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Regulation of Cl^- Secretion by Extracellular ATP in Cultured Mouse Endometrial Epithelium

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Abstract. The present study explored regulation of electrogenic ion transport across cultured mouse endometrial epithelium by extracellular ATP using the short-circuit current (I_{SC}) and the patch-clamp techniques. The cultured endometrial monolayers responded to apical application of ATP with an increase in I_{SC} in a concentration-dependent manner (EC_{50} at 3 μM). Replacement of Cl^- in the bathing solution or treatment of the cells with Cl^- channel blockers, DIDS and DPC, markedly reduced the I_{SC}, indicating that a substantial portion of the ATP-activated I_{SC} was Cl^--dependent. Amiloride at a concentration (10 μM) known to block Na^+ channels was found to have no effect on the ATP-activated I_{SC} excluding the involvement of Na^+ absorption. Adenosine was found to have little effect on the I_{SC} excluding the involvement of P_1 receptors. The effect of UTP, a potent P_{2U} receptor agonist on the I_{SC} was similar to that of ATP while potent P_{2X} agonist, α-β-Methylene adenosine 5′-triphosphate (α-β-M-ATP) and P_{2Y} agonist, 2-methylthio-adenosine triphosphate (2-M-ATP), were found to be ineffective. The effect of ATP on I_{SC} was mimicked by the Ca^{2+} ionophore, ionomycin, indicating a role of intracellular Ca^{2+} in mediating the ATP response. Confocal microscopic study also demonstrated a rise in intracellular Ca^{2+} upon stimulation by extracellular ATP. In voltage-clamped endometrial epithelial cells, ATP elicited a whole-cell Cl^- current which exhibited outward rectification and delayed activation and inactivation at depolarizing and hyperpolarizing voltages, respectively. The results of the present study demonstrate the presence of a regulatory mechanism involving extracellular ATP and P_{2U} purinoceptors for endometrial Cl^- secretion.

Key words: ATP — Endometrium — Epithelium — Ca^{2+} — P_{2U}-receptor — Cl^- secretion

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Introduction

Although it is believed that the electrolyte transport across the endometrial epithelium plays an important role in regulating the fluid environment of the uterus, there is a paucity of information on the endometrial electrolyte transport activity and its regulation. A number of neurohormonal agents have been shown to stimulate electrogenic ion transport across cultured human endometrial epithelium [16–19]. However, the ionic mechanism as well as the regulatory mechanism underlying neurohormonal stimulations are largely unknown. Although the uterus is known to be affected by cystic fibrosis [2, 15, 24, 25], a most common lethal genetic disease associated with impaired Cl^- permeability in most exocrine glands [21], very few evidence for Cl^- secretion and its regulation in the uterus has been reported.

An autocrine mechanism involving ATP in regulating Cl^- channels has recently been put forward for airway epithelial cells [1, 22]. This autocrine mechanism involving ATP may exist in various epithelia since activation of Cl^- channels or stimulation of Cl^- secretion by extracellular ATP has been reported in a number of secretory epithelia including the airway [11, 23], colonic T84 cells [9], breast epithelial cells MCF-7 [12] and the epididymis [4, 27], although the physiological sources of ATP for most of these epithelia are yet to be determined. We undertook the present study to explore regulation of electrogenic ion transport by extracellular ATP across mouse endometrial epithelium using a recently established primary culture grown on permeable supports [7]. Although the mouse endometrial epithelium under unstimulated condition exhibits Na^+ reabsorption predominantly [7], the present results indicate that the response of the endometrial epithelium to ATP stimulation appears to be predominantly attributed to Cl^- secretion which is mediated by a P_{2U}-linked Ca^{2+}-dependent pathway.

Materials and Methods

Materials

Dulbecco's Modified Eagle's medium (DMEM), Hank's balanced salt solution (HBSS), Dulbecco's phosphate buffer solution (D-PBS), F-12 nutrient mixture, trypsin, ATP, DIDS and N-methyl-D-glucamine (NMDG) were purchased from Sigma (St. Louis, MO) while fetal bovine serum from Gibco Laboratories (New York). Diphenylamine-2,2′-dicarboxylic acid (DPC) was from Riedel-de Haen Chemicals (Hannover, Germany), and amiloride hydrochloride from Merck Sharp & Dohme Research Lab. (Rahway, NJ). Millipore filters were purchased from Millipore Corp (Bedford, MA). α-β-Methylene adenosine 5′-triphosphate (α-β-M-ATP) and 2-methylthio-adenosine triphosphate (2-M-ATP) were purchased from Research Biochemical International (Natick, MA).

