y receptor activation may play a critical role. Extracellular ATP, thus, appears to regulate cancer cell growth via P2X or P2Y receptors, depending upon cancer cell types.

Protein kinase C (PKC), that comprises a family of isozymes such as α, βI, βII, γ, δ, ε, θ, μ, η, λ/ι for mouse/human, and ζ, engages multiple signal transductions. Several avenues of evidence have shown the role for PKCs in cell growth and differentiation, and therefore, PKCs could be a potential target for anticancer therapy [17]. PKCs are activated mainly via three pathways relevant to phospholipase C, phospholipase A_2, and phospholipase D [18]. Of P2Y receptors cloned $P2Y_1$, $P2Y_2$, $P2Y_4$, and $P2Y_6$ are linked to Gq-protein involving activation of phospholipase C followed by PKC [19-21].

The present study aimed at understanding a pathway for proliferation of Caco-2 cells, a human colonic cancer cell line, under the control of ATP. The results show that extracellular ATP, at higher concentrations, suppresses Caco-2 cell proliferation at the S phase of cell cycling. The ATP effect is caused by inhibiting PKC and in turn, inhibiting mitogen-activated protein (MAP) kinase as a downstream target of PKC. This may represent regulation of Caco-2 cell proliferation as mediated via an unknown ATP receptor involving PKC inhibition.

Materials and Methods

Materials

Caco-2 cells, a human colonic cancer cell line, and SK-N-SH cells, a human neuroblastoma cell line, were obtained from RIKEN cell bank (Ibaraki, Japan). MTT was purchased from DOJINDO (Kumamoto, Japan). Dulbecco's modified Eagle's medium (DMEM), minimum essential medium (MEM), ATP, 5'-adenylyimido-diphosphate (AMP-PNP), ATPγS, 2-methylthio ATP (2-MeSATP), α,β-methylene ATP (α,β-MeATP), β,γ-methylene ATP (β,γ-MeATP), uridine 5'-triphosphate (UTP), theophylline, GF109203X, PD98059, and 12-*O*-tetradecanoylphorbol-13-acetate (TPA) were from Sigma (St. Louis, MO, USA). Pyridoxalphosphate-6-azophenyl-2',4'-disulfonic acid (PPADS) was from CALBIOCHEM (San Diego, USA). H-89 was from DWTI Research Laboratories (Nagoya, Japan). Suramin, Z-VAD-FMK, Z-YVAD-FMK, Z-DEVD-FMK, Z-IETD-FMK, and Z-LEHD-FMK were from Wako (Osaka, Japan). A VybrantTM apoptosis assay kit containing Hoechst 33342 and propidium iodide was from Molecular Probes (Oregon, USA). PKC substrate peptide was from Peptide Institute (Osaka, Japan). An anti-ERK1 antibody and an anti-phospho-ERK monoclonal antibody were from Santa Cruz Biotechnology (California, USA). A Vector NovaRed substrate kit was from Vector Laboratories (Burlingame, USA). Protein phosphatase inhibitor cocktail, a streptavidin biotin complex peroxidase kit, sodium dodecyl sulfate (SDS), and Sepasol-RNA I Super were from Nacalai Tesque (Kyoto, Japan). SuperScript III reverse transcriptase was from Invitrogen (Carlsbad, USA). Taq polymerase was from Fermentas (Burlington, USA). Primers for reverse transcription-polymerase chain reaction (RT-PCR) were from Hokkaido System Science Co. Ltd. (Hokkaido, Japan).

Cell culture

Caco-2 cells and SK-N-SH cells were cultured in DMEM and MEM-α, respectively, supplemented with 10% heat-inactivated fetal bovine serum, penicillin (final concentration, 100 U/ml), and streptomycin (final concentration, 0.1 mg/ml) in a humidified atmosphere of 5% CO_2 and 95% air at 37°C.

Cell viability

Caco-2 cell viability was evaluated by the method using MTT as previously described [22].

Gene name	Sense primer	Anti-sense primer	Base pair
$P2X_1$	CTCATCAGCAGTGTCTCTGTGAAACTCAAG	TGAAAGAGGCAGGTCTTCATGTGGGCAGC	509
$P2X_2$	GGCCTCTGTCAGCCAATTTCTGGGTACG	CCTGAAGTTGTAGCCTGACGAGGCAGGC	364
$P2X_3$	ACAGCTCTGTGCTCCGGACCTGTGAGATC	TTGGTCAAAGCCGCGATTTTCAGTGTAGTC	701
$P2X_4$	CCCGAGATTCCAGATGCGACCACTGTGTG	ATGCCTCCCTCCACGGCCATGTCCTGG	407
$P2X_5$	CGCTGCCTGCGGAGAGGGAACTTGGCC	TTGTCCAGACGGCTAAAAGAATAGTGAGGG	398
$P2X_6$	TCAAAGGGGTTTCCGTCACTCAGATCAAGG	GAGGTCCCCAATGCGGAACACGGGACAG	504
$P2X_7$	ATGCCGGCCTGCTGCAGCTGCAGTGATG	GGGACACAACCGCTGCTCTTGGCCTTCTG	361
$P2Y_1$	TCATCATCGGCTTCCTGGGCA	ACCACAATGAGCCACACCAGCAC	332
$P2Y_2$	ATGGCACCTGGGATGGGGATG	GCGGGCGTAGTAATAGACCAGCAG	225
$P2Y_4$	TGCCACCCTCACTTCTCCCCTT	TCGGAGGCGGAAGAAGATGAAGAGC	214
$P2Y_5$	GCGACCTCAAGATACAACTGGCAACT	AATACAGTCAACGAGTCCAACCCATAG	371
$P2Y_6$	ACGGCAGCATCCTCTTCCTCACC	CAGCAGGAAGCCGATGACAGTGA	256
$P2Y_7$	GCTGGTCTCAAACTGCTAACATCAAGT	GGTTACCCTGCTGAGTCTGGATTTATG	354
$P2Y_8$	ATTCAACCAGCCCCACCGCC	CTTCTCTATCAGGGACTCACAGAAGCA	367
$P2Y_9$	AGTCCTCAGTGGCGGTATTTCAGCC	CGCACCAGGGCATACAAGAAGAGG	310
$P2Y_{10}$	GCCCTTTGCCTGCTCTGCTTCTAC	GTAATCATCCCGACCAACGCAACT	267
$P2Y_{11}$	GGAACCCAGAGGACGCCAAGAG	GGGTGGGAAAGGCGACTGCTC	244
$P2Y_{12}$	GGTCCTCTTCCCACTGCTCTACACTG	CAAGAGATTTTTGGGGTTGGATGTTTTA	316
$P2Y_{13}$	AACAGTATTTTCCTTTTCAACACATCTATTGAAAG	AACAAACACCCACAGAGCCAAAGTATTC	294
$P2Y_{14}$	TAACATCAAAGAAAACATACCCATCAGTAATTTC	TATTTAGAGAAAGAAGATGCCTGAGTGAGTTGTC	187
$P2Y_{15}$	ATAGCAGCATCCTCTTCCTCACCTGTTT	CTGATCTGTTGGTCCTGTTGGTTGATGT	165

