

P2X7 Receptor-mediated Membrane Blebbing in Salivary Epithelial Cells

Sung-Min Hwang¹, Na-Youn Koo¹, Se-Young Choi¹, Gae-Sig Chun², Joong-Soo Kim¹, and Kyungpyo Park¹

¹Department of Physiology, School of Dentistry, Seoul National University and Dental Research Institute, Seoul 110-749, ²Department of Oral Physiology, School of Dentistry, Dankook University, Cheonan 330-714, Korea

High concentrations of ATP induce membrane blebbing. However, the underlying mechanism involved in epithelial cells remains unclear. In this study, we investigated the role of the P2X7 receptor (P2X7R) in membrane blebbing using Par C5 cells. We stimulated the cells with 5 mM of ATP for 1~2 hrs and found the characteristics of membrane blebbing, a hallmark of apoptotic cell death. In addition, 500 μ M Bz-ATP, a specific P2X7R agonist, induced membrane blebbing. However, 300 μ M of Ox-ATP, a P2X7R antagonist, inhibited ATP-induced membrane blebbing, suggesting that ATP-induced membrane blebbing is mediated by P2X7R. We found that ATP-induced membrane blebbing was mediated by ROCK I activation and MLC phosphorylation, but not by caspase-3. Five mM of ATP evoked a biphasic $[Ca^{2+}]_i$ response; a transient $[Ca^{2+}]_i$ peak and sustained $[Ca^{2+}]_i$ increase secondary to ATP-stimulated Ca^{2+} influx. These results suggest that P2X7R plays a role in membrane blebbing of the salivary gland epithelial cells.

Key Words: P2X7 receptor, Membrane blebbing, ROCKI, Ca^{2+} influx

INTRODUCTION

Apoptosis, a normal process of cell death, is an essential event in a variety of biological systems (Leverrier and Ridley, 2001). Apoptosis is characterized by morphological changes of the cells such as plasma membrane blebbing, cell shrinkage, which is accompanied by several biological events, such as chromatin condensation, nuclear fragmentation, caspase and calpain activation (Coleman et al., 2001; Sebbagh et al., 2001). In the salivary glands, apoptotic cell death has been observed in patients with Sjögren's Syndrome, and plays a major role in salivary gland pathogenesis. However, its precise role in the disease pathogenesis is unknown. Sjögren's Syndrome is a chronic autoimmune disorder characterized by inflammation of the salivary and lacrimal glands; lymphocytic infiltration and destruction of exocrine glands as well as the hyper-reactivity of B cells, which is associated with the production of auto-antibodies, leads to the clinical symptoms of dryness of the mouth and eyes (Delaleu et al., 2005; Sisto et al., 2006).

Extracellular ATP regulates many physiological functions such as excitatory transmitters, cytokine and salivary secretion (Surprenant et al., 1996; Novak, 2003). Salivary acinar cells express purinergic receptors that play a putative role in amylase release, volume changes and ion secretion by increasing membrane permeability to Ca^{2+} and Na^+ (P2X receptor) and/or by modulating Ca^{2+} signaling through

enhanced G-protein-coupled inositol 1,4,5-trisphosphate production (P2Y receptor) (Novak, 2003). These receptors are thought to be activated by neuronal release of ATP, co-packaged with a variety of neurotransmitters (Franke et al., 2001). ATP is also released in response to a variety of stimuli including osmotic swelling, membrane stretching, hypoxia and cell lysis (Novak, 2003). In particular, the P2X7 receptor (P2X7R) is known to have unique characteristics; the prolonged activation of P2X7R leads to the formation of large pores and increases of intracellular Ca^{2+} , which is considered a key factor in the triggering of apoptosis (Nobile et al., 2003). P2X7R mediated membrane blebbing has also been reported in RAW 264.7 macrophages (Pfeiffer et al., 2004).

However, the role of P2X7R in apoptotic cell death and its signaling pathways in epithelial cells has not been rigorously studied. Therefore, we examined whether P2X7R activation induces membrane blebbing, and investigated the underlying mechanism in the salivary epithelial cell line, Par C5.

