Purinoceptor-Mediated Calcium Mobilization and Cellular Proliferation in Cultured Bovine Corneal Endothelial Cells

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ABSTRACT—In the present study, we investigated the effect of adenosine triphosphate (ATP) on cytosolic free calcium mobilization and mitogenic activity in cultured bovine corneal endothelial cells (BCEC). The 
\[ \text{Ca}^{2+} \] was determined using a \text{Ca}^{2+} sensitive indicator, Fura-2/AM, and cell proliferation was evaluated by counting the cell number. ATP, its metabolites and analogs caused transient increase in \[ \text{Ca}^{2+} \]; in a concentration-dependent manner (\(10^{-7} \text{M} \rightarrow 10^{-3} \text{M} \)) and the potency of agonists was ordered as follows: 2-methylthio-ATP > uridine triphosphate > ATP > adenosine diphosphate. Adenosine monophosphate and adenosine did not affect \[ \text{Ca}^{2+} \]. ATP (\(10^{-4} \text{M} \)) also promoted the accumulation of inositol trisphosphate (IP3). The ATP-induced transient \[ \text{Ca}^{2+} \] increase and IP3 accumulation were attenuated by pretreatment with a phospholipase C inhibitor, U-73122 (5 \(\mu \text{M} \)), for 30 min. ATP (\(10^{-5} \text{M} \)) significantly enhanced the proliferation of BCEC. ATP-induced \[ \text{Ca}^{2+} \] increase and cell proliferation were inhibited by a purinoceptor antagonist, suramin (\(10^{-4} \text{M} \)). Thus, the present study indicates that BCEC contain P2 purinoceptors that regulate their proliferation.

Keywords: Corneal endothelium, Adenosine triphosphate, Cytosolic free calcium, Proliferation

It is well known that intracellular adenosine triphosphate (ATP) plays important roles as a source of energy, as a phosphate group donor in phosphorylation reactions, and as a substrate for ATPases or adenylyl cyclase (1). On the other hand, there have been many recent reports about the physiological roles of extracellular ATP in biological responses such as vascular smooth muscle contraction, cardiac function, platelet aggregation, renal gluconeogenesis and glial adenylyl cyclase activity regulation (2–5). There are two classes of purinoceptors: P1- and P2-purinoceotors. P1-receptors are activated by adenosine and are blocked by methylxanthines (6), whereas P2-receptors are activating by ATP and adenosine diphosphate (ADP), and are insensitive to methylxanthines (7).

In ocular tissues, corneal endothelial cells possess essential physiological roles such as maintenance of corneal transparency through their barrier function and water and ion pumping. In several kinds of endothelial cells, extracellular ATP induces a variety of physiological or biochemical responses; for example, \text{Ca}^{2+} mobilization in glomerular

endothelial cells (8), \text{Ca}^{2+} homeostasis and formation of cGMP and prostacyclin in adrenal medullary endothelial cells (9), and stimulation of nitric oxide in cultured bovine aortic endothelial cells (10). However, there are no sufficient reports on the physiological or pharmacological roles of extracellular ATP in corneal endothelial cells.

The present study was performed to clarify the effects of extracellular ATP on cultured bovine corneal endothelial cells (BCEC). This report may extend our knowledge about the pharmacological and/or physiological importance of ATP in cytosolic calcium mobilization and cell proliferation in cultured corneal endothelial cells.

MATERIALS AND METHODS

Materials

The materials used in the present study were purchased from the following sources: ATP, uridine triphosphate (UTP) and ADP from Boehringer (Mannheim, Germany); 2-methylthio (meS)-ATP, \(\alpha,\beta\)-methylene (\(\alpha,\beta\)-me)-ATP and U-73122 from Research Biochemicals, Inc., (Natick, MA, USA); adenosine monophosphate (AMP), adenosine,
Fura-2 and Fura-2/AM from Sigma Chemical (St. Louis, MO, USA); Dulbecco’s modified Eagle medium (DMEM), penicillin-streptomycin, fetal bovine serum and amphotericin B from Gibco (Gaithersburg, MD, USA); suramin from Wako (Osaka). All other chemicals were of the highest available grade.

