Functional expression of purinergic P2X7 receptors in pregnant rat myometrium

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1Graduate School of Biomedical Sciences, Department of Obstetrics and Gynecology, Hiroshima University, Hiroshima; 2Faculty of Health Sciences, Department of Physical Therapy, Hiroshima International University, Hiroshima; 3Department of Obstetrics and Gynecology, Hiroshima Prefectural Hospital, Hiroshima, Japan

Submitted 18 August 2009; accepted in final form 8 January 2010

Miyoshi H, Yamaoka K, Urabe S, Kodama M, Kudo Y. Functional expression of purinergic P2X7 receptors in pregnant rat myometrium. Am J Physiol Regul Integr Comp Physiol 298: R1117–R1124, 2010. First published January 13, 2010; doi:10.1152/ajpregu.00507.2009.—ATP has been reported to enhance the membrane conductance of myometrial cells and uterine contractility. Purinergic P2 receptor expression has been reported in the myometrium, using molecular biology, but the functional identity of the receptor subtype has not been determined. In this study, ATP-induced currents were recorded and characterized in single myometrial cells from pregnant rats using whole cell patch clamping. Extracellular ATP was applied in the range of 10 μM-1 mM and induced currents with an EC50 of 74 μM, with no desensitization, time dependency, or voltage dependency. The currents induced carried multiple monovalent cations, with conductances ranked as K+ > Cs+ > Li+ > Na+. They were activated by P2X receptor agonists, with their effectiveness ranked as 2′,3′-O-(4-benzoylbenzoyl)-ATP >> ATP > αβ-methylene-ATP > 2-methylthio ATP ≅ UTP ≅ GTP > ADP. These currents were blocked by the selective P2X7 receptor antagonist 3-[5-(2,3-dichlorophenyl)-1H-tetrazol-1-yl]methyl pyridine (A-438079). We therefore concluded that ATP-induced currents in rat myometrial cells crossed cell membranes via P2X7 receptors. We further showed that the ATP-induced currents were blocked by extracellular Mg2+ (IC50 = 0.26 mM). Clinically, administering extracellular Mg2+ is known to inhibit uterine contraction. It therefore seems likely that uterine contraction may be induced by raised extracellular Mg2+ and suppressed via Mg2+ inhibiting P2X7 receptors. Further research is needed into the P2X7 receptor as a therapeutic target in abnormal uterine contraction, as a possible treatment for premature labor.

IN GENERAL, SMOOTH MUSCLE cell contraction is controlled by Ca2+ influx through nonselective cation channels (NSCCs) or L-type Ca2+ channels. The presence of receptor-operated NSCCs has been reported in some smooth muscles. For example, norepinephrine- or calcium-activated NSCCs were identified in portal vein smooth muscle cells (21, 35), and ACh-activated NSCCs were reported to induce depolarization to generate contraction of ileal, gastric, and tracheal smooth muscles (13, 15, 29, 34). NSCC-mediated currents have also been found to contribute to the spontaneous depolarization of smooth muscle cells in rabbit pulmonary arteries (2). In a previous report, we identified and characterized NSCC currents in rat uterine smooth muscle cells in which the myometrial NSCCs also showed Ca2+ permeability and were thought to play a certain role in the regulation of myometrial contraction (24). However, the molecular characterization of the NSCCs involved was not complete.

ATP-enhanced ion conductance was reported in cultured primarily smooth muscle cells from pregnant rat myometrium (11). These channels were permeable to many monovalent cations. ATP was also observed to induce an initial hyperpolarization and depolarization to regulate myometrial cell contraction (25). Recently, some P2 receptor agonists were reported to mediate uterine contractions showing that P2 receptors were present in the myometrium (38). The role of ATP in regulating uterine contractility and the ATP receptors present on myometrial cells requires clarification.

