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Research Paper

Activation of chloride current and decrease of cell volume by ATP in nasopharyngeal carcinoma cells

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Abstract: Whole-cell patch clamp and cell volume measurement techniques were used to investigate the ATP-activated chloride current and the ATP effect on cell volume in nasopharyngeal carcinoma cells. Extracellular application of ATP in micromolar concentrations activated a current with the properties of modest outward rectification and negligible time-dependent inactivation in a dose-dependent manner. The current reversed at a potential [(−0.05±0.03) mV] close to the Cl^- equilibrium potential (−0.9 mV). Substitution of Cl^- with gluconate in the extracellular solution decreased the ATP-activated current and shifted the reversal potential positively. NPPB, one of the chloride channel blockers, inhibited the current by (81.03±9.36)%. The current was also depressed by the P2Y purinoceptor antagonist, reactive blue 2, by (67.39±5.06)%. ATP (50 μmol/L) decreased the cell volume under the isotonic condition. Depletion of extracellular and intracellular Cl^- abolished the ATP effect on cell volume. The results suggest that extracellular ATP of micromolar scales can induce a chloride current associated with cell volume regulation by activation of chloride channel through binding to purinoceptor P2Y.

Key words: chloride channels; adenosine triphosphate; receptors, purinergic; patch clamp technique; cell size

ATP 激活鼻咽癌细胞氯电流并减小细胞容积

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摘 要: 采用全细胞膜片钳技术和细胞容积测量技术，在低分化鼻咽癌细胞株 CNE-2Z 上观察 ATP 诱导的 Cl^- 电流的特性及其对细胞容积的影响。细胞外微摩尔水平的 ATP 以剂量依赖性的方式激活一个具有弱外向整流特性,没有时间依赖性失活的电流, 此电流的反转电位 [(−0.05 ± 0.03) mV]接近 Cl^- 的平衡电位(−0.9 mV)。用葡萄糖酸置换细胞外液 Cl^- 后, ATP 激活的电流明显减小并且反转电位发生改变。氯通道抑制剂 NPPB (200 μmol/L)可以抑制这一电流 [(81.03 ± 9.3)%] 。此电流亦可被嘌呤受体 (P2Y) 拮抗剂反应蓝 2 抑制 [(67.39 ± 5.06)%]。50 μmol/L 的 ATP 使在等渗状态下的细胞容积缩小, 替代和耗竭细胞外、内的 Cl^- 后, ATP 的这一作用消失。这些结果提示细胞外微摩尔水平的 ATP 可通过兴奋 P2Y 受体激活氯通道而产生与细胞容积调节相关的 Cl^- 电流。

关键词: 氯通道；腺苷三磷酸；受体,嘌呤的；膜片钳技术；细胞大小
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ATP, besides being as an intracellular energy source, plays diverse physiological roles and is involved in many pathological processes by working as an extracellular messenger, including neurotransmission, secretion, platelet aggregation, control of vascular tone and cardiac contraction, cell volume regulation, cell proliferation and apoptosis[1-3]. ATP activates at least two types of chloride currents, the calcium-dependent and the non-calcium de-

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pendent chloride currents in rat submandibular gland cells [4]. A number of studies indicate that activation of volume-activated chloride channels is one of the key mechanisms involved in cell volume regulation [5]. However, the relationship between ATP-activated chloride currents and the volume regulation and the mechanisms involved in the activation pathway are not clear. The aim of this study was to investigate the ATP-activated chloride current and its roles in cell volume regulation.

1 MATERIALS AND METHODS

1.1 *Cell culture.* The poorly differentiated nasopharyngeal carcinoma cells (CNE-2Z cells) were routinely grown in the culture medium, RPMI 1640 containing 10% new-born calf serum, 100 IU/ml penicillin and 100 μg/ml streptomycin, at 37 ℃ in a humidified atmosphere of 5% CO_2. The cells were subcultured every 2 d.

