The Signaling Mechanism of Contraction Induced by ATP and UTP in Feline Esophageal Smooth Muscle Cells

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P2 receptors are membrane-bound receptors for extracellular nucleotides such as ATP and UTP. P2 receptors have been classified as ligand-gated ion channels or P2X receptors and G protein-coupled P2Y receptors. Recently, purinergic signaling has begun to attract attention as a potential therapeutic target for a variety of diseases especially associated with gastroenterology. This study determined the ATP and UTP-induced receptor signaling mechanism in feline esophageal contraction. Contraction of dispersed feline esophageal smooth muscle cells was measured by scanning micrometry. Phosphorylation of MLC_{20} was determined by western blot analysis. ATP and UTP elicited maximum esophageal contraction at 30 s and 10 μM concentration. Contraction of dispersed cells treated with 10 μM ATP was inhibited by nifedipine. However, contraction induced by 0.1 μM ATP, 0.1 μM UTP and 10 μM UTP was decreased by U73122, chelerythrine, ML-9, PTX and GDPβS. Contraction induced by 0.1 μM ATP and UTP was inhibited by $Gαi_3$ or Gαq antibodies and by $PLCβ_1$ or $PLCβ_3$ antibodies. Phosphorylated MLC_{20} was increased by ATP and UTP treatment. In conclusion, esophageal contraction induced by ATP and UTP was preferentially mediated by P2Y receptors coupled to $Gαi_3$ and Gαq proteins, which activate $PLCβ_1$ and $PLCβ_3$. Subsequently, increased intracellular Ca^{2+} and activated PKC triggered stimulation of MLC kinase and inhibition of MLC phosphatase. Finally, increased $pMLC_{20}$ generated esophageal contraction.

INTRODUCTION

The esophagus is a muscular tube that connects the back of the pharynx to the top of the stomach and ranges from approximately 18-25 cm in length, and 1-2 cm in diameter. The muscles in the upper portion of the esophagus, also called the up-

per esophageal sphincter (UES), are under voluntary control. The lower esophageal sphincter (LES) is a bundle of muscles at the low end of the esophagus, where it connects the stomach. When the LES is closed, it prevents acid and stomach content reflux. The LES consists of smooth muscle like the rest of the digestive tract. Smooth muscle in the gastrointestinal tract is controlled by the autonomic nervous system. Contraction or relaxation of smooth muscle has a key role in gastrointestinal motility. Digestive motility disorders can lead to impaired peristalsis resulting in slow contractions, rapid contractions, or combination of both slow and fast contractions (Vantrappen et al., 1986).

Recently, physiological and pharmacological investigation showed that neurotransmitters other than acetylcholine or noradrenaline are involved in peripheral autonomic transmission. These neurotransmitters are commonly referred to as non-adrenergic, non-cholinergic (NANC) neurotransmitters (Burnstock et al., 1997). Representative NANC neurotransmitters are composed of the peptide mediators including calcitonin gene-related peptide (CGRP) and substance P, and the non-peptide mediators including nitric oxide (NO) and nucleotides such as ATP and UTP (Lundberg, 1996). The nucleotidesin the form of the nucleoside triphosphates ATP and UTP play important roles as essential energy sources and enzyme cofactors in cellular metabolism. Furthermore, ATP and UTP also function as extracellular messengers inducing intracellular signaling through activation of distinct cell surface receptors of the P2 receptor family (Ralevic and Burnstock, 1998).

The P2 receptors for extracellular nucleotides are ubiquitously distributed in various organs of the body and involved in regulation of almost all physiological processes such as the immune, inflammatory, cardiovascular, muscular, and central and peripheral nervous systems (Abbracchio et al., 2006; Surprenant and North, 2009). The term "P2" implies that purine and pyrimidine nucleotides can act as selective ligands of various receptor subtypes (Fredholmet al., 1997). The P2 receptors exist as 2 distinct families. P2X receptors are ligand-gated ionotropic channel family, gating primarily Na^+, K^+, Cl^- and Ca^{2+}. They are composed of 7 subtypes ($P2X_1$, $P2X_2$, $P2X_3$, $P2X_4$, $P2X_5$, $P2X_6$, and $P2X_7$) (Jiang et al., 2003). P2Y receptors are metabotropic, heptahelical G-protein coupled receptor (GPCR) family members with 8 identified subtypes: $P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$, $P2Y_{11}$, $P2Y_{12}$, $P2Y_{13}$, and $P2Y_{14}$ (Abbracchio et al., 1997; Fredholm et al 1997; Lee et al., 2000). Recently, purinergic signaling has begun to attract attention as a potential therapeutic target for a

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variety of diseases (Burnstock, 2006). Exploratory studies focused on purinergic receptors as the future therapeutic targets of GI diseases (Burnstock, 2008; Yiangou et al., 2001).