Cell Isolation and Culture

Epithelial cells were enzymatically isolated from the mouse uterus according to the method described previously [20] with slight modification. Samples of uteri were obtained from immature ICR mice (3.5–4 weeks of age). Uterine tissues were removed and placed into a petri dish containing sterile PBS (without Ca^{2+} and Mg^{2+}). After washing with PBS and trimming off the remained fatty tissue and connective tissues, the uteri were sliced longitudinally. The sliced uteri were incubated in PBS supplemented with 7.5 mg/ml trypsin, 37.5 mg/ml pancreatin, 100 U/ml penicillin and 100 μg/ml streptomycin at 0°C for 60 min and then at room temperature for another 60 min. After the enzyme digestion, the test tube containing D-PBS and the tissues was shaken gently for 30 sec. Uterine tissues were carefully removed and the crude cell solution was passed through a 70 micron fluorocarbon mesh filter (Spectra/Mesh). The filtrate was centrifuged at 1,000 × *g* for 5 min. The supernatant was discarded and the cell pellet was resuspended in 12 ml PBS. The cells were allowed to settle for 5 min, then the top portion (about 2 ml) of the cell suspension was discarded. The cell suspension was centrifuged again at 1,000 × *g* for 5 min. The washing procedures were repeated once more. After centrifugation, the cell pellet was resuspended in Ham's F_{12}/DMEM culture medium containing 10% FBS, 1% NEAA, and 100 U/ml penicillin, 100 μg/ml streptomycin. By now, the isolated cells were ready for subsequent cultures. For the I_{SC} measurements, the isolated endometrial cells were plated at a density of about 1.4×10^6 cells/ml onto floating permeable supports made of Millipore filters (with an area of 0.45 cm^2 for cell growth). Cultures were incubated at 37°C in 5% CO_2/95% air atmosphere, and reached confluence in 3–4 days.

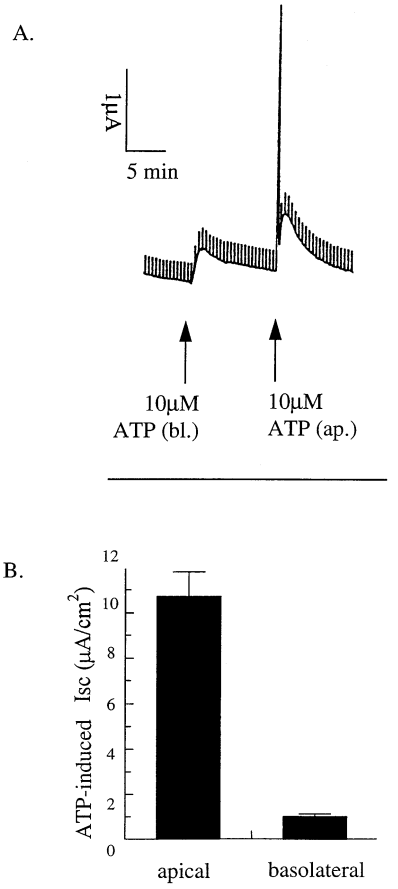

Fig. 1. I_{SC} in response to apical and basolateral addition of ATP. (*A*) I_{SC} recording (representative of 3 experiments) with arrows marking the time at which basolateral and apical ATP (10 μM) was added. The horizontal line represents zero I_{SC}. The transient current pulses were resulted from an intermittently applied voltage of 0.1 mV, from which transepithelial resistance could be calculated. (*B*) Mean current values of peak I_{SC} responses to basolateral and apical addition of ATP. Values are means ±SE.

Short Circuit Current Measurement

The measurement of I_{SC} has been described previously [26, 28]. Monolayers grown on permeable supports were clamped vertically between two halves of the Ussing chamber. Monolayers were bathed in both sides with K-H solution which was maintained at 37°C by a water jacket enclosing the reservoir. The solution was bubbled with 95% O_2 and 5% CO_2 such that the pH of the solution was maintained at 7.4. Drugs could be added directly to the apical or basolateral side of the epithelium. Usually, the epithelium exhibited a basal transepithelial PD which was measured by the Ag/AgCl reference electrodes (Metrohm, Switzerland) connected to a preamplifier that in turn connected to a voltage-clamp amplifier (World Precision Instruction, DVC-1000). In most of the experiments, the change in I_{SC} was defined as the maximal rise in I_{SC} upon agonist stimulation and it could be normalized by the unit area of the epithelial monolayer. In each experiment, a transepithelial PD of 0.1 mV was applied. The change in current in response to the applied potential was used to calculate the transepithelial resistance of the monolayer using the Ohmic relationship.