1.2 *Patch clamp experiments.* Cell suspension was plated onto coverslips in tissue culture dishes and incubated at 37 ℃ for 2~3 h. The coverslip with the prepared cells was then stuck onto the base of the recording chamber (with a volume of 0.5 ml) and perfused with extracellular solutions at a speed of 2 ml/min. Whole-cell currents were recorded by a patch-clamp amplifier (EPC-7, List Electronic, Germany) [6, 7]. The recording pipettes were pulled from glass capillaries of outer diameter 1.5 mm on a two-stage vertical puller (PB-7, Narishige, Japan) and gave a resistance of 5~10 MΩ when filled with the electrode solution. Cells were held at 0 mV and then stepped to ±40, 0 and ±80 mV for 200 ms (each step) with 4 s interval between pulses throughout the experiments. The pulse generation was controlled by a patch-clamp software package (CED, Cambridge, UK). All recordings were carried out at room temperature (20~22 ℃).

The permeability ratios (P_X/P_{Cl}) of various anions (X^-) relative to that of Cl^- were calculated using the modified Goldman-Hodgkin-Katz equation:

$P_X/P_{Cl} = ([Cl^-]_n \exp(-\Delta V_{rev}F/RT)\ [Cl^-]_s)/[X^-]_s$,

where $[Cl^-]_n$ and $[Cl^-]_s$ are the Cl^- concentration in the normal and the substituted bath solutions, $[X^-]_s$ is the concentration of the substituted anion, ΔV_{rev} is the difference of the reversal potentials for Cl^- and X^-, F is the Faraday constant, R is the gas constant and T is the absolute temperature. In anion substitution experiments, an agar bridge was used to connect the reference electrode (Ag-AgCl wire) to the bath solution to minimize the baseline drift caused by anion substitution.

1.3 *Measurements of cell volume.* Cell volume was measured by a method described previously [8]. The coverslip with cells, which had been cultured for 1~2 h, was stuck onto the base of the recording chamber and perfused with isotonic solution at a speed of 2 ml/min for 5 min. Cell images were then acquired every 60 s by a CCD digital camera (4910, Cohu Inc. USA) which was attached to the microscope (IMT-2, Olympus, Japan) and controlled by the Scion image processor and analysis software (Scion Corporation, USA). Cell area and diameter were obtained by analyzing cell images with the Scion software and cell volume was computed from cell diameter. All experiments were carried out at room temperature (20~22℃). Cell volume was standardized.

1.4 *Solutions.* The isotonic bath solution contained (in mmol/L): 70 NaCl, 0.5 $MgCl_2$, 2 $CaCl_2$, 10 HEPES and 140 D-mannitol. The pipette solution contained (in mmol/L): 70 N-methyl-D-glucamine chloride (NMDG-Cl), 1.2 $MgCl_2$, 10 HEPES, 1 EGTA, 140 D-mannitol and 2 ATP. The pH of bath and pipette solutions was adjusted to 7.4 and 7.2, respectively, with Tris solution. The osmolarity of both solutions was detected by a freezing point osmometer (Osmomat 30, Gonotec, Berlin, Germany) and adjusted to 300 mOsmol/L.

1.5 *Statistics.* Data are expressed as mean±SEM and ANOVA was used to test significance of differences.

2 RESULTS

2.1 ATP-activated currents

A background current was recorded with the whole-cell patch clamp technique in CNE-2Z cells (Fig. 1*A*). The density of the current was (5.07±1.16) pA/pF at + 80 mV and (−4.96 ± 1.22) pA/pF at −80 mV (Fig. 1*C*, n = 13). Extracellular application of ATP (50 μmol/L) activated a current in 30~60 s, with the properties of weak outward rectification. The current did not show significant time-dependent inactivation and varied between ± 10.0 pA/pF and ± 60 pA/pF at ± 80 mV, with mean outward current of (51.66 ± 2.6) pA/pF (at +80 mV, n = 13) and mean inward current of (−42.53 ± 2.93) pA/pF (at −80 mV, n =13) (Fig. 1*B* and 1*C*). The difference between outward and inward current was significant ($P < 0.01$). The activation of the current was reversible and repeatable by depleting or reapplying extracellular ATP (Fig. 1*D*).