METHODS

Reagents

ATP, Bz-ATP, oxidized ATP (ox-ATP), UTP and ADP were obtained from Sigma (St. Louis, Mo., USA). ROCK I inhibitor (Y-27632) was obtained from CALBIOCHEM (San Diego, CA., USA). Texas Red-X phalloidin and fura-2

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Corresponding to: Kyungpyo Park, Department of Physiology, School of Dentistry, Seoul National University, 28, Yeongeon-dong, Chongno-gu, Seoul 110-749, Korea. (Tel) 82-2-740-8658, (Fax) 82-2-762-5107, (E-mail) kppark@snu.ac.kr

ABBREVIATIONS: ATP, adenosine triphosphate; ROCK, rho-associated kinase; $[Ca^{2+}]_i$, intracellular Ca^{2+} concentration; ox-ATP, oxidized ATP; Ab, antibody; MLC, myosin light chain; Bz-ATP, 3'-O-(4-benzoyl)benzoyl-ATP; SS, Sjögren's syndrome.

A/M (fura-2) were obtained from Molecular Probes (Eugene, OR., USA). pMLC and MLC antibodies were purchased from Cell Signaling (Beverly, MA., USA). ROCK I antibody was obtained from BD Biosciences (Palo Alto, CA., USA).

Cell cultures

Par C5 cells were cultured in 60 mm culture dishes in DMEM/F12 (1 : 1 mixture) supplemented with 2.5% fetal calf serum, 5 $\mu\text{g ml}^{-1}$ transferrin, 1, 1 μM hydrocortisone, 0.1 μM retinoic acid, 2.0 nM T3, 5 $\mu\text{g ml}^{-1}$ insulin, 80 ng ml^{-1} epidermal growth factor, 4 mM L-glutamine, 50 $\mu\text{g ml}^{-1}$ gentamicin sulfate, and a trace element mixture.

Fluorescence microscopy

Par C5 cells, plated on glass coverslips, were treated with nucleotides in the presence or absence of the P2X7 antagonist, ox-ATP, or in non- Ca^{2+} -containing solution. Following treatment, the cells were washed once with phosphate-buffered saline (PBS), fixed in 4% formaldehyde solution, for 10 min at room temperature, and washed with PBS+0.1% Triton. The cells were then stained for actin filaments using 66 nM Texas red X-phalloidin in PBS for 20 min at room temperature and washed with PBS solution. The cells were analyzed by fluorescence or differential-interference contrast microscopy (Zeiss, Chester, VA., USA).

Intracellular Ca^{2+} measurement

The intracellular Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) was measured in the Par C5 cells loaded with fura-2. The cells were loaded with 2 μM Fura-2 in 2 ml of culture medium for 30 min at 37°C. The cells were gently washed once by replacing the medium, and incubated in normal solution for 10 min prior to the experiments in order to allow for maximal de-esterification of the dye. Fura-2-loaded cells were excited at 340/380 nm and changes in $[\text{Ca}^{2+}]_i$ were measured under the inverted microscope (Olympus IX70, Japan). The normal solutions contained 140 mM NaCl, 5 mM KCl, 1 mM MgSO_4 , 1 mM CaCl_2 , 10 mM HEPES, and 10 mM glucose. The Ca^{2+} -free solution was prepared by replacing CaCl_2 with EGTA.

Immunoblotting

The Par C5 cells were homogenized in lysis buffer (50 mM Tris pH 7.5, 1% Triton X-100, 100 mM NaCl, 10 mM tetrasodium pyrophosphate, 10 mM NaF, 1 mM EDTA, 1 mM Na_3VO_4 , 1 mM EGTA, 1 mM phenylmethylsulphonyl fluoride (PMSF) and 1 $\mu\text{g ml}^{-1}$ aprotinin, leupeptin and pepstatin) followed by gentle sonification in ice. Following protein concentration determination, the proteins were separated on a SDS-polyacrylamide gel and transferred electrophoretically to polyvinylidene fluoride (PVDF) membranes and incubated with primary antibodies.

Statistical analysis

Data are expressed with mean \pm S.E.M. values. Statistical significance was evaluated by the Student's unpaired *t* test. A probability below 0.05 ($p < 0.05$) was considered significant.