**BCEC culture**

Cultures of BCEC were established as previously described (11). The corneoscleral shell was excised from a bovine eyeball, and the Descemet’s membrane was carefully stripped using Jeweller’s forceps under a dissecting microscope (Topcon, Tokyo). The Descemet’s membrane was then cut into 1- to 2-mm squares using spring scissors and placed on 60-mm tissue culture dishes (Corning, Corning, NY, USA) containing DMEM medium with 10% fetal bovine serum, penicillin (100 U/ml), streptomycin (100 μg/ml) and amphotericin B (1.25 mg/ml). Cells were incubated at 37°C in 5% CO₂ and allowed to reach confluence before being passaged. At the time of passage, the cells were treated with 2 ml of 0.25% trypsin-EDTA, and incubated for 10 min at 37°C. The cells were then centrifuged for 5 min at 1000 rpm, resuspended and replated onto culture dishes. In this experiment, the BCEC that had been passaged 2–4 times were used.

**Measurement of the intracellular free calcium concentration ([Ca²⁺]ᵢ)**

The method for [Ca²⁺]ᵢ measurement was as previously described (4, 12). Cells between the second and fourth passages were harvested after trypsinization at five days before performing the experiments and seeded onto 22 × 22 mm cover glasses at a concentration of 10⁴ cells/dish. The cover glasses (2-cm diameter) were attached to the bottom of 35-mm plastic culture dishes. Cells were then washed with modified Hanks’ solution consisting of the following components: 127 mM NaCl, 0.8 mM MgSO₄, 0.33 mM Na₂HPO₄, 0.44 mM KH₂PO₄, 1 mM MgCl₂, 10 mM HEPES and 1 mM CaCl₂ (pH 7.4) and loaded with Fura-2/AM (10 μM) for 45 min at 37°C. Fluorescence-loaded cells were washed three times with the same solution to exclude the unloaded Fura-2/AM. The fluorescence in BCEC was measured at room temperature using the InCa™ Imaging System from Intracellular Imaging, Inc. (Cincinnati, OH, USA). [Ca²⁺]ᵢ was calculated from the standard curve generated in situ (13).

**Measurement of inositol trisphosphate (IP₃) accumulation**

For the measurement of IP₃, BCEC were metabolically labeled for 20 h in an inositol-free medium containing 0.5% heat-inactivated fetal bovine serum and 1 mM [³H]-myo-inositol (NEN, Boston, MA, USA). The cells were incubated in HBSS containing 10 mM LiCl and 0.2% bovine serum albumin with or without the specified additives. Inositol phosphates and free inositol were extracted with cold 10% perchloric acid. The extraction solution was neutralized using 10% KOH and then loaded onto AG 1-X* (formate form) columns. The inositol monophosphate (IP₁) and inositol bisphosphate (IP₂) were excluded by eluted serially by 0.2 M ammonium formate/0.1 M formic acid (for IP₁) and 0.4 M ammonium formate/0.1 M formic acid (for IP₂). The [³H]myo-inositol trisphosphate was eluted sequentially using 1 M ammonium formate/0.1 M formic acid (14).

**Determination of cell proliferation**

Cells were seeded at a concentration of 10⁵ cells/well to a six-well plate and incubated for 1 day for cell attachment to the bottom. After confirmation of cell attachment, agonists and an antagonist were applied once a day at the final concentration of 10⁻⁵ M for 7 days. To determine cell proliferation, cells were detached using trypsin-EDTA solution (0.25%) and the cell number was counted using trypan blue.

**Statistical analyses**

Statistical analyses were performed using Student’s t-test and the ANOVA test for two-group comparison and the comparison of multiple data, respectively. A P value less than 0.05 was considered significant.