The ATP-activated current reversed at the potential of

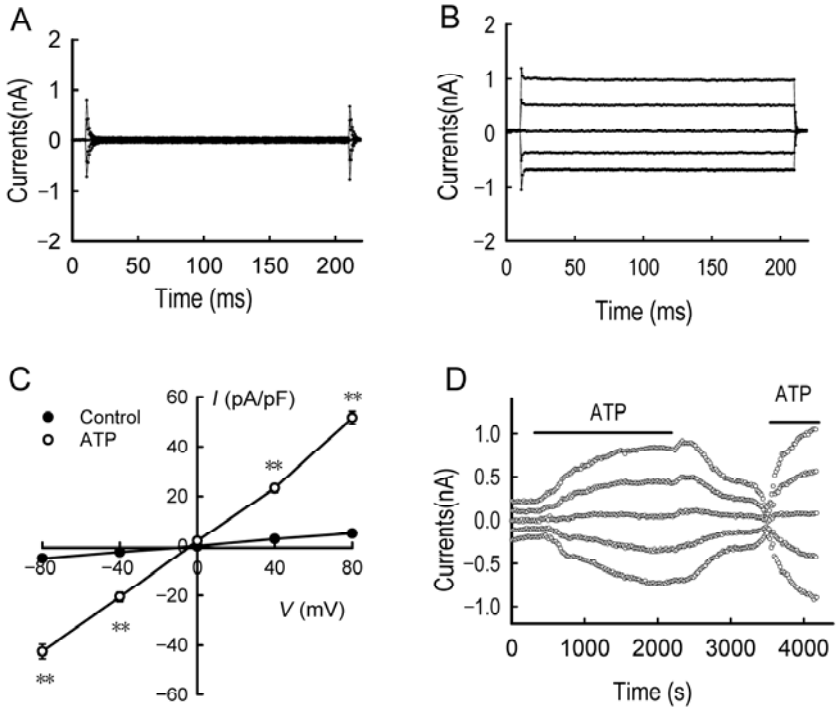

Fig. 1. ATP-activated current in CNE-2Z cells. Typical current traces recorded under isotonic bath condition and after extracellular application of ATP (50 μmol/L) are shown in *A* and *B*. The voltage was held at 0 mV and then stepped to 0, ± 40, ± 80 mV with an interval of 4 s between steps. The current-voltage relationship is presented in *C* (mean ± SEM, $n = 7$). *D* shows a typical time course of the ATP-activated current. $^{**}P < 0.01$ *vs* control.

(−0.05 ± 0.03) mV ($n = 13$), which was close to Cl^- equilibrium potential (−0.9 mV). In this study, there was no K^+ in the intracellular and extracellular solutions. This indicates that the ATP-activated current is a chloride current.

Substitution of extracellular sodium chloride with sodium gluconate inhibited the ATP-activated chloride current by (80.48 ± 1.57) % at + 80 mV ($n = 4$, $P < 0.01$) and by (44.93 ± 2.56)% at −80 mV ($n = 4$, $P < 0.01$), and altered the reversing potential of the current [from (−0.05 ± 0.03) mV to (24.08 ± 1.28) mV, $P < 0.01$] (Fig. 2). Calculation of ionic permeability by the modified Goldman-Hodgkin-Katz showed that the ATP-activated channel was less permeable to gluconate than to chloride, with a ratio of 0.34 ± 0.02 ($P_{gluconate}/P_{Cl}$).