Krebs-Henseleit (K-H) solution was used, which contained (in mM): NaCl, 117; KCl, 4.5; $CaCl_2$, 2.5; $MgSO_4$, 1.2; $NaHCO_3$, 24.8; KH_2PO_4, 1.2; glucose, 11.1. The solution was gassed with 95% O_2, 5% CO_2 at 37°C and the pH of the solution was 7.4. In some experiments, ambient Cl^- was removed by substituting with gluconate.

Patch-clamped Whole-cell Current Measurement

For these studies cells were grown in culture dishes instead of permeable supports. After 3–4 days in culture, the cells formed colonies in dish. To isolate single cells for patch-clamp study, the cells were immersed in a bath NaCl solution containing low Ca^{2+} concentration (120 nM) for 10–20 min. The dissociated cells were then allowed to recover from low Ca^{2+} treatment in a solution with similar composition except for 1 mM Ca^{2+} for another 10–15 min before whole-cell current measurements.

Current recordings were obtained using the whole-cell patch-clamp technique as described by Hamill and others [13] with a patch-clamp amplifier (Axopatch-200 or Axopatch-1D, Axon Instruments, Foster City, CA). Patch pipettes, made from borosilicate glass (Vitrex, Modulohm I/S, Herlev, Denmark), were prepared as previously described [3, 14]. After formation of whole-cell configuration, the series resistance and cell capacitance were compensated. The control of command voltages was carried out using an IBM-AT compatible computer equipped with interface (TL-1-125, Axon Instruments) and utilizing the software *p*Clamp Version 6. The output current signals, after being filtered through an 8-pole Bessel filter (AI-2040, Axon Instruments) at a cutoff frequency of 1 kHz, were displayed on a chart recorder (Graphic, Yokohama, Japan).

The following pipette solutions were used (in mM): 140 NaCl (or 70 NMDGCl), 1 $MgSO_4$, 1.2 NaH_2PO_4, 10 HEPES, 16 glucose and 0.1 EGTA (pH 7.2). The bath solutions contained: 40 NaCl (or 70 NMDGCl), 1 $MgSO_4$, 1.2 NaH_2PO_4, 10 HEPES, 16 glucose and 1 $CaCl_2$ (pH 7.4). Osmolarity of the solutions was raised to isotonic (300 mOsm) by addition of mannitol, using a vapour pressure osmometer (Wescor 5500, Logan, UT).

Confocal Scanning Microscopy

Cells grown on glass coverslips were loaded with the Ca^{2+}-sensitive fluorescence dye Fluo 3-AM (3 μM) from a 1 mM stock in dry DMSO to the culture medium without serum. The cells were then incubated at 37°C for 45 min in dark. After loading, the cells were washed three times with medium and placed in the incubation chamber with 0.4 ml physiological saline solution containing (in mM): 130 NaCl, 2 $CaCl_2$, 5 KCl, 1 $MgCl_2$, 10 glucose, and 20 HEPES, pH 7.4.

Fluorescence images were captured by a laser scanning confocal microscope (MRC 1000 UV confocal imaging system, Bio-Rad) equipped with an Argon-ion UV laser and connected to an inverted microscope (Nikon Diaphot, Japan) fitted with a 100 × 1.4 NA objective. Fluo-3 was excited at 488 nm and Ca^{2+}-dependent fluorescence was captured at 520 nm and collected at intervals of 1.2 sec.

Statistical Analysis

Results are expressed as mean ± *SE*. Comparisons between groups of data were made by Student's unpaired *t*-test. A 'P' value of less than 0.05 was considered statistically significant.

Results

ATP-activated I_{SC}

The cultured endometrial epithelium responded to extracellular ATP, both apical and basolateral applications,

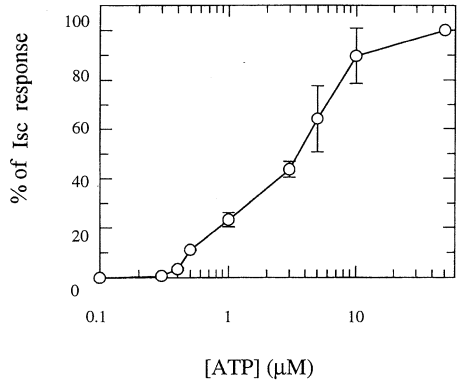

Fig. 2. Dose-response curve of ATP. Percentage of I_{SC} response is plotted against ATP concentrations. The percentage of response is obtained by dividing individual I_{SC} by the maximum response obtained at 50 μM. Data were obtained in normal K–H solution. The EC_{50} is about 3 μM.