Table 1. Primers used for RT-PCR.

Fluorescent cytochemistry

After washing with cold phosphate-buffered saline (PBS), Caco-2 cells untreated or treated with ATP (1 mM) for 48 h were incubated in 1 ml PBS containing each 1 µl of the fluorescent dyes, propidium iodide (1 mg/ml) and Hoechst 33342 (5 mg/ml), at 0°C for 30 min. Reactions to each dye were detected with a fluorescent photomicroscope (ECLIPSE TE300, NIKON Co., Tokyo, Japan) equipped with an epifluorescence device.

Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay

Caco-2 cells were fixed with 4% (v/v) paraformaldehyde. After removing inactivate endogenous peroxidase with PBS containing 0.3% (v/v) H_2O_2, a Permeabilization Buffer was applied to cells and stood on ice for 5 min. Then, a Labeling Reaction Mixture was added and incubated in a humidified chamber at 37°C for 60 min. Reactive cells were stained with 3, 3'-diaminobenzidine and detected with a light microscope. Both TUNEL-negative and positive-cells were counted in the area of 0.37 mm^2 selected at random.

Cell cycle analysis

After treatment with ATP (1 mM) for 48 h, Caco-2 cells were harvested by a trypsinization, centrifuged at 1500 x g for 5 min, washed with PBS, and fixed in 70% (v/v) ethanol at 4°C overnight. Fixed cells were washed twice with PBS and incubated in PBS containing 1.5 µg/ml ribonuclease A at 37°C for 1 h, followed by staining with 5 µl of propidium iodide on ice for 20 min. Then, cells were collected on a nylon mesh filter (pore size, 40 µm), and cell cycles for 2 x 10^4 cells were assayed with a flow cytometer (FACSCalibur; Becton Dickinson, Franklin Lakers, NJ, USA) at an excitation of 488 nm and an emission of 585 nm, and analyzed using a Mod Fit LT software (Verity Software House Inc., Topshan, NE, USA).

In situ PKC assay

In situ PKC activity was assayed by the modified method as previously described [23]. Caco-2 cells or SK-N-SH cells were plated in 96-well plates (1 x 10^4 cells/well). Cells were untreated and treated with ATP, ATPγS, AMP-PNP, GF109203X, TPA, PD98059, TPA plus GF109203X, TPA plus ATP, GF109203X plus PD98059, or ATP plus PD98059 in a serum-free medium at 37°C for 60 min. Then, cells were rinsed with 100 µl of Ca^{2+}-free PBS and incubated in 50 µl of extracellular solution [137 mM NaCl, 5.4 mM KCl, 10 mM $MgCl_2$, 5 mM EGTA, 0.3 mM Na_2HPO_4, 0.4 mM K_2HPO_4, 20 mM HEPES (pH 7.2)] containing 50 µg/ml digitonin, 25 mM glycerol 2-phosphate, 200 µM ATP, and 100 µM synthetic PKC substrate peptide (Pyr-Lys-Arg-Pro-Ser-Gln-Arg-Ser-Lys-Tyr-Leu)(Peptide Institute, Osaka, Japan) at 30°C for 15 min. The supernatants were collected and boiled at 100°C for 5 min to terminate the reaction. Aliquot of the solution (20 µl) was loaded onto a reversed-phase high-performance liquid chromatography (HPLC)(LC-10ATvp, Shimadzu, Co., Kyoto, Japan). A substrate peptide peak and a new product peak were detected at an absorbance of 214 nm (SPD-10Avp UV-VIS detector; Shimadzu Co., Kyoto, Japan). In the analysis of matrix-assisted laser desorption ionization time of flight mass spectrometry (Voyager ST-DER; PE Biosystems Inc., Foster City, CA, USA), it was confirmed that each peak corresponds to unphosphorylated and phosphorylated substrate peptide. Areas for unphosphorylated and phosphorylated substrate peptide were measured (total area corresponds to a concentration of PKC substrate peptide used here), and the amount of phosphorylated substrate peptide was calculated. Phosphorylated substrate peptide (pmol/min/cell protein weight) was used as an index of PKC activity.

MAP kinase assay

Caco-2 cells untreated or treated with PD98059, GF109203X, ATP, PD98059 plus GF109203X, PD98059 plus ATP,

or GF109203X plus ATP in a serum-free medium for 60 min were lysed with a glass homogenizer in a cold PBS. Homogenates were centrifuged at 1000 x g for 10 min, and the supernatant (30 μg protein) was loaded on 10% (v/v) SDS-polyacrylamide gel and electrophoresed. Separated proteins were electrophoretically transferred onto a PVDF membrane. The membrane was then incubated in an anti-ERK1 antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA)(1:1000) or an anti-phospho-ERK antibody (Santa Cruz Biotechnology)(1:500) followed by a biotinylated anti-rabbit IgG antibody (1:5000) or an anti-mouse IgG (1:5000), respectively, and a streptavidin biotin complex peroxidase kit. Immunoreactive bands were visualized with a Vector NovaRED substrate kit and quantified by a densitometry using an NIH Image 1.52 software. ERK activity was normalized by calculating the ratio of phosphorylated ERK intensity/unphosphorylated ERK1 at 41 and 42 kDa.