Our studied also demonstrated that activation of the chloride current by extracellular ATP was dose-dependent

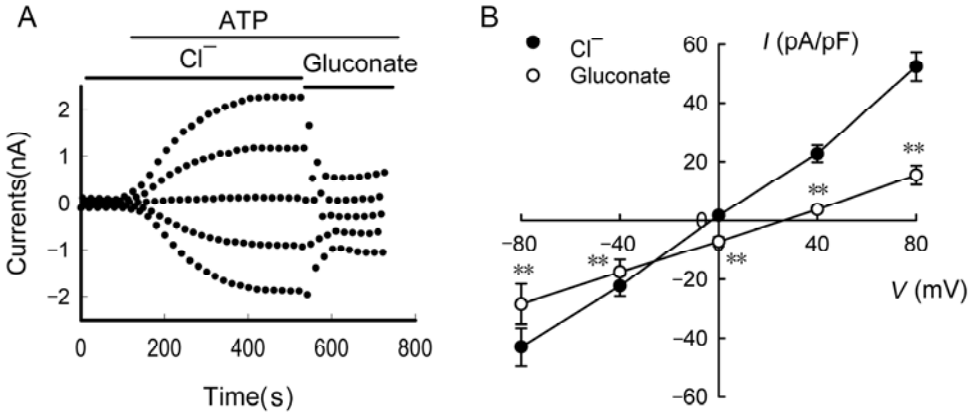

Fig. 2. Response of ATP-activated current to anion substitution. When the ATP-activated current, induced by 50 μmol/L ATP, reached the peak and levelled off, the solution containing 70 mmol/L NaCl was substituted by a solution contained equimolar sodium gluconate. Gluconate shifted the reversal potential positively. A typical time course of the experiments is shown in *A*. The current-voltage relationship is presented in *B* (mean ± SEM, $n = 4$). $^{**}P < 0.01$ *vs* Cl^-.

with an EC_{50} of 5.3 μmol/L (Fig. 3).

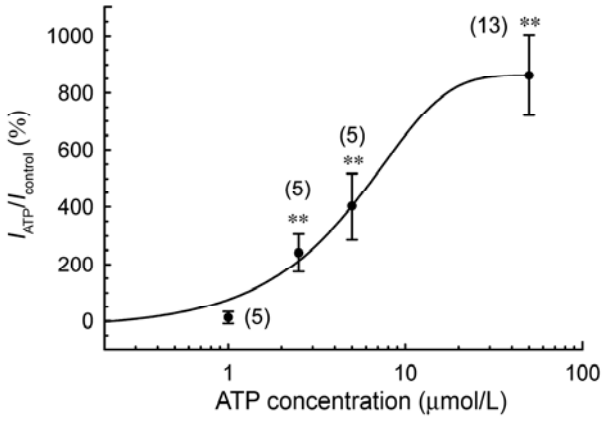

Fig. 3. Dose-dependent activation of the current by ATP. ATP activated a current in a dose-dependent manner. I_{ATP} is the current activated by ATP and $I_{control}$ is the current recorded before application of ATP. Data are mean ± SEM. The numbers in parentheses represent the number of cells recorded. $^{**}P < 0.01$ *vs* control.

2.2 Inhibition of ATP-activated currents by chloride channel blockers

To investigate whether the ATP-activated current is mediated by chloride channels, the effects of chloride channel blocker 5-nitro-2-(3-phenylpropylamino)benzoic acid (NPPB) on the current were observed. Our results demonstrated that NPPB (200 μmol/L) inhibited the ATP-activated current (Fig. 4). The outward current at +80 mV decreased from (47.1 ± 4.4) pA/pF to (15.2 ± 1.4) pA/pF (n = 4, P <0.01) and the inward current at −80 mV decreased from (−40.3 ± 5.2) pA/pF to (−11.1 ± 2.2) pA/pF (P <0.01). The outward and inward currents were inhibited by (81.03 ± 9.36)% and (86.60 ± 9.41)%, respectively. There was no significant difference in the inhibition between the outward current and the inward current (P > 0.05).