Extracellular ATP regulates many cellular functions via specific purinergic P2 receptors. P2 receptors are divided into two superfamilies, ionotropic P2X and metabotropic P2Y receptors. Seven subtypes of P2X and eight subtypes of P2Y receptors have been cloned and characterized (1, 6). P2X receptors are present in most organs, and their roles in pain, inflammation, apoptosis, and the regulation of smooth muscle have been investigated. The expression profile of P2X receptors in the human bladder has also been investigated (27). In addition, an ATP-gated channel has been characterized in toad gastric smooth muscle cells, with the properties of a P2X7 (previously known as P2Z) channel (31). P2X purinoceptors have been identified in the canine colon, in which depolarization of the cell membrane enhanced muscle excitability (20). P2X7 receptors have been identified in human saphenous vein smooth muscle, and its activation has been shown to induce muscle contractions (4).

We have reported that P2X4 and P2X7 receptors were predominantly expressed in rat myometrium using real-time RT-PCR (32). In this study, the electrophysiological and pharmacological properties of ATP-induced ion fluxes were investigated in myometrial smooth muscle cells isolated from pregnant rat. From these observations, we determined the molecular identities of the ATP-activated NSCCs in rat myometrium.

MATERIALS AND METHODS

This study was approved by the Research Ethics Committee of Hiroshima University (license A05-91).

Isolation of myometrial cells. Pregnant, primigravida, Sprague-Dawley rats, mated at 9 wk of age, were purchased from Charles River Japan (Yokohama, Japan). They were killed under diethyl ether anesthesia according to guidelines issued by the Research Facilities Committee for Laboratory Animal Science, Hiroshima University. Single myometrial cells were isolated enzymatically, from the longitudinal muscle layer of the rat uterus at 19–21 days gestation, as previously reported (23). In brief, myometrial strips were dissected from the uterus and cut into pieces <1 mm long in ice-cold Hanks’
solution (GIBCO-BRL, Gaithersburg, MD). The tissue was washed for 15 min in 5 ml low-Ca\(^{2+}\) Hank's solution [10 \(\mu\)M CaCl\(_2\) added to Ca\(^{2+}\)- and Mg\(^{2+}\)-free Hank's solution (GIBCO-BRL)] at 37°C and then shaken with enzyme solutions at 37°C, in four consecutive incubations, each lasting 20 min. The first two incubations were with 0.1 U/ml collagenase (Collagenase, V. alginolyticus; Boehringer Mannheim Biochemica, Indianapolis, IN) and 2.3 U/ml dispase (Dispase I, B. polyoxymyx; Boehringer in low-Ca\(^{2+}\)-Hanks' solution containing 10 mM HEPES (pH 7.5; Wako, Tokyo, Japan). The last two incubations were with 0.07% collagenase (type 4; Worthington), and 0.1% FBS albumin (Sigma Chemical, St. Louis, MO), at pH 7.4. After the enzyme incubations, the tissue suspension was gently drawn up and down in a large-bore pipette in low-Ca\(^{2+}\)-Hanks' solution. After settling for 5 min at room temperature, the solution was removed, and the isolated cells were resuspended in modified solution containing (in mM) 80 KCl, 20 K-HPO\(_4\), 5 MgSO\(_4\), 5 pyruvate, 20 taurine, 5 creatine, 5 succinate, 5 K\(_2\)ATP, 10 glucose, and 0.04 EGTA (pH 7.4) (14). After a minimum of 1 h at 4°C, but within 8 h of isolation, the cells were used in experiments.

Patch-clamping experiments. The isolated myometrial cells were put into 0.5-ml recording chambers mounted on an inverted microscope (TMS; Nikon, Tokyo, Japan). The conventional whole cell patch-clamp method (9) was applied to single myometrial cells. Patch pipettes were made from capillary tubes (GC150TF-15; Harvard Apparatus, Kent, UK) using a pipette puller (PC-10; Narishige, Tokyo, Japan) and were heat polished. The tip resistance of the patch electrode. The current recordings were filtered through a four-pole amplifier (Axopatch 200B; Axon Instruments, Burlingame, CA), test pulses were generated, and the currents were recorded using a computer connected to a DNA interface (DigiData 1200; Axon) using pCLAMP software (Axon). The recording chamber contained an Ag-AgCl\(_2\) pellet as the ground electrode. The current recordings were filtered through a four-pole analog low pass Bessel filter in the Axopatch 200B with a 2-KHz cut-off frequency. All experiments were carried out at room temperature.