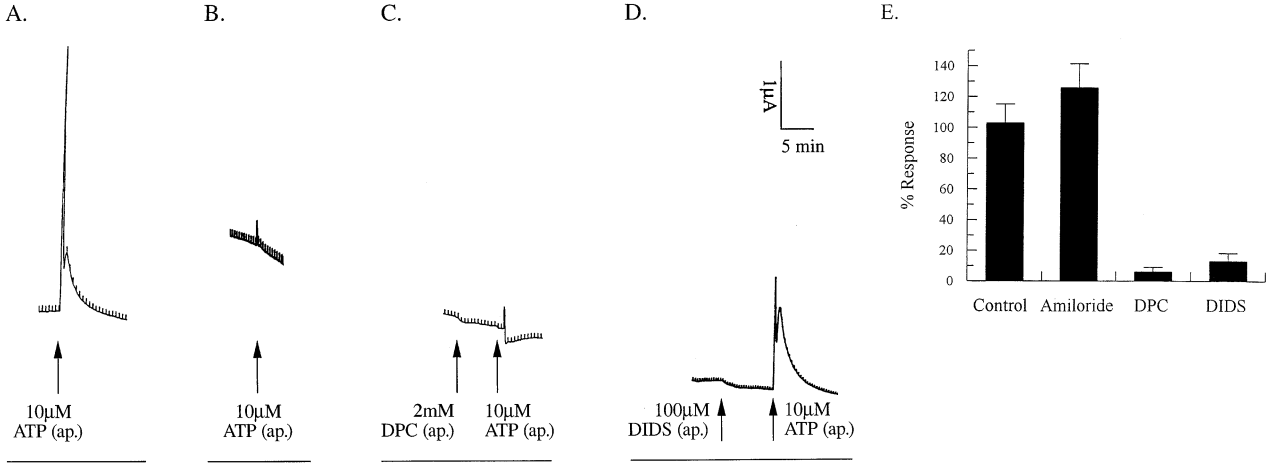

Fig. 3. Effect of Cl^- removal and channel blockers on the ATP-activated I_{SC}. (*A*) I_{SC} recording ($n = 12$) in response to 10 μM ATP (apical) in normal K–H bathing solution. (*B*) ATP-induced I_{SC} in Cl^--free solution ($n = 3$). (*C*) Recording of the ATP-induced I_{SC} obtained from epithelium ($n = 6$) pretreated with the Cl^- channel blocker, DPC (2 mM, apical). (*D*) Recording of the ATP-induced I_{SC} obtained in K–H bathing solution from epithelium ($n = 5$) pretreated with the Cl^- channel blocker, DIDS (100 μM, apical). (*E*) Comparison of effects of amiloride (10 μM), DPC (2 mM) and DIDS (100 μM) on the ATP-activated I_{SC}. Mean values of I_{SC} measured at the first peak of the response to 10 μM ATP (apical) in K–H bathing solution are shown (means ± SE, $n > 5$).

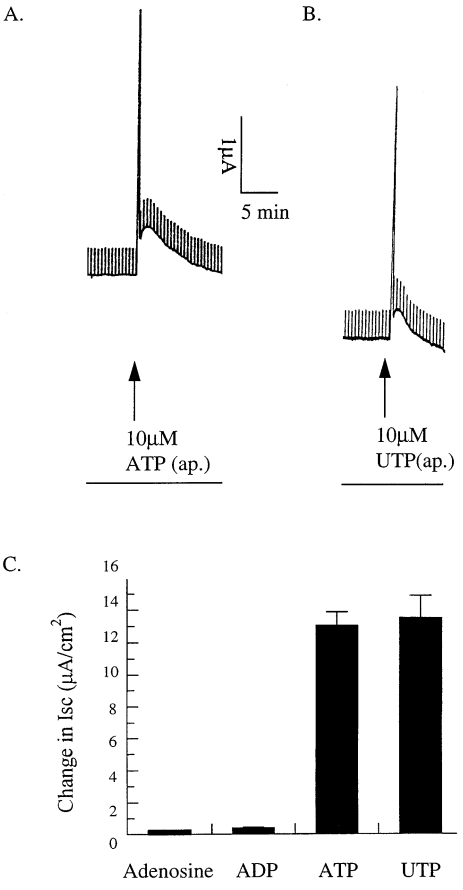

Fig. 4. Comparison of effects of different nucleotides/nucleoside on the I_{SC}. (*A*) and (*B*) I_{SC} recordings (representative of at least 3 experiments) show similar responses to apical addition of ATP (10 μM) and UTP (10 μM). (*C*) Comparison of effects of UTP, ATP, ADP and adenosine on the I_{SC} (at 10 μM). Mean I_{SC} measured at the peak responses in K–H bathing solution are shown (means ± SE, $n = 3$).