RT-PCR

Total RNAs of Caco-2 cells and SK-H-SH cells were purified by an acid/guanidine/thiocyanate/chloroform extraction method using a Sepasol-RNA I Super kit. After purification, total RNAs were treated with RNase free-DNase I (2 unit) at 37°C for 30 min to remove genomic DNAs, and 10 μg of RNAs were resuspended in water. Then, oligo dT primers, dNTP, 5x First Strand buffer, and SuperScript III RNase H-Reverse Transcriptase were added to the RNA solution and incubated at 65°C for 5 min followed by 60°C for 1 min, 56°C for 60 min, 58°C for 60 min, 85°C for 5 min to synthesize the first strand cDNA. Subsequently, 1 μl of the reaction solution was diluted with water and mixed with 10x PCR reaction buffer, dNTPs, $MgCl_2$, oligonucleotide, dimethylsulfoxide [final concentration, 5% (v/v)] and 1 unit of Taq polymerase (final volume, 20 μl). RT-PCR was carried out with a Takara Thermal cycler Dice programmed as follows: the first one step at 94°C for 2 min and the ensuing 30 cycles at 94°C for 1 s, 62°C for 15 s, and 72°C for 30 s using primers shown in Table 1. PCR products were stained with ethidium bromide and visualized by 2% (w/v) agarose electrophoresis.

Results

Extracellular ATP suppresses Caco-2 cell proliferation

In the MTT assay, 48-h treatment with extracellular ATP reduced Caco-2 cell viability in a concentration (10 μM-10 mM)-dependent manner (Fig. 1A). Likewise, AMP-PNP, an unhydrolyzed ATP analogue, exhibited an effect similar to ATP (Fig. 1B), indicating that ATP by itself, but not its metabolites such as ADP, AMP, and adenosine, is endowed with reduced Caco-2 cell viability.

The ATP effect on Caco-2 cell viability was not inhibited by caspase inhibitors such as Z-VAD-FMK, a pan-caspase inhibitor, Z-YVAD-FMK, a caspase-1 inhibitor, Z-DEVD-FMK, a caspase-3 inhibitor, Z-IETD-FMK, a

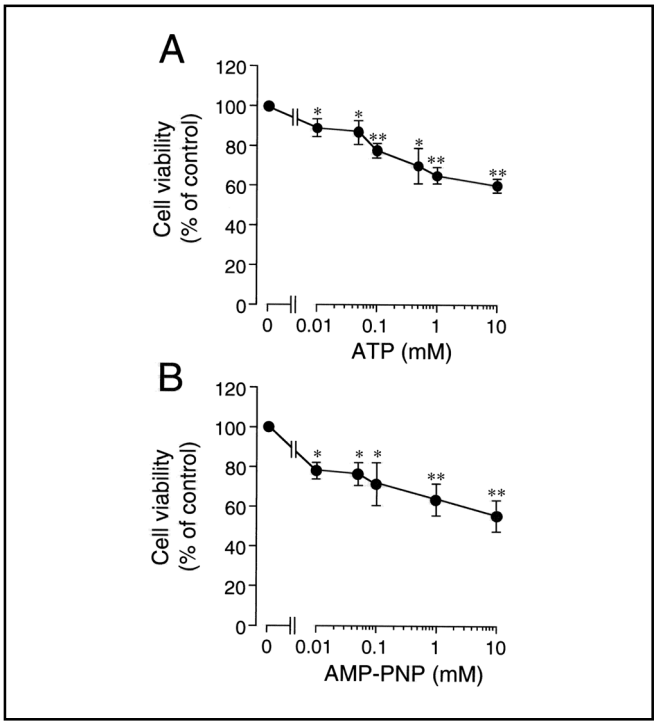


Fig. 1. The effect of extracellular ATP on Caco-2 cell viability. Caco-2 cells were treated with ATP (A) or AMP-PNP (B) at concentrations as indicated in a serum-free medium for 48 h, and cell viability was monitored with an MTT assay. In the graph, each point represents the mean (± SEM) percentage of control (MTT intensities for untreated cells)(n=4). **P*<0.05, ***P*<0.01 as compared with control, paired *t*-test.

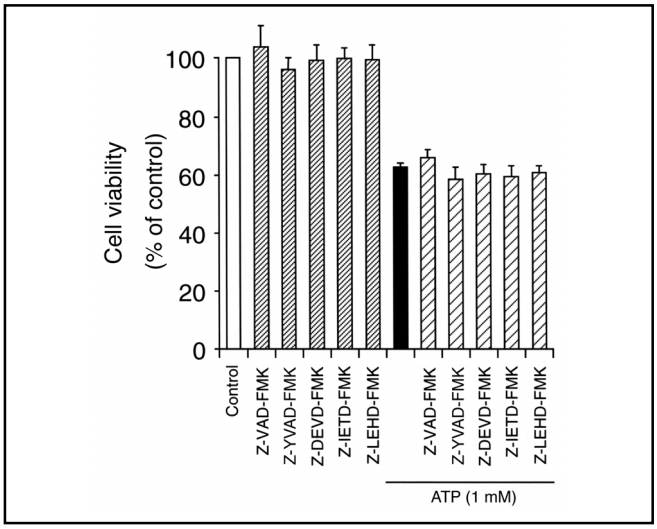


Fig. 2. The effect of caspase inhibitors on extracellular ATP-induced reduction of Caco-2 cell viability. Caco-2 cells were untreated and treated with ATP (1 mM) in a serum-free medium for 48 h in the presence and absence of Z-VAD-FMK, Z-YVAD-FMK, Z-DEVD-FMK, Z-IETD-FMK, or Z-LEHD-FMK at a concentration of 10 μM. In the graph, each column represents the mean (± SEM) percentage of control (MTT intensities for untreated cells)(n=4).