A step pulse or ramp pulse protocol was used for recording the ATP-induced ion currents. The holding potential was set at −40 mV in all experiments. The ramp pulse protocol used a constant preconditioning pulse of +40 mV for 100 ms and successive ramp pulses from +40 to −100 mV for 350 ms. The preconditioning pulse inactivated voltage-dependent Ca\(^{2+}\) and K\(^{+}\) channels and minimized their effects. The data were plotted as the current generated in response to the ramp pulse against the membrane potential from −100 to +40 mV, without subtracting the leak current.

A selective P2X7 receptor antagonist, 3-(5-(2,3-dichlorophenyl)-1H-tetrazol-1-yl)phenyl pyridine (A-438079), was purchased from Tocris (Bristol, UK). Other P2 receptor agonists and antagonists were purchased from Sigma.

Solutions for patch-clamping experiments. The standard external solution contained (in mM) 125 NaCl, 20 tetraethylammonium chloride (TEA-Cl), 0.1 MgCl\(_2\), 11 glucose, and 5 HEPES (pH 7.4), with 5 \(\mu\)M nifedipine (Sigma), 30 \(\mu\)M LaCl\(_3\), and 1 \(\mu\)M CaCl\(_2\). Because divalent ions block ATP-induced currents in our preparations, we carried out most experiments with low concentrations of Mg\(^{2+}\) (0.1 mM) and Ca\(^{2+}\) (1 \(\mu\)M) in the external solution, to generate sufficient current. Except for Na\(^{+}\) permeation experiments, KCl, CsCl, and LiCl in the external solutions were replaced with equimolar NaCl. In Na\(^{+}\) permeation experiments, Na\(^{+}\) in the external solutions was substituted by equimolar N-methyl-D-glucamine (NMDG; Sigma).

The internal solution, in the pipette, contained (in mM) 125 cesium aspartate, 20 TEA-Cl, 5 MgATP, 1.2 CaCl\(_2\), 5 EGTA, and 10 HEPES (pH 7.2). In all experiments, the voltage-dependent and Ca\(^{2+}\)-activated K\(^{+}\) channels were blocked using a combination of Cs\(^{+}\) in the internal solution and TEA in the external solution. The L-type Ca\(^{2+}\) currents were inhibited using 5 \(\mu\)M nifedipine in the external solution. The background NSCC currents were blocked using 30 \(\mu\)M LaCl\(_3\) (24).

Data analysis. The permeability for each monovalent cation relative to that of internal Cs\(^{+}\) was calculated using the equation:

\[
P_{i} = \frac{P_{\text{rev}}}{P_{\text{ions}}} = \frac{RT/F}{P_{\text{mol}}C_{i}}
\]

where \(P_{i}\) is the permeability of each ion, \(P_{\text{rev}}\) is the reversal potential, \(R\) is the gas constant, \(T\) is the absolute temperature, \(F\) is Faraday’s constant, and \(P_{\text{mol}}C_{i}\) is the product of concentration and permeability. Therefore, relative permeability for each ion can be estimated by measuring the reversal potential.

Results from the patch-clamping measurements were expressed as means ± SD (n = no. of observations).

RESULTS

ATP-induced ionic currents in myometrial cells. The external application of ATP to myometrical cells induced ion conductance at concentrations >10 \(\mu\)M. A group of the currents generated in response to 0.1 mM ATP with step pulses between −120 and +40 mV and at a holding potential of −40 mV are shown in Fig. 1A. The currents seen were rectangular, showed no time-dependent changes, and had conductance with a linear relationship to membrane voltage (see Fig. 1B). The activation of these currents was directly dependent on ATP concentration, in the range of 10 \(\mu\)M to 1 mM. The EC\(_{50}\) of ATP was estimated to be 74 \(\mu\)M (n = 7), as shown in Fig. 2B.

The change in the holding current at −40 mV after a simple application of 0.1 mM ATP is shown in Fig. 1C. The ATP-induced currents were maintained throughout the period of ATP treatment and did not decay. No desensitization to ATP was seen for up to 15 min. The constant components of the currents were analyzed further, as described below.