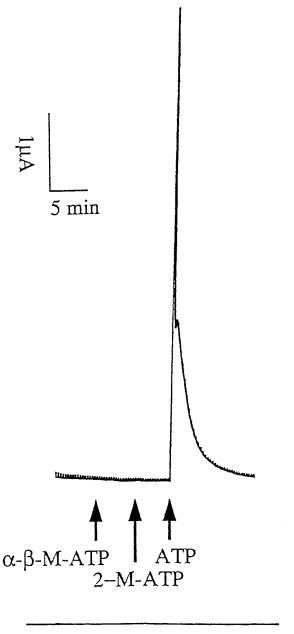

Fig. 5. Ineffectiveness of P_{2X} and P_{2Y} agonists in activating the I_{SC}. Representative I_{SC} recording ($n = 4$) with arrows indicating the time of apical addition of agonists. P_{2X} and P_{2Y} agonists, α-β-M-ATP and 2-M-ATP (10 μM), respectively, and ATP were added at a concentration of 10 μM.

with a rapid increase in the I_{SC} (Fig. 1). While the I_{SC} response to basolateral ATP (10 μM) was small (increase of 1.1 ± 0.1 μA/cm^2, $n = 3$), the response to apical ATP (10 μM) was much larger (increase of 10.7 ± 1.1 μA/cm^2). The responses were measured at the peak of the I_{SC} after a challenge. The apical response often, but not always (77%, total $n = 145$), exhibited biphasic characteristic with first peak at 0.1 min and second peak (37% of the first peak) at 0.6 min. The subsequent study was focused on the apical ATP response. The value of the first peak was used for analysis throughout the study. Figure 2 shows concentration-dependent effect of apical addition of ATP on the I_{SC}, with a value of EC_{50} at about 3 μM.

Cl^--dependence of the ATP-activated I_{SC}

Ion substitution experiments were performed to study the involvement of Cl^- in the ATP-induced I_{SC} response. The ATP-activated I_{SC} was greatly reduced (90.9%) in the absence of extracellular Cl^- ($n = 3$, Fig. 3*A* and *B*), indicating a Cl^- dependence of the I_{SC}. In another set of experiments, the monolayers were treated with Cl^- channel blockers, DIDS or DPC, prior to the addition of ATP. At a concentration of 2 mM, DPC almost completely inhibited the ATP-activated I_{SC} (93.9%, $n = 5$, Fig. 3C). While DIDS at a concentration of 100 μM substantially inhibited the first peak of the ATP-induced I_{SC} (86.9%, $n = 6$), it did not affect the second peak significantly (Fig. 3*D*). The effect of Cl^- channel blockers was compared to that of a Na^+ channel blocker, amiloride (Fig. 3*E*). Addition of amiloride (10 μM) to the apical aspect had no effect on the ATP-induced I_{SC} ($n = 7$), excluding the participation of Na^+ reabsorption.

Involvement of P_{2U} Receptors

The effects of different adenosine nucleoside or nucleotides on the I_{SC} were compared to that of ATP. As shown in Fig. 4, the effect of UTP on the I_{SC} was similar to that of ATP; however, the effect of ADP (100 μM, $n = 3$) and adenosine (500 μM, $n = 6$) were much less prominent. The order of potency appeared to be UTP ≥ ATP >> ADP = adenosine, consistent with that for P_{2U}-purinoceptors [10]. The effect of a more potent P_{2X} agonist, α-β-Methylene adenosine 5′-triphosphate (α-β-M-

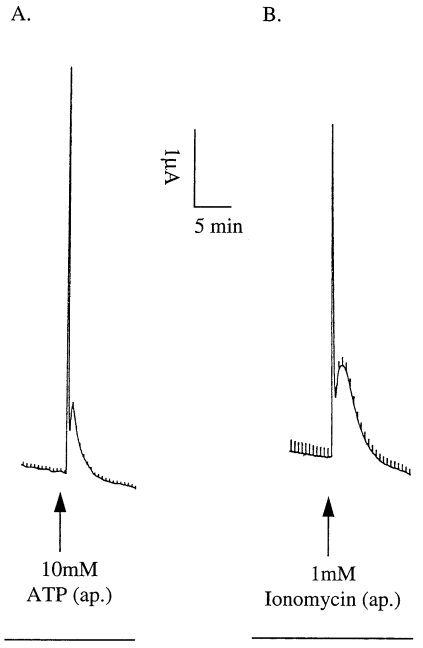

Fig. 6. Mimicking the ATP-activated I_{SC} by ionomycin. Recordings (representative of 4) of the ATP-activated I_{SC} (*A*, 10 μM) and ionomycin-induced I_{SC} (*B*, 1 μM). Note the presence of biphasic characteristic in both responses.