RESULTS

P2X7 receptor induced membrane blebbing

We examined whether 5 mM ATP induced membrane blebbing in the Par C5 cells. The images were obtained using differential interference contrast (DIC) and fluorescence microscopy. Five mM of ATP was used for 2 hrs to induce membrane blebs (Fig 1A, indicated by arrows in the second panel). However, blebs were not observed following ATP stimulation in cells pretreated with 300 μM ox-ATP (the third panel) or in Ca^{2+} -free bath solution (the fourth panel). We went on further to examine whether other types of P2X or P2Y receptor agonists could induce membrane blebbing. 500 μM Bz-ATP, a specific P2X7R agonist, and 5 mM of ATP induced blebbing in 43.0 \pm 0.1% and 61.0 \pm 0.1% from total of Par C5 cells, respectively (Fig. 1B). Neither 1 mM UTP nor 1 mM ADP induced membrane blebbing. The membrane blebbing induced either by 5 mM ATP or 500 μM Bz-ATP was not observed following pretreatment with 300 μM ox-ATP, a specific P2X7R antagonist or in Ca^{2+} -free solution.

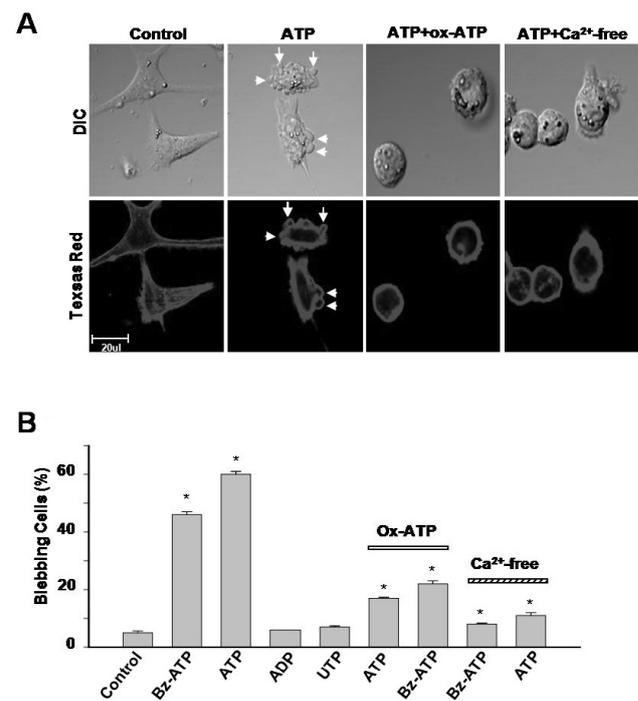


Fig. 1. Membrane blebbing induced by ATP. (A) Par C5 cells were treated with 5 mM ATP, 5 mM ATP with 300 μM ox-ATP for 2 hrs, and 5 mM ATP in Ca^{2+} -free solution. The cells were stained with Texas Red-X phalloidin. In addition, 5 mM of ATP for 2 hrs induced several membrane blebs per cell (arrows in the second panel). Membrane blebbing was reduced following pretreatment with 300 μM ox-ATP or 5 mM ATP in a Ca^{2+} -free bath solution (the third and fourth panels). (B) The histogram summarizes the results of the percent of cells demonstrating membrane blebbing following treatment with 1 mM Bz-ATP, 5 mM ATP, 1 mM ADP, or 1 mM UTP. Membrane blebbing was also quantified following ATP or Bz-ATP stimulation following 2 hrs of incubation with 300 μM ox-ATP or in Ca^{2+} -free solution. The data are the means of five separate experiments. * $p < 0.05$ is derived from comparisons with untreated control cells.