Na\(^{+}\) permeability of the ATP-activated channels. The permeability of the ATP-activated channels on myometrial cells to Na\(^{+}\) was examined by looking at changes in conductance in response to different external Na\(^{+}\) concentrations, between 15 and 125 mM. The ATP-induced currents were recorded using the ramp pulse protocol at voltages between −100 and +40 mV, as shown in Fig. 3, inset. Increasing the external Na\(^{+}\) concentration substantially increased the amplitude of the current, indicating that the currents were mainly due to Na\(^{+}\) flux in the standard external solution (see Fig. 3A) and shifted the reversal potential of the current to the right. The presence of a residual current when the extracellular Na\(^{+}\) was completely replaced with NMDG\(^{+}\) indicated that the channels were permeable to other ions, as well as Na\(^{+}\), such as intracellular Cs\(^{+}\) or extracellular NMDG\(^{+}\) (data not shown). The substantial currents that persisted in the absence of external Na\(^{+}\) interfered with measurements of Ca\(^{2+}\) conductance through these ATP-activated channels, since it was extremely difficult to detect small Ca\(^{2+}\) currents in the presence of relatively large monovalent cation fluxes.

Selectivity for monovalent cations. Because ATP-induced currents of relatively large conductance persisted in the absence of external Na\(^{+}\), indicating that these channels were permeable to other monovalent cations, we investigated the selectivity for different ions. To do this, the reversal potential was measured under different ionic condition, using Li\(^{+}\), Cs\(^{+}\), or K\(^{+}\) in the external solution, by determining the potential where the current-voltage curves in the presence and absence of ATP intersected. The reversal potentials with K\(^{+}\), Cs\(^{+}\), Li\(^{+}\)，
or Na⁺ in the external solution were 10.5 ± 2.4 (n = 11), 7.7 ± 1.6 (n = 12), 6.4 ± 4.5 (n = 8), and 1.5 ± 2.4 (n = 14) mV, respectively, as shown in Fig. 3, B–E. The permeability of the channels for each monovalent cation could therefore be ranked as K⁺ > Cs⁺ > Li⁺ > Na⁺.

**Effects of agonists.** Rat myometrial cells were not only sensitive to ATP, but also to other nucleotide compounds, including ADP, GTP, and UTP, and to the ATP analogs 2-methylthio-ATP (2-MeSATP), αβ-methylene-ATP (αβ-MeATP), and 2',3'-O-(4-benzoylbenzoyl)-ATP (Bz-ATP). The currents activated by ADP, GTP, and UTP were 13 ± 14% (n = 12), 40 ± 42% (n = 12), and 44 ± 26% (n = 12), respectively, of the control, ATP-induced currents (Fig. 4A) in the same concentration of 0.1 mM. These currents were relatively low compared with those induced by ATP. The ATP analogs 2-MeSATP, αβ-MeATP, and Bz-ATP induced currents that were 45 ± 28% (n = 8), 60 ± 36% (n = 9), and 266 ± 107% (n = 10), respectively, of those induced by ATP (Fig. 4, B and C). The receptor was most sensitive to Bz-ATP, which is characteristic of P2X7 receptors, indicating that these are the major channels carrying ATP-induced currents in rat myometrial cells.

**Effects of antagonists.** When we looked at the effects of P2 receptor antagonists on ATP-induced currents, we found that suramin and pyridoxal-phosphate-6-azophenyl-2',4'-disulphonic acid (PPADS) blocked them, but Cibacron Blue 3GA did not (data not shown). At concentrations of 0.1 mM, suramin blocked the currents by 47 ± 10% (n = 6), and PPADS...
The extracellular application of ATP to myometrial smooth muscle cells from pregnant rat induced ionic currents in a dose-dependent manner. These currents were recorded under conditions in which La³⁺-sensitive NSCCs and conventional Ca²⁺ and K⁺ channels were completely blocked by specific inhibitors. The currents were not affected by Cibacron Blue 3GA, which excluded P2Y receptors as the channels transmitting the currents. The remaining possibility was that these currents were conducted through P2X receptors. The notion that ATP induced P2X currents in myometrium was supported by our previous report, where P2X4 and P2X7 receptors were indicated to be predominantly expressed and enhanced toward the delivery in pregnant rat myometrium (32).