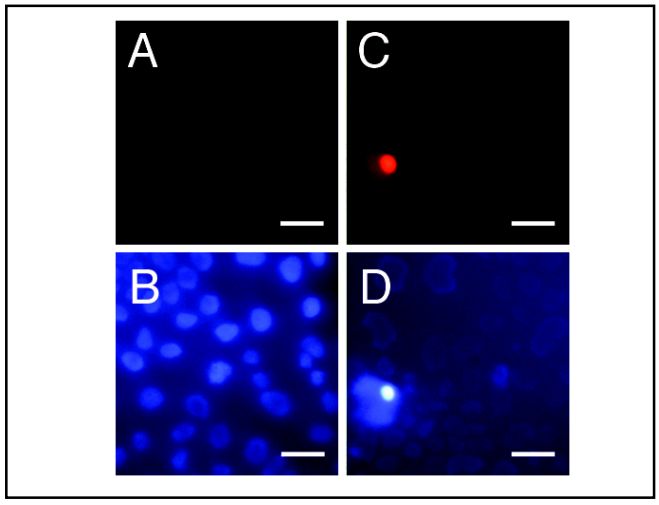


Fig. 3. Fluorescent cytochemistry. Caco-2 cells were untreated (Control) or treated with ATP (1 mM) for 48 h, and then stained with the fluorescent dyes propidium iodide (PI) and Hoechst 33342 (Hoechst). Note that similar results were obtained with 4 independent experiments. Bars, 50 μm.

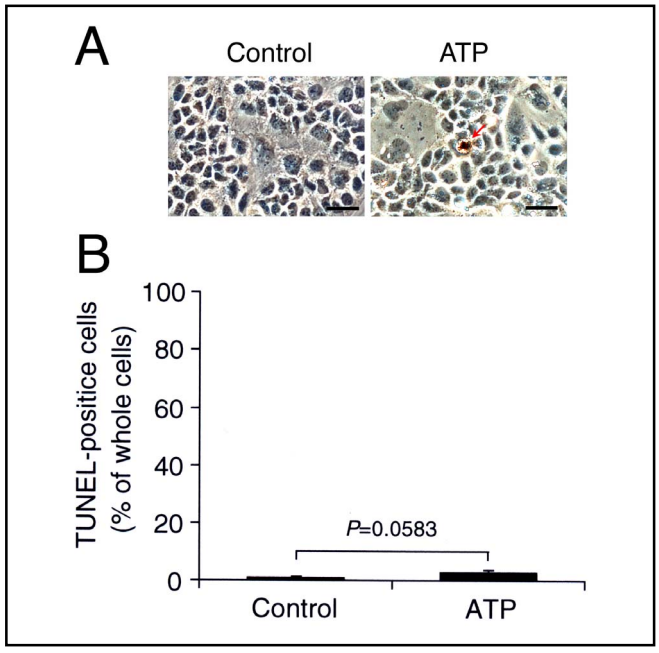


Fig. 4. TUNEL assay. Caco-2 cells were untreated and treated with ATP (1 mM) in a serum-free medium for 48 h, and TUNEL-positive cells were counted. (A) TUNEL staining for cells untreated (Control)) and treated with ATP. Arrow, TUNEL-positive cell. Bars, 50 μm. (B) In the graph, each column represents the mean (± SEM) percentage of whole cells (n=6 independent experiments). *P* values, unpaired *t*-test.

caspase-8 inhibitor, or Z-LEHD-FMK, a caspase-9 inhibitor (Fig. 2), suggesting that ATP-induced reduction of Caco-2 cell viability is not due to apoptotic cell death. In the fluorescent cytochemistry, nuclei of Caco-2 cells treated with ATP (1 mM) for 48 h were little reactive to propidium iodide, a marker of cell death, and Hoechst 33342, a marker of apoptosis (Fig. 3), suggesting less apoptotic cell death induced by ATP. In the TUNEL assay, TUNEL-positive Caco-2 cells were 0.87 ± 0.50 and 2.81 ± 0.76% of whole cells after non-treatment and treatment with ATP (1 mM) for 48 h, respectively, and there was no significant difference between two groups (Fig. 4A,B). It is suggested from these results that extracellular adenosine-induced reduction of Caco-2 cell viability is not mainly ascribed to apoptotic cell death. In the cell cycle analysis, 48-h treatment with ATP (1 mM) significantly decreased proportion of cells in the G_0/G_1 and G_2/M phase of cell cycling, but instead increased the proportion in the S phase (Fig. 5). Overall, extracellular ATP appears to suppress Caco-2 cell proliferation by arresting cell cycling at the S phase.

Extracellular ATP suppresses Caco-2 cell proliferation via a PKC and MAP kinase pathway

We next asked a question as to what signals underlie the ATP action. The PKC inhibitor GF109203X (100 nM) reduced Caco-2 cell viability to an extent similar to that for ATP (1 mM), and no further reduction was obtained with co-treatment with ATP (Fig. 6). TPA

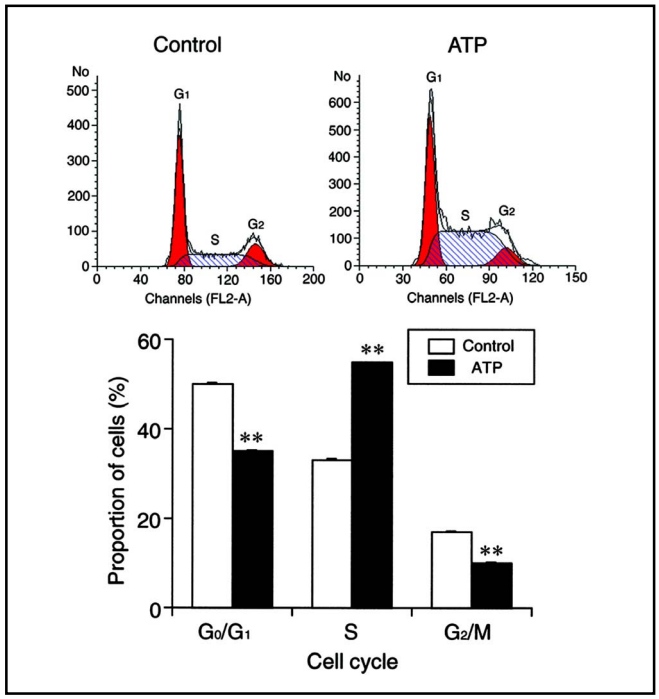


Fig. 5. The effect of extracellular ATP on Caco-2 cell cycling. Caco-2 cells were untreated (Control) or treated with ATP (1 mM) in a serum-free medium for 48 h, and proportion of cells in each phase of cell cycling was assayed. In the graph, each column represents the mean (± SEM) percentage for each phase as indicated (n=4). **P<0.01, unpaired *t*-test.