ATP) and a P_{2Y} agonist, 2-methylthio-adenosine triphosphate (2-M-ATP), were also examined. As shown in Fig. 5, only ATP (10 μM), but not the other two agonists, α-β-M-ATP and 2-M-ATP (10 μM), exerted a prominent effect on the I_{SC} ($n = 4$).

Involvement of Ca^{2+} in the ATP Response

To see whether the ATP-stimulated I_{SC} response was mediated through changes in intracellular free calcium, the effect of a Ca^{2+} ionophore, ionomycin (1 to 2 μM), on the I_{SC} was compared to that of ATP. As shown in Fig. 6, the ionomycin-induced I_{SC} was similar to that induced by ATP in the current magnitude as well as the biphasic characteristic ($n = 4$), suggesting that the ATP-induced I_{SC} could be mediated by Ca^{2+}. A rise in intracellular Ca^{2+} in response to extracellular ATP was also demonstrated by confocal microscopy, ATP-induced time dependent changes in intracellular Ca^{2+} was recorded, as shown in Fig. 7 ($n = 3$), further indicating the involvement of intracellular Ca^{2+} in mediating the ATP response.

ATP-activated Whole Cell Current

The ATP-activated current was further studied using the whole-cell voltage-clamp technique. The whole-cell currents were elicited by applying a series of command voltage pulses from a holding potential of −30 mV to potentials between −120 to 120 mV with 20 mV increment. NMDG-Cl-containing pipette and bathing solutions were used so that Cl^- was the major permeant ion species. Most of the ATP-activated whole-cell currents recorded were transient in nature but the time of current inactivation varied from cell to cell. The present study focused on the peak current response, usually recorded 1–2 min after stimulation. The whole-cell current obtained in symmetrical NMDG-Cl solutions in response to 100 μM ATP exhibited outward rectification and delayed activation and inactivation at depolarizing and hyperpolarizing voltages, respectively (Fig. 8, $n = 4$). Whole-cell currents with similar characteristics were also observed in NaCl-containing pipette and bathing solutions (140:40 mM, respectively, *not shown*). The averaged reversal potential, 24.2 ± 1.8 mV ($n = 6$), was close to the calculated Cl^- equilibrium potential, 31 mV, demonstrating the activation of Cl^- current by ATP. The ATP-activated currents could be inhibited by 78.9 ± 9.7% with a Cl^- channel blocker, DIDS (100 μM, $n = 5$).

Discussion

Although a number of neurohormonal agents have been shown to stimulate electrogenic ion transport across the endometrial epithelium in isolated human culture cells [16–19], the ion species mediating the response and the regulatory mechanism underlying neurohormonal stimulations have not been investigated extensively. The present study has provided the first evidence for the stimulation of Cl^- secretion across the cultured mouse endometrial epithelium cells by extracellular ATP. While both cultured human [16] and mouse [7] endometrial epithelia exhibited Na^+ absorption predominantly under unstimulated condition, the present study shows that the response of mouse endometrial epithelium to ATP is almost entirely mediated by Cl^- secretion. The supporting evidence came from the experiments in which replacement of Cl^- in the bathing solutions greatly reduced the I_{SC} and the ATP-activated I_{SC} was blocked by Cl^- channel blockers, DIDS and DPC, applied to the apical membrane. The possibility of Na^+ absorption involved in ATP-stimulated I_{SC} was excluded based on the observation that amiloride, at a concentration known to block apical Na^+ channels, produced little effect on the ATP-stimulated I_{SC}. Activation of Cl^- conductance by ATP was also demonstrated by whole-cell patch-clamp experiments, further supporting a role in Cl^- in mediating the ATP response in the mouse endometrial epithelium.