2.3 Inhibition of ATP-activated Cl^- currents by purinoceptor antagonists

It has been known that ATP activates purinoceptors.

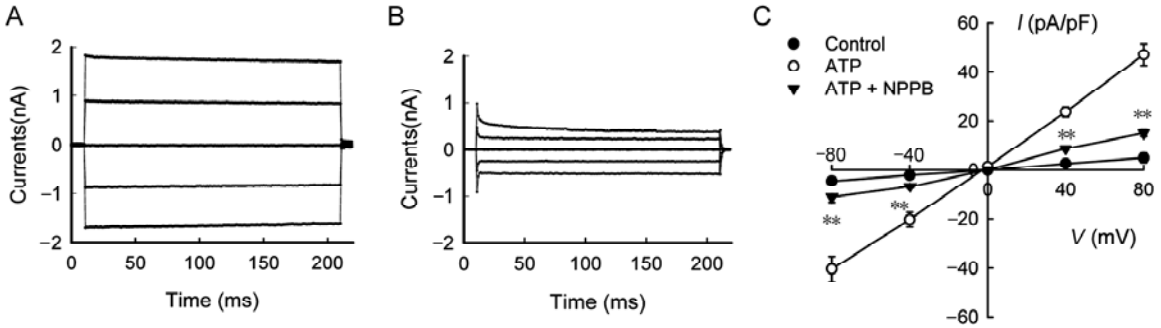

Fig. 4. Inhibition of the ATP-activated current by NPPB. Extracellular application of ATP (50 μmol/L) activated a current (*A*) that was inhibited by chloride channel blocker NPPB (200 μmol/L, *B*). The current-voltage relationship is presented in *C* (mean ± SEM, $n = 4$). $^{**}P <$ 0.01 *vs* ATP.

To clarify the involvement of purinoceptor in the activation of the chloride current induced by ATP, the effects of the purinoceptor antagonist, reactive blue 2, on the ATP-activated chloride current were studied. Blue reactive 2 was added to the perfusion solution in a final concentration of 200 μmol/L when the ATP-activated chloride current reached the peak and levelled off. Figure 5 shows that extracellular application of reactive blue 2 inhibited the outward current at + 80 mV by (67.39 ± 8.77)% and the inward current at −80 mV by (64.79 ± 10.36)%.

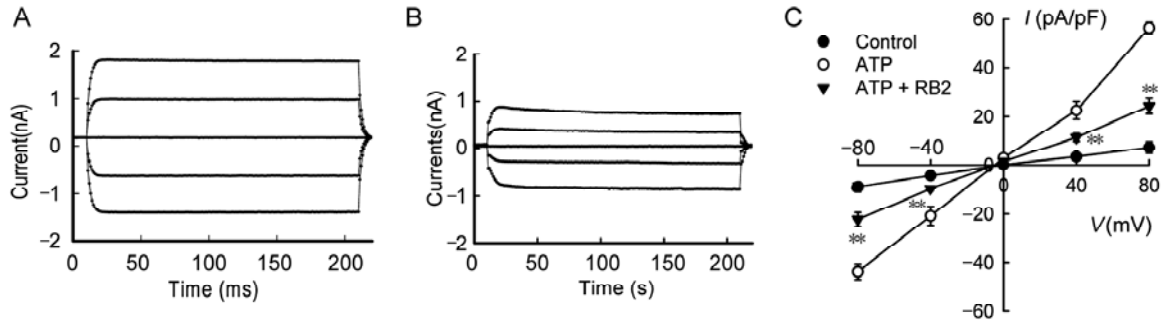

Fig. 5. Inhibition of the ATP-activated current by reactive blue 2. *A*: ATP-activated current induced by extracellular application of ATP (50 μmol/L). The current was inhibited by purinergic receptor antagonist reactive blue 2 (RB2, 200 μmol/L, *B*). The current-voltage relationship is presented in *C* (mean ± SEM, $n = 4$). $^{**}P < 0.01$ *vs* ATP.