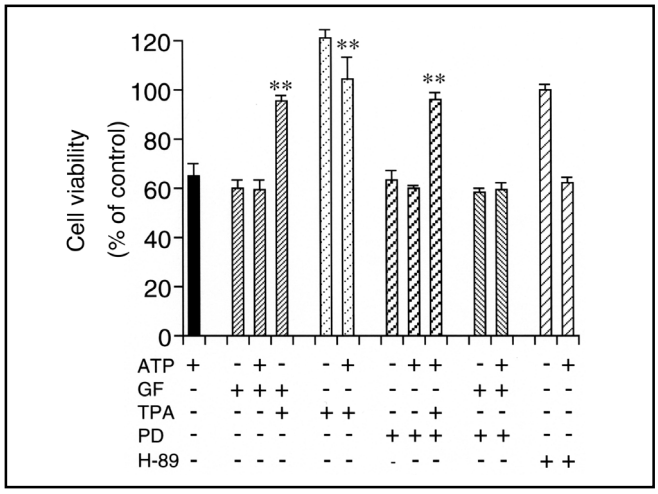


Fig. 6. The effect of a PKC inhibitor or a MEK inhibitor on Caco-2 cell viability. Caco-2 cells were untreated and treated with various combinations of ATP (1 mM), GF109203X (GF) (100 nM), TPA (100 nM), PD98059 (PD) (10 μM), and H-89 (1 μM) as indicated in a serum-free medium for 48 h. In the graph, each column represents the mean (± SEM) percentage of control (MTT intensities for untreated cells)(n=4). $^{**}P<0.01$ as compared with viability of cells treated with GF or ATP, unpaired *t*-test.

(100 nM), a PKC activator, enhanced Caco-2 cell viability to approximately 120% of basal levels (Fig. 6). The effect of GF109203X or ATP on Caco-2 cell viability was reversed by TPA (Fig. 6), suggesting that ATP suppresses Caco-2 cell proliferation by inhibiting PKC. Notably, PD98059 (10 μM), an inhibitor of MAP kinase kinase (MEK), also reduced Caco-2 cell viability to an extent similar to that for ATP (1 mM) or GF109203X (100 nM), and no further reduction was found with co-treatment with ATP, GF109203X, or ATP plus GF109203X (Fig. 6). The effect of PD98059 plus ATP was neutralized by TPA (Fig. 6). In contrast, H-89 (1 μM), an inhibitor of protein kinase A (PKA), did not affect basal Caco-2 cell viability and ATP-induced reduction of Caco-2 cell viability (Fig. 6). Taken together, these results indicate that extracellular ATP suppresses Caco-2 cell proliferation in a PKC- and MAP kinase-dependent manner.

Extracellular ATP inhibits PKC in Caco-2 cells as mediated via an unknown receptor

To see whether ATP actually inhibits PKC in Caco-2 cells, we carried out in situ PKC assay. TPA (100 nM) increased an area for phosphorylated substrate peptide (Fig. 7A), i.e., TPA activated PKC (Fig. 7B), and the increase was abolished by GF109203X (100 nM) (Fig. 7A), i.e., TPA-induced PKC activation was prevented

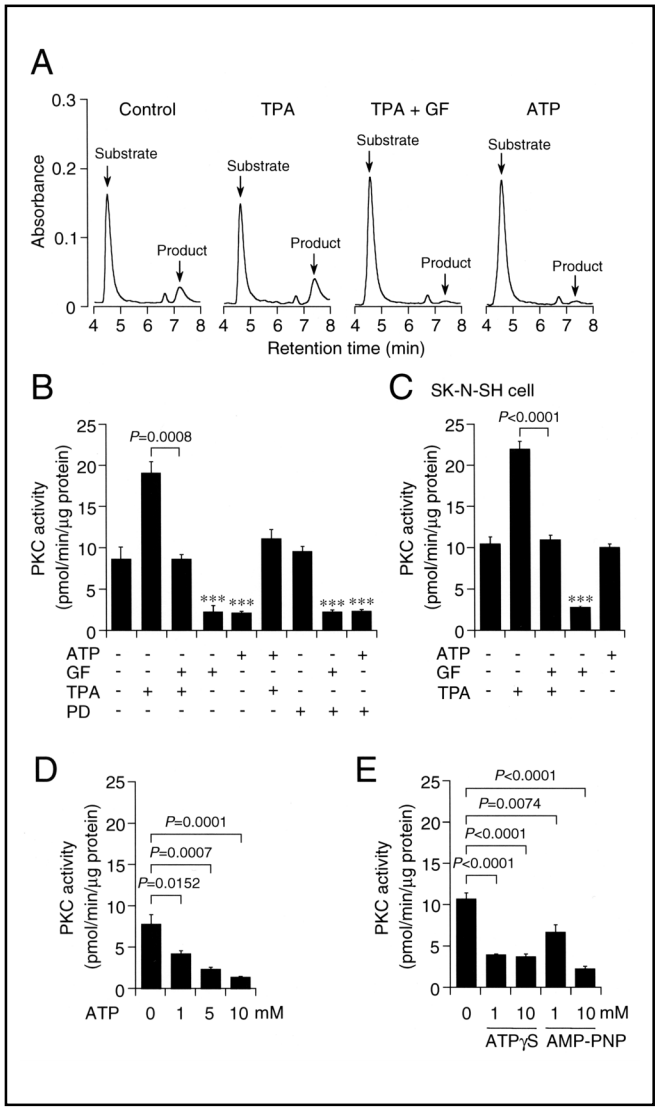
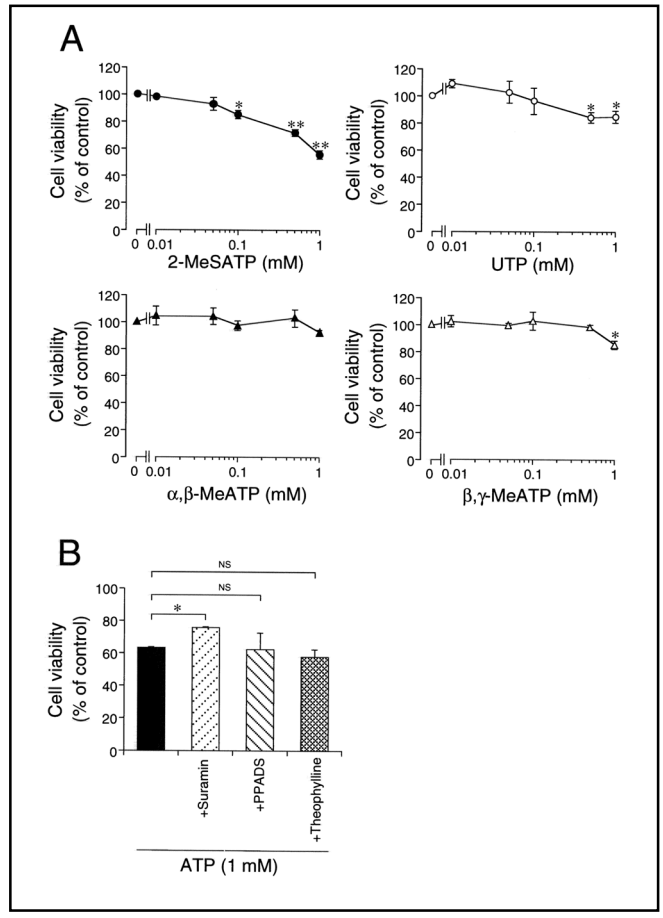