Activation of chloride channels or stimulation of Cl^- secretion by extracellular ATP has also been reported in a number of other secretory epithelia [4, 9, 11, 12, 23,

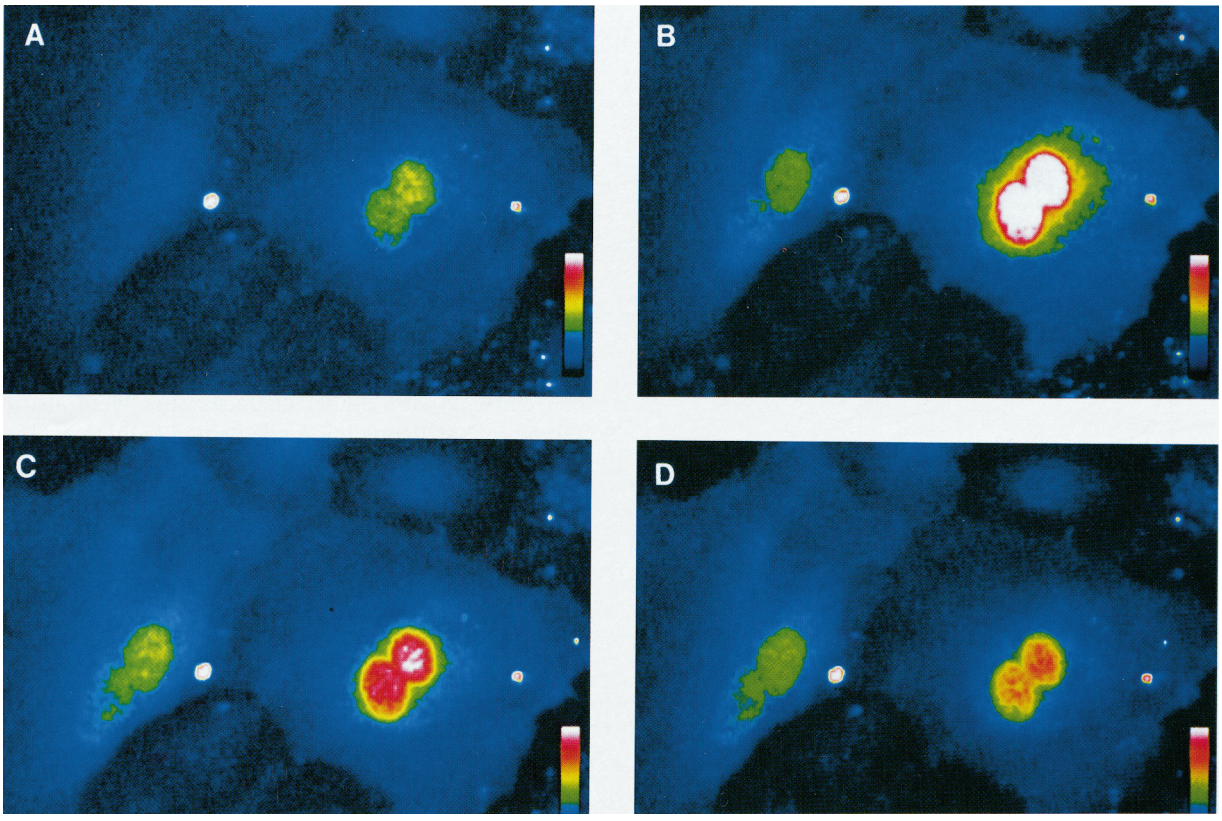

Fig. 7. Confocal micrograph showing changes in intracellular Ca^{2+}. Data were collected before (*A*) and 5 secs (*B*), 30 secs (*C*), 65 secs (*D*) after ATP (10 μM) challenge (representative of 3). Cells grown on glass coverslips were loaded with the Ca^{2+}-sensitive fluorescence dye Fluo 3-AM (3 μM) for 45 mins. Fluorescence images were captured by a laser scanning confocal microscope with a 100 × 1.4 NA objective. Fluo-3 was excited at 488 nm and Ca^{2+}-dependent fluorescence was captured at 520 nm.

27]. However, the action of ATP (direct or indirect via different receptors) and the signal transduction pathways involved in mediating the ATP-induced Cl^- secretion are quite different in the tissues and cells examined. The present study indicates that the effect of ATP on the mouse endometrial epithelium was mediated by P_{2U}-purinoceptors. It was shown that the effect of ATP was much greater than that exerted by other adenosine nucleotides but similar to that exerted by UTP. Specific P_{2Y} agonist, 2-M-ATP, and P_{2X} agonist, α-β-M-ATP, were found to be ineffective in activating the I_{SC}, excluding the involvement of P_{2Y} and P_{2X}. The involvement of P_{2U} receptors in mediating ATP-stimulated Cl^- secretion has also been reported in a number of epithelia including the epididymis [4, 9, 12] and CF pancreatic duct cells [6].