The outward current was decreased from (56.4 ± 2.4) pA/pF to (24.4 ± 3.2) pA/pF (+80 mV; $n = 4$, $P < 0.01$) and the inward current from (−43.7 ± 3.1) pA/pF to (−22.3 ± 2.9) pA/pF (−80 mV; $P < 0.01$). The inhibition of outward currents was not significantly different from that of inward currents ($P > 0.05$).

2.4 Effects of ATP on cell volume

Analysis of cell images indicated that extracellular perfusion of ATP decreased cell volume in CNE-2Z cells. Cells were bathed in the isotonic solution for 5~10 min and then ATP was added to the perfusion solution in a concentration of 50 μmol/L. Cell volume decreased gradually after application of ATP. The mean shrinkage was (5.81 ± 0.67)% ($n = 23$, $P < 0.01$) in 30 min. Cells regained their volume gradually when ATP was washed out. In control cells that were perfused continuously with isotonic solution for 2 h, the cell volume did not change significantly.

The effect of ATP on cell volume was abolished by depletion of extracellular and intracellular Cl^-. The depletion of intracellular Cl^- was achieved by bathing cells in isotonic solution containing sodium gluconate instead of sodium chloride for 2 h [8]. The cells were then perfused with sodium gluconate solution containing 50 μmol/L of ATP for 30 min. The results showed that cells shrank only by (0.6 ± 0.2)% (n=28). This change was not significantly different from that of the control cells ($P > 0.05$).

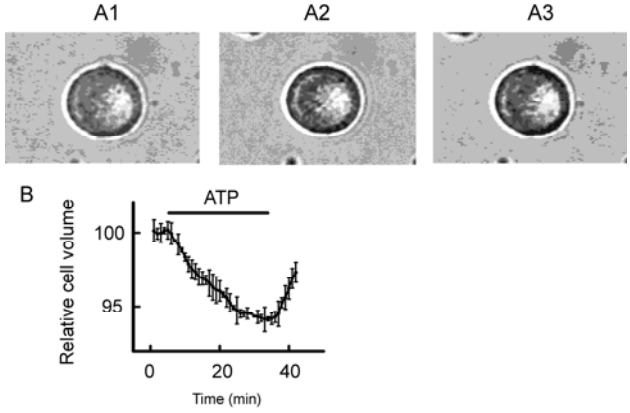

Fig. 6. Effects of ATP on Cell volume. The CNE-2 cell was bathed in isotonic solution (control) for 5 min (*A1*), perfused with 50 μmol/L ATP in isotonic solution for 30 min (*A2*) and then returned to the control solution (*A3*). *B:* Time course of experiments (mean ± SEM, $n = 23$). The data indicated that extracellular application of ATP (50 μmol/L) decreased cell volume.

3 DISCUSSION

More and more attention has been paid to chloride channels, but less is known about the ATP-activated chloride current and its mechanisms. Our results indicate that ATP activats a current with the property of weak rectification and a reversal potential close to 0 mV. Under the experimental conditions in this study, Cl^-, Na^+, Mg^{2+} and Ca^{2+} were the main ions in the extracellular and intracellular solutions. The equilibrium potential for Cl^- was about 0 mV and that for Na^+, Mg^{2+} or Ca^{2+} was far from 0 mV. The fact that the reversal potential of ATP-activated current is close to the equilibrium potential for Cl^- implicates that the ATP-activated current is a Cl^- current. This postulation is supported by the data obtained in this study, i.e., substitution of sodium chloride in the bath solution with sodium gluconate decreased the ATP-activated current and altered the reversal potential. Chloride channel blocker NPPB inhibited the current.