Fig. 7. The effect of extracellular ATP on PKC activity. Caco-2 cells or SK-N-SH cells were untreated and treated with various combinations of ATP, ATPγS, AMP-PNP, GF109203X (GF), TPA, and PD98059 (PD) as indicated in a serum-free medium at 37°C for 60 min, and PKC activity was quantified with a reversed phase HPLC. (A) HPLC profiles. In the graphs (B,C,D,E), data represent the mean (± SEM) PKC activity. (B) Effect of ATP on PKC activity in Caco-2 cells (n=8). ATP, 10 mM; GF, 100 nM; TPA, 100 nM; PD, 10 μM. P value between PKC activity in the presence of TPA alone and that in the presence TPA plus GF; $^{***}P<0.001$ as compared with basal PKC activity in the absence of ATP, GF, TPA, and PD, unpaired *t*-test. (C) Effect of ATP on PKC activity in SK-N-SH cells (n=8). ATP, 10 mM; GF, 100 nM; TPA, 100 nM. *P* value between PKC activity in the presence of TPA alone and that in the presence TPA plus GF; $^{***}P<0.001$ as compared with basal PKC activity in the absence of ATP, GF, and TPA, unpaired *t*-test. (D) Concentration-dependent inhibition of ATP on PKC activity in Caco-2 cells (n=8). *P* values, unpaired *t*-test. (E) Effect of ATPγS or AMP-PNP on PKC activity in Caco-2 cells (n=8). P values, unpaired *t*-test.

Fig. 8. The effect of P_2 purinoceptor agonists on Caco-2 cell viability and the effect of purinoceptor antagonists on extracellular ATP-induced reduction of Caco-2 cell viability. (A) Caco-2 cells were treated with 2-MeSATP, UTP, α,β-MeATP, or β,γ-MeATP at concentrations as indicated in a serum-free medium for 48 h. In the graphs, each point represents the mean (±SEM) percentage of control (viability of cells untreated with ATP) (n=4). **P*<0.05; ***P*<0.01, unpaired *t*-test. (B) Cells were treated with ATP (1 mM) in the presence and absence of suramin (50 μM), PPADS (50 μM), or theophylline (50 μM) in a serum-free medium for 48 h. In the graphs, each column represents the mean (±SEM) percentage of control (C)(MTT intensities for untreated cells)(n=4). **P*<0.05, unpaired *t*-test.



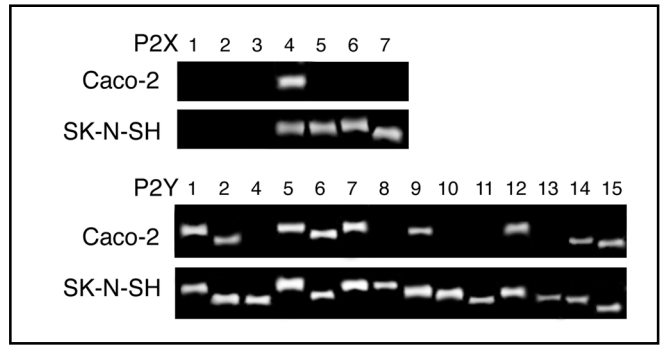


Fig. 9. RT-PCR analysis for P2X and P2Y receptors expressed in Caco-2 and SK-N-SH cells. PCR product for $P2X_1$ (1), $P2X_2$ (2), $P2X_3$ (3), $P2X_4$ (4), $P2X_5$ (5), $P2X_6$ (6), and $P2X_7$ receptor (7) in the upper 2 columns and $P2Y_1$ (1), $P2Y_2$ (2), $P2Y_4$ (4), $P2Y_5$ (5), $P2Y_6$ (6), $P2Y_7$ (7), $P2Y_8$ (8), $P2Y_9$ (9), $P2Y_{10}$ (10), $P2Y_{11}$ (11), $P2Y_{12}$ (12), $P2Y_{13}$ (13), $P2Y_{14}$ (14), and $P2Y_{15}$ receptor (15) in the lower 2 columns.

by GF109203X (Fig. 7B). This confirms that PKC assay used here is reliable. GF109203X (100 nM) inhibited basal PKC activation in Caco-2 cells (Fig. 7B). Likewise, ATP (10 mM) inhibited basal PKC activation in Caco-2 cells to an extent similar to GF109203X, and the inhibition was neutralized by TPA (Fig. 7B). This accounts for ATP-induced PKC inhibition in Caco-2 cells. PD98059 (10 μM) did not affect basal PKC activation or PKC inhibition induced by GF109203X or ATP (Fig. 7B), indicating that MAP kinase is not implicated in the PKC inhibition.