In our preparations, ATP-induced currents were not inactivated after 10 min exposure to ATP, and no desensitization to ATP was observed. In most smooth muscle cells, ATP-induced currents have been reported to desensitize, with rapid desensitization reported in artery, vas deferens, and bladder smooth muscles (3, 7, 12). In contrast, ATP-induced currents in toad gastric smooth muscle cells did not desensitize, which characterized them as P2Z-like currents (31). ATP-induced currents that did not desensitize have been reported previously in cultured rat myometrial cells, but the subtype of the receptor involved was not identified (11). P2X receptor subtypes can be classified by observing the time course of desensitization during ATP application. P2X1 and P2X3 have been shown to desensitize rapidly, within 1 s, whereas P2X2, P2X4, and P2X5 gradually desensitize over 30–60 s. However, the P2X7 receptor has been shown not to desensitize (26, 30). Because in our experiments the amplitude of ATP-induced currents remained constant for at least 10 min, this suggested that the
channels responsible in rat myometrial cells were P2X7 receptors. A concentration of at least 10 μM ATP was necessary to induce currents in rat myometrial cells with the EC_{50} calculated to be 74 μM (Fig. 3B). However, much higher concentrations of ATP (150 μM) were required to activate P2Z-like receptors in toad gastric smooth muscles (31). With respect to the EC_{50}, a value of 780 μM has been estimated for the human recombinant P2X7 receptor (5), whereas a 10-fold smaller EC_{50} value has been reported for the rat recombinant P2X7 receptor (28). Therefore, the relatively low EC_{50} value that we found indicates that the P2X7 receptors in rat myometrium belong to a group of highly sensitive receptors.

P2X receptor currents are induced by various nucleotides and ATP analogs. Bz-ATP is widely recognized as a P2X7-selective receptor agonist with a much higher potency than ATP (18). In rat microglia, the potency of Bz-ATP has been reported to be 4.8-fold higher than that of ATP (33). Similarly, Bz-ATP has been observed to have five- and twofold greater effects than ATP in a neuroblastoma cell line (19) and in rat dorsal root ganglia cells, respectively (37). We observed 2.6-fold higher currents induced by Bz-ATP compared with ATP in this study. This result provided further evidence confirming that ATP-sensitive, nondesensitizing currents in rat myometrial cells were carried by P2X7 receptors. In addition, the application of αβ-MeATP and 2-MeATP induced currents in our preparation, but these currents were smaller than those induced by ATP, with relative amplitudes of 60% for αβ-MeATP and 45% for 2-MeATP, compared with ATP. Our results ranked the effectiveness of ATP analogs in the order Bz-ATP >> ATP > αβ-MeATP > 2-MeATP, which is in good agreement with that reported for other P2X7 receptors (19, 37).

The P2X receptor antagonists suramin and PPADS inhibited the ATP-induced currents in rat myometrial cells. These currents were relatively insensitive to both antagonists, and the blockade was partial, even at higher concentrations of 0.3 mM suramin and 1 mM PPADS. The P2X7 receptor has previously been reported to be blocked by suramin and PPADS but to be relatively insensitive to them (26, 30). Both suramin and PPADS at 0.1 mM blocked P2X7 currents in NG108-15 cells by ~60% (36) and the human recombinant P2X7 was inhibited by these antagonists in a similar manner (5).

A-438079, a newly developed inhibitor more specific to P2X7 receptor, was tested. ATP-induced currents in the presence of 30 μM La^{3+} and 5 μM nifedipine were blocked by ~40% with 1 μM A-438079. The sensitivity was comparable to the report of McGaraughty et al. (22) in which Bz-ATP-evoked currents were inhibited by A-438079 with concentrations of 0.3–3 μM in nonneuronal cells in dorsal root ganglia. These findings should support that these currents were carried through P2X7 receptors.