The present study has indicated the involvement of Ca^{2+} in mediating the ATP response in the mouse endometrial epithelium. While confocal microscopy demonstrated a rise in intracellular Ca^{2+} in response to extracellular ATP, the I_{SC} measurement demonstrated that the ATP-activated I_{SC} was mimicked by the Ca^{2+} ionophore, ionomycin, both in current magnitude and current kinetics, e.g., biphasic characteristic of the response. It seems likely that the stimulation of Cl^- secretion by ATP in mouse endometrial epithelial cells is mediated by P_{2U}-linked Ca^{2+} mobilization which in turn activates Cl^- channels, presumably, the Ca^{2+}-dependent Cl^- channels. This notion is supported by the results obtained from whole-cell patch-clamp experiments demonstrating that the ATP-activated whole-cell currents exhibit characteristics of delayed activation and inactivation at depolarizing and hyperpolarizing voltages, respectively, which have been described for the Ca^{2+}-activated Cl^- channels found in various epithelial cells [8, 14]. ATP-activated whole-cell currents with similar characteristics have also been observed in epididymal cells, which were demonstrated to be Ca^{2+}-dependent Cl^- currents [5]. It should be noted that the ATP-induced I_{SC} in the mouse endometrial epithelium exhibits a biphasic characteristic similar to that observed in the rat epididymal epithelium [4, 27]. This biphasic nature of the ATP-induced response in the epididymal epithelium has been shown to involve both Ca^{2+} and cAMP-dependent regulatory pathways [4, 5]. The present study has also demonstrated differential

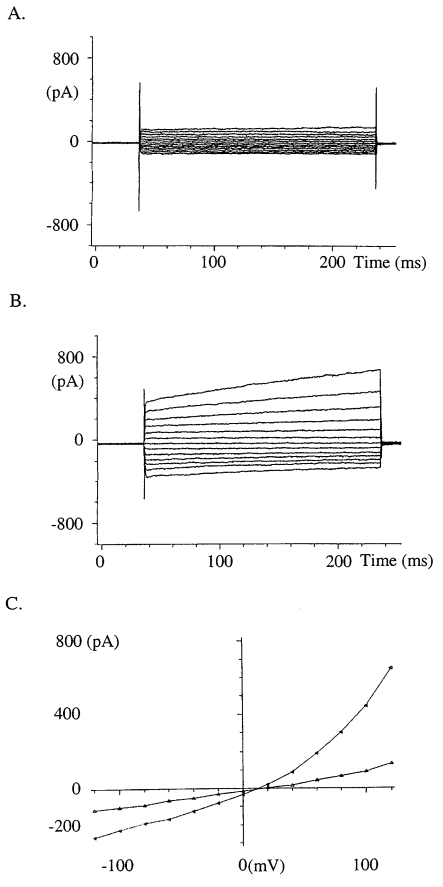

Fig. 8. ATP-activated whole-cell currents. Whole-cell currents obtained prior to (*A*) and 1 min (*B*) after stimulation with ATP (100 μM) in 140 mM symmetrical NMDG-Cl-containing solutions ($n = 4$). (*C*) Corresponding *I–V* relationship obtained at peak current, 220 msec after voltage pulse. Currents were elicited by voltage pulses from a holding potential of −30 mV to potentials between −120 to 12 mV, 20 mV increment.

sensitivity of the two peaks of the ATP-induced I_{SC} to Cl^- channel blockers, DIDS and DPC, indicating possible involvement of different Cl^- channels. Further investigation into detail signal transduction mechanisms involved in mediating the biphasic ATP-induced response in endometrial epithelial cells may provide information pertinent to the regulation of Cl^- secretion in the uterus. Although ATP appears to have effects on electrolyte transport in many epithelia, it remains to be elucidated the physiological role of this transient response of ATP in controlling Cl^- secretion in the uterus.

An autocrine regulatory mechanism involving ATP has recently been put forward for airway epithelial cells [1, 22]. The present study has shown that apical application of ATP is much more effective than basolateral application in activating Cl^- secretion across the endometrial epithelium. It is possible that the regulation of endometrial Cl^- secretion in the mouse may also involve release of ATP from the epithelium to the lumen. The details of how ATP release and Cl^- secretion are coupled and regulated in the endometrium require further investigation. The present finding that ATP could alter endometrial ion transport activity, e.g., from predominantly Na^+ absorption under unstimulated condition to predominant Cl^- secretion upon stimulation, may imply different roles of different ion transport processes in endometrial functions, and suggests the importance of ATP in regulating the internal environment of the uterus.

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