To prove that ATP-induced PKC inhibition in Caco-2 cells is not a non-specific effect, we assayed PKC activity in SK-N-SH cells, a human neuroblastoma cell line. TPA (100 nM) still activated PKC in SK-N-SH cells, that is eliminated by GF109203X (100 nM) (Fig. 7C). GF109203X attenuated basal PKC activation in SK-N-SH cells, yet no PKC inhibition was obtained with ATP (10 mM) (Fig. 7C). This confirms the specific inhibitory action of ATP on PKC activation in Caco-2 cells. The ATP effect on PKC in Caco-2 cells was concentration (1-10 mM)-dependent (Fig. 7D), although no significant inhibition was obtained with lower concentrations of ATP (10-100 μM) (data not shown). Like ATP, the unhydrolyzed ATP analogues, ATPγS and AMP-PNP, inhibited PKC activation in a concentration (1-10 mM)-dependent manner (Fig. 7E). It is suggested from these results that extracellular ATP inhibits PKC activation in Caco-2 cells as mediated via an extracellular receptor in Caco-2 cells.

Then, the question addressing is what receptor mediates the ATP action. P2 purinoceptor agonists such as 2-MeSATP, UTP, and β,γ-MeATP reduced Caco-2 cell viability in a concentration (10 μM-1 mM)-dependent manner, with the order of potency: 2-MeSATP> UTP>β,γ-MeATP, but no effect was found with α,β-MeATP (Fig. 8A). ATP (1 mM)-induced reduction of Caco-2 cell viability, on the other hand, was partially inhibited by suramin (50 μM), a non-selective inhibitor of P_2 purinoceptors, but no inhibition was obtained with

PPADS (50 μM), another non-selective inhibitor of P2 purinoceptors, or theophylline (50 μM), a broad inhibitor of adenosine receptors (P_1 purinoceptors) (Fig. 8B). Taken together, these results suggest that a P_2 purinoceptor, but not a P_1 purinoceptor, might participate in the ATP action. In the RT-PCR analysis for the P2X receptors $P2X_{1,2,3,4,5,6,7}$ and the P2Y receptors $P2Y_{1,2,4,5,6,7,8,9,10,11,12,13,14,15}$, Caco-2 cells expressed mRNAs for P2X4 receptor and P2Y receptors such as $P2Y_{1,2,5,6,7,9,12,14,15}$, while SK-N-SH cells expressed mRNAs for P2X receptors such as $P2X_{4,5,6,7}$ and all the P2Y receptors examined here (Fig. 9). This implies that an identified ATP receptor does not mediate extracellular ATP-induced PKC inhibition in Caco-2 cells, since SK-N-SH cells expressed all the ATP receptor mRNAs that Caco-2 cells did.

Extracellular ATP inhibits MAP kinase downstream PKC in Caco-2 cells

To see whether ATP inhibits PKC followed by MAP kinase in Caco-2 cells, we monitored activity of ERK, a MAP kinase, with a Western blotting. PD98059 (10 μM) decreased normalized phosphorylated ERK both at 41 and 42 kDa (Fig. 10), i.e., PD98059 inhibited ERK activation. GF109203X (100 nM), ATP (10 mM), or GF109203X plus ATP inhibited ERK activation to an extent similar to that for PD98059 alone (Fig. 10). The inhibitory effect of PD98059 on ERK activation was not enhanced by co-treatment with GF109203X or ATP (Fig. 10). Accordingly, the results indicate that extracellular ATP inhibits PKC and in turn, MAP kinase activation as a downstream target of PKC in Caco-2 cells.

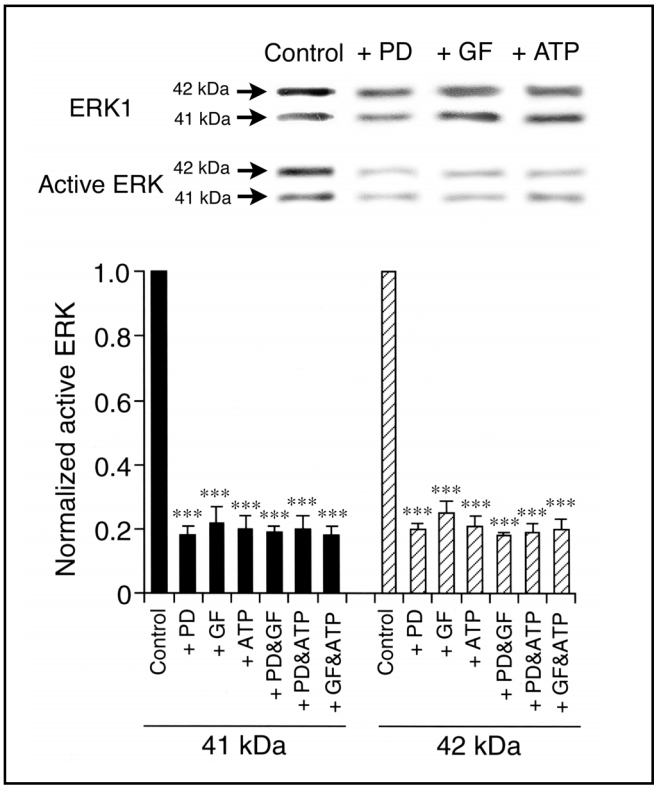


Fig. 10. The effect of extracellular ATP on MAP kinase activity. Caco-2 cells were untreated or treated with various combinations of PD98059 (PD)(10 μM), GF109203X (GF)(100 nM), and ATP (10 mM) as indicated in a serum-free medium for 60 min, and then Western blotting was carried out using an anti-ERK1 antibody (ERK1) and an anti-p-ERK antibody (Active ERK). ERK activity was normalized by calculating the ratio of phosphorylated ERK intensity/unphosphorylated ERK1 at 41 and 42 kDa. In the graph, each column represents the mean ratio against control regarding as 1 (normalized ERK activity for untreated cells)(n=6 independent experiments). ***P<0.001 as compared with each control ERK activity at 41 and 42 kDa, unpaired *t*-test.

Discussion

The results of the present study clearly demonstrate that extracellular ATP or the unhydrolyzed ATP analogue AMP-PNP reduced Caco-2 cell viability in a concentration (10 μM-10 mM)-dependent manner. The ATP effect was not affected by caspase inhibitors, suggesting less possibility for ATP-induced Caco-2 cell apoptosis. In support of this note, Caco-2 cells were little reactive to propidium iodide and Hoechst 33342 or little positive to TUNEL after 48-h treatment with ATP (1 mM). In the cell cycle analysis, extracellular ATP (1 mM) significantly increased proportion of cells in the S phase of cell cycling, while it decreased the proportion in the G0/G1 and G2/M phase. This indicates that extracellular ATP, at higher concentrations (mM range), reduces Caco-2 cell viability by inhibiting Caco-2 cell growth at the S phase of cell cycling. A study shows that more than one-third of Caco-2 cells had apoptotic nuclei after 48-h treatment with ATP (2 mM) in the TUNEL assay [24]. In the present study, however, TUNEL-positive Caco-2 cells were only 3% of whole cells after 48-h treatment with ATP (1 mM). The reason for this discrepancy is presently unknown, but the results of the present study strongly suggest that higher concentrations of extracellular ATP favor suppression of Caco-2 cell proliferation rather than induction of apoptotic cell death.