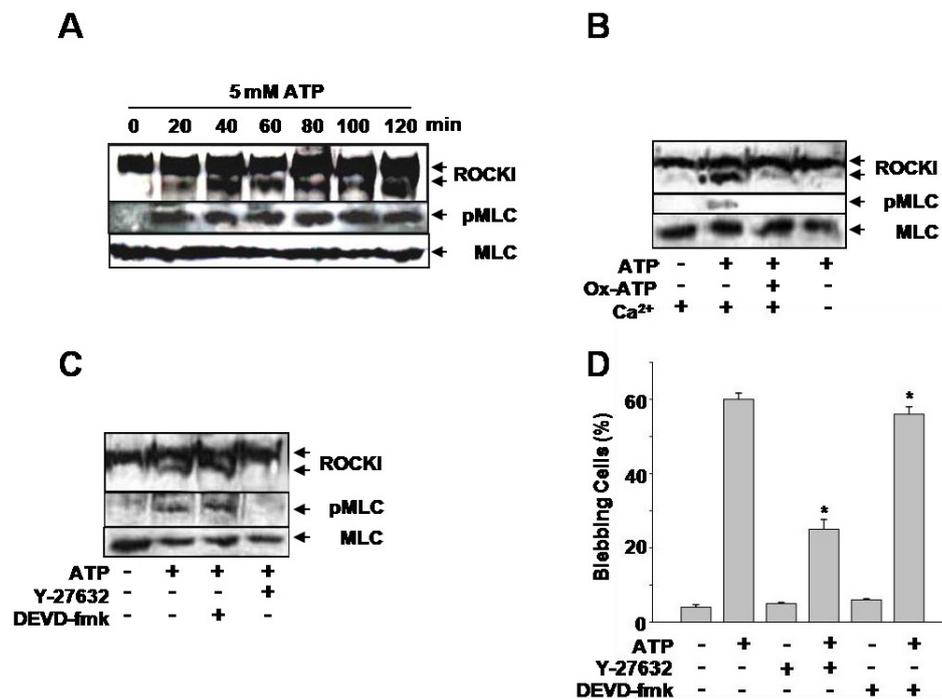


Fig. 2. ATP induced ROCK I activation and MLC phosphorylation in Par C5 cells. (A) ROCK I cleavage and phosphorylated-MLC (pMLC) from whole cell lysates (50 μ g) were resolved by SDS-PAGE and blotted with specific antibodies. The blots were probed with antibodies against ROCK I, pMLC, and MLC (Control). The cells were treated with ATP for up to 2 hrs. (B) ATP-induced ROCK I cleavage and pMLC after pretreatment with 300 μ M ox-ATP or in Ca²⁺-free solution. (C) ATP-induced ROCK I cleavage and pMLC after pretreatment with 300 μ M caspase-3 inhibitor (DEVD-fmk) or 10 μ M ROCK I inhibitor (Y-27632). (D) The effects of Y-27632 and DEVD-fmk on the percent of cells demonstrating membrane blebbing following 5 mM ATP stimulation. The number of cells containing membrane blebs was determined from a total of 500 cells. The data are means from five separate experiments * $p < 0.05$ compared to untreated control cells.

Intracellular transduction mechanism of membrane blebbing

We next investigated the signaling pathway of ATP-induced membrane blebbing. We examined whether 5 mM ATP, which had induced membrane blebbing in our experiments, can activate ROCK I and MLC, the main target molecules in neurons and immune cells (Pfeiffer et al., 2004; Croft et al., 2005; Minambres et al., 2006). Fig. 2A shows a cleaved, active form of ROCK I and MLC phosphorylation 20 min after ATP stimulation. Five mM of ATP stimulation following pretreatment with 300 μ M ox-ATP for 2 hrs or in Ca²⁺-free bath solution completely blocked ROCK I activation and MLC phosphorylation (Fig. 2B). Pretreatment with 10 μ M Y-27632, a ROCK I inhibitor, blocked both ATP-induced ROCK I activation and MLC phosphorylation. However, 300 μ M of DEVE-fmk, a caspase-3 inhibitor, did not inhibit ATP-stimulated activation or phosphorylation (Fig. 2C). Finally, we examined whether Y-27632 or DEVE-fmk inhibits the formation of membrane blebbing. In the presence of Y-27632, ATP-stimulated membrane blebbing was inhibited. By contrast, the percent of cells exhibiting ATP-stimulated membrane blebbing was not altered in the presence of DEVE-fmk (Fig. 2D).