**RESULTS**

**Dose-response curves of agonists-induced increase in [Ca²⁺]ᵢ**

In order to investigate the effects of ATP on BCEC, ATP-, its metabolite- and ATP-analogs-induced increases of [Ca²⁺]ᵢ were monitored. The basal [Ca²⁺]ᵢ, with 1 mM CaCl₂ in the bathing solution was 87 ± 9 nM (mean ± S.E.M., n = 16). As shown in Fig. 1, ATP increased [Ca²⁺]ᵢ in a concentration-dependent manner, and significant effects of ATP were observed at concentrations higher than 10⁻⁷ M. Not only ATP but also ADP, UTP and 2-meS-ATP induced a [Ca²⁺]ᵢ increase in a concentration-dependent manner. An ATP analog, αβ-m-m-ATP, also induced a [Ca²⁺]ᵢ increase, but only at a high dosage (10⁻³ M). In contrast, AMP and adenosine did not change [Ca²⁺]ᵢ. The maximal response occurred at a concentration of 10⁻⁴ M, except with 2-meS-ATP (10⁻⁷ M). ATP induced a prompt and transient increase of [Ca²⁺]ᵢ, to a peak with a 5- to 20-s lag after application of ATP and a steady decrease to a plateau was achieved between 90–120 s after the peak. From the concentration-response curves, the half-maximal response (EC₅₀) concentrations were as follows: ATP, 2.1 × 10⁻⁶ M; ADP, 6.3 × 10⁻⁶ M; 2-meS-ATP, 3.9 × 10⁻⁷ M and UTP, 7 × 10⁻⁷ M.
ATP Affects \([Ca^{2+}]\), and Proliferation in BCEC

Source of ATP-mediated calcium mobilization

To determine whether the source of an \([Ca^{2+}]\) increase was intracellular calcium stores or the extracellular milieu across plasma membranes, ATP-evoked \([Ca^{2+}]\) increase was measured under the \(Ca^{2+}\)-free condition. To remove \(Ca^{2+}\) from the medium, 1 mM EGTA was added to the medium 60 s before ATP application. EGTA did not affect the resting level of \([Ca^{2+}]\). As shown in Fig. 2, when BCEC was incubated in the modified Hanks’ solution not containing extracellular free calcium, ATP (10^{-4} M)-mediated transient \([Ca^{2+}]\) increase could also be observed. The peak height, however, was significantly attenuated about 20% compared to that in the normal condition (normal condition: 587.6 ± 28% and \(Ca^{2+}\)-free condition: 388.8 ± 25%). Moreover, the ATP-induced sustained phase disappeared under the \(Ca^{2+}\)-free condition.

Inhibitory effects of U-73122 on ATP-induced \([Ca^{2+}]\), increase and IP3 accumulation

To evaluate the possible involvement of phospholipase C (PLC) in ATP-induced \([Ca^{2+}]\) increase, ATP-induced IP3 accumulation was measured. ATP-induced IP3 accumulation increased in a time (to 3 min)- and concentration (from 10^{-6} M to 10^{-4} M)-dependent manner. As shown in Fig. 3, the transient \([Ca^{2+}]\) increase induced by ATP was significantly inhibited by pretreatment with 5 x 10^{-6} M of U-73122 for 30 min (basal \([Ca^{2+}]\): 85.3 ± 9.4 nM, N = 8; ATP-treated: 591 ± 36% and U-73122 + ATP-treated: 220 ± 70%, compared to basal value), and ATP-induced IP3 accumulation (control: 154.1 ± 11.6 cpm) was attenuated by the pretreatment with 5 x 10^{-6} M of U-73122 or 10^{-4} M of suramin (U-73122 + ATP-treated: 124.9 ± 5.4% and suramin + ATP-treated: 143 ± 9.7%) (Fig. 4). alpha, beta-Me-ATP (10^{-4} M) failed to induce IP3 accumulation.

Antagonistic effect of suramin on ATP-induced increase in \([Ca^{2+}]\)

Next we investigated effects of suramin, a P2 receptor antagonist, on ATP-induced \([Ca^{2+}]\) increase. Suramin did not affect the basal \([Ca^{2+}]\). However, as shown in Fig. 5, ATP (10^{-4} M)-induced \([Ca^{2+}]\) increase was significantly inhibited by the pretreatment with suramin for 2 min. This antagonistic effect was concentration-dependent (ATP-treated: 572 ± 32%, suramin 10^{-6} M-pretreated: 483.6 ± 23% and suramin 10^{-4} M-pretreated: 272.7 ± 14%).