Notably, the PKC inhibitor GF109203X reduced Caco-2 cell viability to an extent similar to that for extracellular ATP (1 mM). The GF109203X effect was not

enhanced by co-treatment with ATP, and reduction of Caco-2 cell viability induced by ATP or GF109203X was reversed by the PKC activator TPA. This suggests that extracellular ATP, at higher concentrations, suppresses Caco-2 cell proliferation by inhibiting PKC. In the in situ PKC assay, higher concentrations (mM range) of extracellular ATP indeed inhibited PKC activation in Caco-2 cells in a fashion that mimics the GF109203X. Like ATP, the unhydrolyzed ATP analogues, ATPγS and AMP-PNP, inhibited PKC activation in Caco-2 cells, suggesting that ATP by itself, but not ATP metabolites, inhibits PKC activation as mediated via an extracellular receptor.

Extracellular ATP regulates a wide-range of cellular functions via the P2 purinoceptors such as P2X and P2Y receptors. ATP is shown to inhibit cell growth via those receptors [11-16]. P2X receptors contain non-selective cation channels and transmit signals through ion influx and efflux. Interestingly, $P2X_7$ receptors mediate activation of protein kinase D, PKC-δ, and ERK1/2 in freshly isolated rat parotid acinar salivary cells [25] or ATP aggregates platelets via a $P2X_1$ receptor/PKC/ERK2 pathway [26]. P2Y receptors are classified as G protein-linked receptors. $P2Y_1$, $P2Y_2$, $P2Y_4$, and $P2Y_6$ are linked to G_q protein involving phospholipase C activation followed by PKC activation, $P2Y_{11}$, $P2Y_{12}$, and $P2Y_{13}$ to G_s protein involving adenylate cyclase activation followed by PKA activation, and $P2Y_{14}$ to G_i protein involving adenylate cyclase inhibition followed by PKA inhibition [19-21]. No evidence, however, has been provided for P2X and P2Y receptors involving PKC inhibition, although a study suggests that extracellular ATP inhibits HL-60 cell growth via a P_2 purinoceptor insensitive to UTP and α,β-MeATP, perhaps bearing PKC inhibition [27]. In the present study, P_2 purinoceptor agonists such as 2-MeSATP, UTP, and β,γ-MeATP, but not α,β-MeATP, reduced Caco-2 cell viability in a concentration (10 μM-1 mM)-dependent manner, with the order of potency: 2-MeSATP>UTP>β,γ-MeATP, and extracellular ATP (1 mM)-induced reduction of Caco-2 cell viability was partially inhibited by the non-selective P_2 purinoceptor inhibitor suramin, but not PPADS. This suggests that a P_2 purinoceptor might mediate the ATP action. The ATP effect was not affected by the broad P_1 purinoceptor inhibitor theophylline. This, taken together with the finding that the unhydrolyzed ATP analogue AMP-PNP exhibited an effect similar to ATP, excludes the implication of a P_1 purinoceptor in the ATP action. In the in situ PKC assay, extracellular ATP (10 mM) did not inhibit PKC activation in SK-N-SH cells, although SK-N-SH cells expressed all the mRNAs for P2X and P2Y receptors that Caco-2 cells did. This would interpret that an as of yet unidentified P_2 purinoceptor participates in extracellular ATP-induced PKC inhibition in Caco-2 cells. Previous studies indicate that well-recognized P_2 purinoceptors on the plasma membrane are activated by ATP or their agonists at concentrations ranging from nM to μM. ATP here, however, suppressed Caco-2 cell proliferation and inhibited PKC at higher concentrations (mM range), while no significant effect was obtained with lower concentrations (μM range). This might also support the note for the implication of an unknown ATP receptor.

The MEK inhibitor PD98059 also reduced Caco-2 cell viability to an extent similar to that for extracellular ATP (1 mM) or GF109203X, and the PD98059 effect was not enhanced by co-treatment with ATP, GF109203X, or ATP plus GF109203X. Reduction of Caco-2 cell viability induced by co-treatment with ATP and PD98059 was neutralized by TPA. These results indicate that extracellular ATP, at higher concentrations, suppresses Caco-2 cell proliferation by inhibiting PKC and the ensuing MAP kinase. PKC is recognized to regulate MAP kinase activity by targeting c-Raf in a MAP kinase activation pathway linked to growth factor receptors/tyrosine kinase (or Src family)/Ras/c-Raf/MEK [28]. In the MAP kinase assay, ATP (10 mM), GF109203X, PD98059 plus GF109203X, PD98059 plus ATP, and GF109203X plus ATP decreased the active form of ERK in Caco-2 cells to an extent similar to that achieved by PD98059 alone. This, in the light of the finding that PD98059 did not affect PKC activity in Caco-2 cells, implies that extracellular ATP, at higher concentrations (mM range), inhibits PKC followed by MAP kinase as a downstream target of PKC. Lower concentrations (μM range) of extracellular ATP, alternatively, are show to activate MAP kinase via a P2Y purinoceptor [29]. This may account for the stimulatory and inhibitory biphasic actions of extracellular ATP on MAP kinase, depending upon its concentrations. Overall, extracellular ATP, at higher concentrations, might suppress Caco-2 cell proliferation by inhibiting MAP kinase downstream PKC via an unknown ATP receptor. To address the responsible receptor, we are currently carrying out further experiments.

In conclusion, the results presented here show that extracellular ATP, at higher concentrations, suppresses proliferation of Caco-2 human colonic cancer cells at the S phase of cell cycling by inhibiting PKC, possibly as mediated via an unknown ATP receptor, followed by MAP kinase. This may extend our knowledge of ATP/its receptor signaling relevant to colonic cancer cell proliferation.

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