It was reported that the ATP-activated Cl^- current in vascular endothelia cells was associated with the purinoceptor P_2. The current was outward rectified, time-independent and was involved in the regulation of Ca^{2+} movement [9]. ATP activates at least two channels, the Ca^{2+}-activated Cl^- channel and the Ca^{2+}-independent Cl^- channel, possibly cystic fibrosis transmembrane regulator (CFTR), by stimulating purinoceptor P_2 in rat salivary submandibular gland cells [4]. However, it was demonstrated that the ATP-activated chloride current in mouse parotid acinar cells was not associated with P_2. Reactive blue 2 is a specific antagonist of purinergic receptor P_2, especially P2Y [11] and is normally used to block P2Y receptors [12,13]. The results of the present study demonstrated that the ATP-activated chloride current was inhibited by reactive blue 2. This suggests that induction of the chloride current was the result of activation of chloride channels via stimulating receptor P2Y in CNE-2Z cells.

ATP is found both inside and outside of the cells. The concentration of intracellular ATP (in mmol/L scale) is much higher than that of extrcellular ATP (in μmol/L scale) [1,2]. There are three sources of extracellular ATP, as an extracelllular signaling molecule. ATP is released from cells via channels, by ATP transporters, or in the form of exocytosis [1,2]. Extracellular ATP is easily degraded into adenosine by ectoenzyme. ATP can act directly on receptor P_2 or indirectly on P_1 via adenosine [14]. In the present study, ATP was prepared freshly and applied to cells through perfusion solutions which did not contain enzymes. This suggests that ATP activates chloride currents by directly acting on P_2 rather than indirectly on P_1 via adenosine.

The cell membrane is highly permeable to water. Water movement across the cell membrane is driven by an osmotic pressure gradient. In isotonic solutions, cell volume is relatively stable. Exposure of cells to hypotonic solution

results in swelling of the cells and activation of the K^+ and Cl^- channels. The release of ions and the passive outflow of water result in the recovery of cell volume[5]. The results of the present study indicate that the properties of the ATP-activated chloride current in CNE-2Z cells are similar to those of the volume-activated chloride current in ciliary epithelial cells and CNE-2Z cells reported by us previously [15-17]. Both of the currents are inhibited by NPPB. The data suggest that the ATP-activated chloride current may be involved in the regulation of cell volume. We demonstrated previously that Cl^- outflow through the Cl^- channels was one of the key mechanisms of volume decrease of the ciliary epithelial cells and CNE-2Z cells[18,19]. In this study, extracellular application of ATP decreased the cell volume in isotonic solution, the effect was abolished by depletion of extracellular and intracellular Cl^-. The results indicate that the effect of ATP on cell volume is Cl^- dependent. Activation of Cl^- channels by ATP induces release of Cl^- from cells and passive outflow of water, and therefore results in a decrease in cell volume. Osmotic cell swelling has been found to induce the release of ATP from many cell types [20]. The extracellular ATP concentration in the immediate vicinity of the cell surface was observed to reach over 10 μmol/L, which is sufficiently high enough to stimulate purinergic receptors [20]. However, whether the ATP-activated chloride channel is the volume-activated chloride channel is far from clear. The relationship between the two channels remains to be studied.

As a summary, we found in the present study that extracellular ATP of micromolar scales induces a chloride current by activation of chloride channel through binding to purinoceptor P2Y in CNE-2Z cells. The current shares the properties of the volume-activated chloride current and may be involved in cell volume regulation.

REFERENCES

1 Huang YH (黄亿华), Weng XH, Zhou ZQ. Extracellular ATP: effects, sources and fate. Prog Physiol Sci(生理科学进展) 1998; 29:115-119 (Chinese, English abstract).

2 Vassort G. Adenosine 5'-triphosphate: a P2-purinergic agonist in the myocardium. Physiol Rev 2001; 81: 767-806.

3 Chiozzi P, Murgia M, Falzoni S, Di Virgilio F. Role of the purinergic P2Z receptor in spontaneous cell death in J774 macrophage cultures. Biochem Biophys Res Commun 1996; 218: 176-181.