Purinoceptor-mediated cell proliferation

To investigate the physiological role of ATP on BCEC, we examined the mitogenic activity of ATP. As shown in Fig. 6, at 5 days after ATP (10^{-6} M) treatment, significant cell proliferation started to occur. Maximal increase of the cell number was observed on day 7 (control: 79.3 ± 4.1 x 10^6 cells and ATP-treated: 127.7 ± 3.0 x 10^6 cells), but adenosine and alpha, beta-Me-ATP had no effect on cell proliferation (81.7 ± 4.2 x 10^6 and 85.0 ± 5.1 x 10^6 cells, respectively). This increase of cell number due to ATP was significantly inhibited by the concomitant treatment of the cell with 10^{-4} M suramin (control: 82.0 ± 9.9 x 10^6 cells, ATP-treated: 119.8 ± 5.1 x 10^6 cells and suramin + ATP-treated: 95.8 ± 5.1 x 10^6 cells). Addition of suramin alone, had no effect on cell proliferation (control: 81.8 ± 12.4 x 10^6 cells) (Fig. 7).

DISCUSSION

The results of our present study revealed that extracellular ATP exhibits cytosolic calcium mobilization and mitogenic activity in cultured BCEC. Corneal endothelium is a single layer of cells lining the inner surface of the cornea that is bathed by the aqueous humor. The endothelium has a principal role in maintaining corneal transparency through its active pumping of water and ions. Since this cell layer has limited reproductive capacity in humans, if the endothelium is damaged by aging, inflammation,
Fig. 2. Effect of the extracellular calcium-free condition on ATP-induced \( [\text{Ca}^{2+}] \), increase. Left panels show representative signalings and the right panel shows change of peak value. To ensure the extracellular calcium-free condition, 1 mM EGTA was added to the media not containing calcium 60 s before addition of ATP. The results represent the mean ± S.E.M. of 4 experiments.

Fig. 3. The effect of U-73122 on ATP-induced \( [\text{Ca}^{2+}] \), increase. Representative signalings showing effect of U-73122 on ATP-induced \( [\text{Ca}^{2+}] \), increase in BCEC after the pretreatment of BCEC with U-73122 for 30 min (left panel). The effect of U-73122 on ATP-induced increase in \( [\text{Ca}^{2+}] \), in BCEC (right panel). The results represent the means ± S.E.M. of 4 experiments.
trauma or surgery, neighboring cells migrate and cover the damaged area. The average endothelial cell density is decreased in this damaged area and the decreased density is compensated by increased cell size. The pump function of the human corneal endothelium seems to be maintained regardless of age, but conditions that increase endothelial permeability, such as corneal guttata, can cause a compensatory increase in pump site density and probably pump function (15). In rabbits, upon corneal wound healing,

physiological functions of corneal endothelium are gradually compensated with the restoration of Na⁺,K⁺-ATPase density (16). For proper functioning of Na⁺,K⁺-ATP-

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**Fig. 4.** Effects of ATP and/or U-73122 on IP₃ accumulation in BCEC. BCEC were incubated with 10⁻⁵ M ATP for 5 min, and [³H]-IP₃ concentration was determined. U-73122 was added 30 min prior to ATP. The results are presented as the mean ± S.E.M. of 3 experiments.

**Fig. 5.** Effect of suramin on ATP-induced increase in [Ca²⁺] in BCEC. Cells were pretreated with suramin for 2 min before ATP addition. The results are presented as the mean ± S.E.M. of 4 experiments.

**Fig. 6.** Mitogenic effect of ATP or adenosine on BCEC proliferation. Cells (10⁷ cells/well of 6-well plate) were seeded. After 24 h, agonists were added once a day for 7 days. All wells, in triplicate, were trypsinized and the cells were counted. The results are presented as the mean ± S.D. in triplicate from three different experiments.

*P<0.05 and **P<0.01. ○: Control, □: ATP, ■: Adenosine, △: αβ-Me ATP.

**Fig. 7.** Effect of suramin on ATP-induced mitogenic effect. Suramin was added concomitantly with ATP. Cells were counted on the 7th day. The results are presented as the means ± S.D. in triplicate wells from three different experiments.
ase, adenosine plays important roles in maintaining normal intracellular ATP levels and exhibits a protective effect on Na⁺,K⁺-ATPase in the presence of glutathione (17).

Adenosine seems to act on adenosine receptors that stimulate adenylate cyclase in cultured bovine corneal endothelium (18). Its derivatives, ADP, ATP as well as bradykinin and histamine are also known to induce intracellular calcium mobilization in cultured bovine and human corneal endothelial cells (19). These results represent that adenosine and its derivatives regulate corneal endothelial cell functions.