The increase of [Ca²⁺]_i mediated by P2X7R in Par C5

Our results from the membrane blebbing studies suggest that membrane blebbing is dependent on [Ca²⁺]_i. Thus, we characterized the P2X7R-mediated Ca²⁺ mobilization in Par C5 cells. Five mM ATP increased [Ca²⁺]_i in a biphasic manner. The initial increase in [Ca²⁺]_i was followed by a sustained elevation of [Ca²⁺]_i, which remained higher than the basal levels. Mg²⁺ is known to nonselectively inhibit cation channels; we examined whether Mg²⁺ could inhibit

the Ca²⁺ influx. Although 1 mM Mg²⁺ had little effect on the sustained elevation of [Ca²⁺]_i, high concentrations of 20 mM Mg²⁺, a nonselective cation channel blocker, significantly inhibited the sustained elevation of [Ca²⁺]_i (Fig. 3A). Then we examined whether the sustained increase of [Ca²⁺]_i was due to the Ca²⁺ influx from the extracellular medium. Both 5 mM ATP and 1 mM UTP transiently increased [Ca²⁺]_i in the Ca²⁺-free medium, but a sustained elevation of [Ca²⁺]_i was not observed (Fig. 3B). Furthermore, pretreatment of the cells with 300 μ M ox-ATP for 2 hrs, resulted in blocking sustained elevation in [Ca²⁺]_i following ATP stimulation (Fig. 3C). Thus the results of this study suggest that the sustained increase of [Ca²⁺]_i was due to Ca²⁺ influx mediated by P2X7R.

DISCUSSION

Intracellular Ca²⁺ overload is an important factor involved in inducing apoptotic conditions (Zhang et al., 2005). Our results suggest that Ca²⁺ plays an important role in the initiation and maintenance of membrane blebbing, a hallmark of apoptotic cell death. A lower concentration of ATP (100 μ M), transiently induced [Ca²⁺]_i but was not associated with a sustained elevation of Ca²⁺, and did not induce membrane blebbing (data not shown). In addition, five mM of ATP-induced apoptotic membrane blebbing was not observed in the Ca²⁺-free medium, suggesting that an influx of Ca²⁺ is a prerequisite for inducing membrane blebbing. Furthermore, the findings showed that the sustained elevation of Ca²⁺ was mediated by the P2X7 receptor (P2X7R), since only high concentrations of ATP or Bz-ATP, a specific P2X7R agonist, induced a sustained increase of [Ca²⁺]_i. Moreover, it was inhibited in the presence of ox-ATP, a specific P2X7R antagonist. While, other types of P2Y or P2X receptor agonists did not induce membrane