4 Zeng W, Lee MG, Muallem S. Membrane-specific regulation of Cl channels by purinergic receptors in rat submandibular gland acinar and duct cells. J Biol Chem 1997; 272:32956-32965.

5 Lang F, Busch GL, Ritter M, Volkl H, Waldegger S, Gulbins E, Haussinger D. Functional significance of cell volume regulatory mechanisms. Physiol Rev 1998; 78: 247-306.

6 Chen LX, Wang LW. Volume-activated chloride current in pigmented ciliary epithelial cells. Acta Physiol Sin(生理学报) 2000; 52: 421-426.

7 Wang L, Chen L, Jacob TJC. Antisense to MDR1 mRNA reduces P-glycoprotein expression, swelling-activated Cl current and volume regulation in bovine ciliary epithelial cells. J Physiol (Lond) 1998; 511: 33-44.

8 Wang L, Chen L, Zhu L, Rawle M, Nie S, Zhang J, Ping Z, Cai K, Jacob TJC. Regulatory volume decrease is actively modulated during the cell cycle. J Cell Physiol 2002; 193: 110-119.

9 Wei WL, Guang YY, He H, Deng CY, Qian WM, Ruan HM, Sun JJ. ATP induced chlorine current in vascular endothelial cells and its relationship with Ca^{2+} movement. Chin J Pharmacol Toxicol (中国药理与毒理学杂志) 2001;15: 125-130 (Chinese, English abstract).

10 Arreola J, Melvin JE. A novel chloride conductance activated by extracellular ATP in mouse parotid acinar cells. J Physiol (Lond) 2003; 15:547:197-208.

11 Li ZW (李之望), Li CY. P2X purinoceptors and their regulations. Prog Physiol Sci(生理科学进展) 1999;30:349-352 (Chinese).

12 Darby M, Kuzmiski JB, Panenka W, Feighan D, MacVicar BA. ATP released from astrocytes during swelling activates chloride channels. J Neurophysiol 2003; 89: 1870-1877.

13 Burnstock G, Warland JJ. P2-purinoceptors of two subtypes in the rabbit mesenteric artery: reactive blue 2 selectively inhibits responses mediated via the P2y-but not the P2x-purinoceptor. Br J Pharmacol 1987;90: 383-391.

14 Burnstock G. Potential therapeutic targets in the rapidly expanding field of purinergic signalling. Clin Med 2002;2: 45-53.

15 Chen L, Wang L, Zhu L, Nie S, Zhang J, Zhong P, Cai B, Luo H, Jacob TJC. Cell cycle dependent expression of volume-activated chloride currents in nasopharyngeal carcinoma cells (CNE-2Z). Am J Physiol 2002; 283: C1313-C1323.

16 Wang L, Chen L, Jacob TJC. The role of ClC-3 in volume-activated chloride currents and volume regulation in bovine epithelial cells demonstrated by antisense inhibition. J Physiol (Lond) 2000;524: 63-75.

17 Chen LX, Wang LW, Jacob Tim. The role of MDR1 gene in volume-activated chloride currents in pigmented ciliary epithelial cells. Acta Physiol Sin(生理学报) 2002;54:1-6.

18 Chen L, Wang L, Zhu L, Nie S, Zhang J, Ping Z, Cai B, Luo H, Jacob TJC . The role of Cl in regulatory volume decrease of nasopharyngeal carcinoma cells. Chin J Pathophysiol(中国病理生理杂志) 2002;18:490-493 (Chinese, English abstract).

19 Chen L, Wang L. The Role of the multiple drug resistance gene in the volume regulation of ciliary epithelial cells. Chin J Pathophysiol (中国病理生理杂志) 1999;15:365-368 (Chinese, English abstract).

20 Okada Y, Maeno E, Shimizu T, Dezaki K, Wang J, Morishima S. Receptor-mediated control of regulatory volume decrease (RVD) and apoptotic volume decrease (AVD). J Physiol (Lond) 2001; 532:3-16.