In our experiment, extracellular ATP exhibited mitogenic activity in cultured BCEC. Cytoplasmic ATP concentration in most cells is assumed to be over 5 mM, and a significant proportion can be released as a result of loss of cell viability. The concentration of percellular ATP upon endothelial cell death could definitely reach the stimulatory range for ATP receptor(s) (3).

It is well known that ATP, ATP metabolites, ATP analogs and UTP induce intracellular free calcium mobilization and IP₃ accumulation mediated by purinoceptor stimulation in various cells (4, 8, 9). The present study provides the evidence of cytosolic free calcium mobilization and cell mitogenesis with ATP treatment in BCEC. ATP increased [Ca²⁺], in a concentration-dependent manner (Fig. 1), and the minimum effective concentration of ATP was between 10⁻⁸ and 10⁻⁷ M. Since P₂-purinoceptors had several kinds of subtypes that were classified into types P₂X (P₂X₁ – P₂X₃) and P₂Y (P₂Y₁ – P₂Y₃), and these subtypes are known to exhibit different sensitivities to ATP-related agonists (20), we compared the potency of ATP with those of its metabolites and analogs on cytosolic Ca²⁺ mobilization. In our results, the rank order for the potency of nucleotides was 2-meS-ATP > UTP > ATP > ADP.

The results of the extracellular calcium-free experiment showed that ATP-induced [Ca²⁺], increase consisted of two compartments, an initial rapid rise and a sustained phase. Removal of calcium from the medium resulted in an attenuation of the sustained phase of [Ca²⁺], and reduction in the height of the initial [Ca²⁺], peak. Furthermore, in order to clarify the second messenger in the signal transduction pathway, we studied ATP-induced IP₃ accumulation and the effects of U-73122, a PLC inhibitor, on ATP-induced cytosolic Ca²⁺ mobilization and IP₃ accumulation. In addition, suramin, a purinoceptor antagonist, also inhibited ATP-stimulated IP₃ accumulation. From the results of pretreatment with 5 × 10⁻⁶ M U-73122 for 30 min, ATP-induced cytosolic Ca²⁺ mobilization was shown to be associated with PLC activation.

Although U73122 inhibited ATP-induced calcium mobilization, some part of the cytosolic calcium increment remained. This result means that calcium influx was also induced by extracellular ATP stimulation.

From the present results on [Ca²⁺], changes by agonists, it is speculated that the ATP-related receptor subtypes in cultured BCEC are P₂Y₁, P₂Y₂ and P₂X purinoceptors. However, we cannot concluded at present which P₂ subclasses dominantly contributes to the responses observed in BCEC.

Recently, the mitogenic effect of extracellular ATP has been well established. Unfortunately, there has been no investigation about the effect of ATP on BCEC proliferation. It is well known that elevation of intracellular calcium concentration occurs as an initial response to receptor activation, resulting in downstream effects on cellular differentiation and proliferation. In vascular smooth muscle cells, calcium influx from the extracellular environment modulates DNA synthesis (21). The degree of proliferation and terminal differentiation of cultured keratinocytes is dependent on the concentration of extracellular calcium (22–24), which influences intracellular free calcium levels directly (25). In addition, extracellular Ca²⁺ and HCO₃⁻ influence the epidermal growth factor-induced DNA synthesis in cultured rat hepatocytes (26). In other cases, extracellular ATP and UTP are capable of activating the mitogen-activated protein kinase pathway in different cell types (27–29) and also increase [³H]thymidine incorporation. This action is dependent on PLC activation, which is mediated by P₂ purinoceptors.

Extracellular ATP is rapidly degraded to adenosine by ecto-nucleotidases. Because of this enzyme activity, we administered ATP every 24 h. According to our results, ATP induces the cell proliferation through the receptor-mediated response and this phenomenon was supported by the experiment on the antagonistic effect of suramin. In contrast to ATP, adenosine does not have any effect on cytosolic Ca²⁺ mobilization and cell proliferation. Therefore, the participation of the P₁-purinoceptor can be excluded in the cell proliferation of cultured BCEC.

In conclusion, ATP-induced cytosolic calcium mobilization and cell proliferation in BCEC is a suramin-sensitive purinoceptor-mediated response. To determine the exact subclasses of purinoceptors involved, further studies including the analysis of mRNA expression may be necessary.

REFERENCES

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