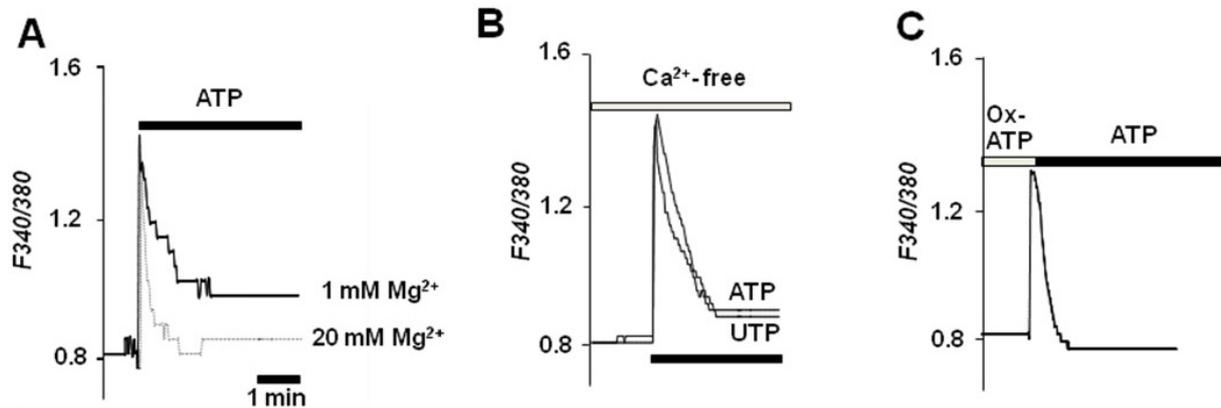


Fig. 3. Measurement of cytoplasm free Ca^{2+} concentrations ($[\text{Ca}^{2+}]_i$) mediated by P2X7R activation in fura-2 loaded Par C5 cells. (A) In the presence of 1 mM Mg^{2+} , 5 mM ATP induced a transient and sustained elevation in $[\text{Ca}^{2+}]_i$. In addition, 20 mM Mg^{2+} completely blocked the sustained increase in $[\text{Ca}^{2+}]_i$. (B) In a Ca^{2+} -free bath solution, the transient peak increase in $[\text{Ca}^{2+}]_i$ was measured following stimulation with ATP (5 mM) or UTP (1 mM). The sustained increase was absent in the Ca^{2+} -free media. (C) Two hours following preincubation with 300 μM ox-ATP, a peak increase in $[\text{Ca}^{2+}]_i$ stimulated by 5 mM of ATP was present without evidence of a sustained increase. Each tracing is a typical representative sample of at least 10 separate experiments using different cell preparations.

blebbing. Neither 1 mM UTP, which is known as P2Y2, P2Y4 and P2Y6 agonists, nor 1 mM ADP, which is known as P2Y1 and P2Y12 agonists, induced membrane blebbing. In our experiments, high concentrations of Mg^{2+} , also inhibited the sustained influx of Ca^{2+} , suggesting that the divalent cation acts as an allosteric modulator, altering the affinity of ATP-binding to P2X7R (Acuna-Castillo et al., 2007). The profile of our ATP-induced rapid increase of $[\text{Ca}^{2+}]_i$ suggest that P2X7R, in Par C5 cells, is preassembled in mouse parotid duct cells (Li et al., 2003).

One of the morphological characteristics of apoptotic cells, membrane blebbing, is observed during apoptosis through specific pathway activation (Coleman et al., 2001; Sebbagh et al., 2001). In particular, the formation of membrane blebbing is induced by the Rho kinase (ROCK) pathway. Activation of ROCK regulates the phosphorylation of the myosin light chain (MLC), resulting in membrane blebbing (Leverrier and Ridley, 2001). In our experiments, P2X7R stimulation induced ROCK activation, which subsequently increased phosphorylated MLC levels (Fig. 2). There are two types of ROCK isoforms, ROCK I and ROCK II (Coleman et al., 2001). In our experiments ROCK I was activated by 5 mM of ATP. Membrane blebbing induced by anti-Fas antibody and $\text{TNF-}\alpha$ in Jurkat cells and NIH 3T3 mouse fibroblasts, respectively, was also associated with ROCK I activation. In addition, membrane blebbing was the result of caspase-mediated activation of ROCK I (Coleman et al., 2001; Sebbagh et al., 2001). By contrast, our experiments indicated that caspase-3 was not involved in the cleavage of ROCK I, MLC phosphorylation or P2X7R-induced membrane blebbing. Similarly, cleavage of ROCK I or MLC phosphorylation, not mediated by caspase-3, has also been previously reported in HEK293 cells (Morelli et al., 2003). Therefore, it seems that signaling pathways for membrane blebbing depend on the involved receptors and cell type. The results of our study demonstrated that ATP-induced ROCK I activation and phosphorylation of MLC depends on changes in $[\text{Ca}^{2+}]_i$. These findings suggest that ROCK I cleavage may be mediated by one of the calcium-activating protease, e.g., calpain. We speculate that unknown Ca^{2+} -dependent proteases mediate ATP-induced

membrane blebbing in Par C5 cells, since a calpain inhibitor did not block the ROCK I cleavage in our experiments (unpublished results).

In summary, stimulation of Par C5 cells with 5 mM ATP induced membrane blebbing. The membrane blebbing was mediated by ROCK I activation and MLC phosphorylation, but not by caspase-3. Our results may provide an important clue to the mechanism involved in ATP-induced membrane blebbing in salivary gland epithelial cells.

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