Mg^{2+} is known to suppress uterine contractions and is used clinically to treat preterm labor. In our previous report, the expression of P2X4 and P2X7 receptors was dramatically enhanced in the inflammatory preterm labor model by the application of lipopolysaccharide (32). This enhancement may lead to the increase of uterine contractility. We also have reported that Mg^{2+} blocked ATP-insensitive NSCC currents in rat myometrial cells with an IC_{50} of 0.28 mM (24). Similarly, Mg^{2+} suppressed ATP-activated NSCCs in our preparation in this study, with an IC_{50} of 0.26 mM. Thus blocking NSCCs with Mg^{2+} was thought to suppress their spontaneous depolarization and so lead to uterine contraction. Perhaps blocking ATP-induced currents with Mg^{2+} may be a mechanism by which uterine contractions are inhibited in preterm labor.

The external application of ATP to mouse uterine muscle has been reported to induce membrane depolarization by increasing Na^{+} conductance (25). Normally, depolarization results in muscle contraction as a result of the successive ac-
vation of voltage-dependent Ca$^{2+}$ channels. However, there is very little direct evidence of the activation of uterine contraction by ATP (10). On the other hand, the mechanisms by which ATP induces bladder contraction have been elucidated. Neu-

rrogenic contraction in the bladder has been shown to be mediated through P2X receptor activation (17). Gap junctions may also contribute to the initiation of uterine contraction. For example, the expression of gap junctions in the myometrium is

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Fig. 5. Inhibition of ATP-induced currents by P2X antagonists. The effect of the P2X antagonists suramin, pyridoxal-phosphate-6-azophenyl-2',4'-disulphonic acid (PPADS), and 3-[5-(2,3-dichlorophenyl)-1 H-tetrazol-1-yl]methyl pyridine (A-438079) in the presence of 0.1 mM ATP was investigated using the ramp-pulse protocol. Inhibition by suramin (A) and PPADS (B) was only partial, with ~50% inhibition by suramin at the highest concentration of 300 μM and 60% inhibition by PPADS at 100 μM. On the other hand, A-438079 blocked these currents by ~40% at 1 μM and 70% at 100 μM (C). D–F: the amplitudes of the currents measured at −80 mV at each concentration of suramin (D), PPADS (E), and A-438079 (F) are shown relative to those in the absence of antagonists (Cont).

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Fig. 6. Effect of extracellular Mg$^{2+}$ on ATP-induced currents. A: the effect of extracellular Mg$^{2+}$ on ATP-induced currents was investigated at various concentrations using the ramp-pulse protocol. The inhibition by Mg$^{2+}$ was dose dependent. B: the amplitudes of the currents measured at −80 mV are shown relative to that in the presence of 0.01 mM Mg$^{2+}$, and mean amplitudes were plotted as a function of the Mg$^{2+}$ concentration (n = 9 observations; error bars represent SDs). IC$_{50}$ was calculated to be 0.26 mM from the curve shown, which was obtained by fitting the data to the Boltzmann equation.
well known to be dramatically enhanced in the late stages of gestation and to have a role in inducing synchronized uterine contractions (8). Indeed, ATP release through connexin 43 hemichannels has been observed in hippocampal astrocytes (16). Thus we suggest that the coordinated release of ATP through gap junctions on myometrial cells may occur at the late stages of gestation.

In conclusion, ATP-induced ion currents were observed in freshly isolated myometrial cells from pregnant rat, and the channel implicated was permeable to multiple monovalent cations, indicating that it was a NSCC. The electrophysiological and pharmacological evaluation of these currents demonstrated that P2X7 receptor was the main channel involved. The expression profile of P2X receptor mRNA supported this notion in our previous report (32). In future, characterization of cloned myometrial P2X receptors expressed in cultured cells will be helpful to determine the main functional P2X receptor in rat myometrium.

**Perspectives and Significance**

We suggest that, in the myometrium, ATP signaling generates uterine contraction through the activation of P2X7 receptors. Blocking of P2X7 receptors by Mg2+ may be one of the mechanisms for inhibiting uterine contraction in tocolysis. Thus future studies targeted to P2X7 receptors would lead to developing new treatments on preventing uterine contraction in preterm delivery.

**GRANTS**

This study was supported by Grant-in-Aid for Scientific Research No. 20591917 from the Ministry of Education, Science and Culture of Japan.

**DISCLOSURES**

No conflicts of interest are declared by the authors.

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