It was previously demonstrated that both P2X and P2Y receptors exist in esophageal smooth muscle and mediate esophageal contraction (Cho et al., 2010). However, the detailed signaling mechanism of ATP- and UTP- induced contraction via P2X and P2Y receptors in feline esophageal smooth muscle has not been studied. Understanding the signaling mechanism of purinergic receptors on esophagus can contribute to the treatment of esophageal diseases. In addition, coexistence of P2X and P2Y receptors raises the question of which receptor subtype preferentially mediates the action of the endogenous ligand. The purpose of this study was to investigate which receptor preferentially induces contraction activated by ATP and UTP and the signaling mechanism involved in feline esophageal smooth muscle cells. The nucleotides ATP and UTP were utilized to activate P2 receptors and to identify preferentially activated receptor and the signaling pathways. Selective G-protein antibody was utilized to identify the coupling of specific G-proteins to effector enzymes, and selective inhibitors were used to characterize the pathways involved in MLC_{20} (20 kDa, regulatory light chain of Myosin II) phosphorylation and esophageal smooth muscle cell contraction.

MATERIALS AND METHODS

Materials and reagents
G protein antibodies ($G_{\alpha i1}$, $G_{\alpha i2}$, $G_{\alpha i3}$, $G_{\alpha q}$, $G_{\alpha s}$, $G_{\alpha o}$, and G_β) and PLC antibodies ($\beta1$, $\beta3$, $\gamma1$) from Santa Cruz Biotechnology (USA); Chelerythrine chloride from Research Biochemicals (USA); goat anti-rabbit IgG-HRP from Bethyl Laboratories Inc. (USA); rainbow molecular weight marker from Amersham (USA); enhanced chemiluminescence (ECL) agents from PerkinElmer Life Sciences (USA); sodium dodecyl sulfate (SDS) sample buffer from Owl scientific Inc. (USA); nitrocellulose membrane, Tris/Glycine/SDS buffer and Tris/Glycine buffer from BioRad (USA); phosphate-buffered saline (PBS) from Roche Diagnostics Co. (USA); Restore™ Western Blot Stripping Buffer from Pierce (USA); and 4-(2-hydroxyethyl)-1-piperazine-N′-2-ethane sulfonic acid (HEPES), collagenase type F, ammonium persulfate, ponceau S, bovine serum albumin (BSA), leupeptin, aprotinin, β-mercaptoethanol, N,N,N′,N′-tetramethylethylene diamine (TEMED), ethylene glycol-bis-(β-aminoethylether)-N,N,N′,N′-tetraacetic acid (EGTA), ethylenediaminetetraacetic acid (EDTA), phenylmethyl-sulfonylfluoride (PMSF) and other reagents from Sigma Chemical Co. (USA).

Tissue dissection and dispersion of smooth muscle cells
Adult male cats weighing between 3 and 5 kg were anesthetized and esophageal smooth muscle tissue was prepared as previously described (Biancaniet al., 1987; Murthy and Makhlouf, 1998; Nam et al., 2013). The tissue was digested overnight maintaining a temperature of 4°C with HEPES-buffered solution thatcontained 1 mg/ml papain, 1 mM dithiothreitol, 1 mg/ml BSA and 0.5 mg/ml collagenase (type F, Sigma). The HEPES-buffered solution contained 1 mM $CaCl_2$, 250 μM EDTA, 10 mM glucose, 10 mM HEPES, 4 mM KCl, 131 mM NaCl, 1 mM $MgCl_2$ and 10 mM taurine.

Next day, the tissue was warmed in HEPES-buffed solution at room temperature (15-20°C) for 30 min and then heated in a water bath at 31°C for 30 min. After heating, the digested tissue was poured out over a 400 μm nylon mesh, rinsed in collagenase-free HEPES buffer to remove any trace of collagenase

and then incubated in this solution at 31°C, which was gassed with 95% O_2-5% CO_2. The cells were allowed to dissociate freely for 10 to 20 min. Before beginning the experiment, the cells were kept at 31°C for at least 10 min to relax the cells. Throughout the procedure, care was taken not to agitate the fluid in order to avoid cell contraction in response to mechanical stress.

The experiments were performed in accordance with the guidelines of the Institutional Animal Care and Use Committee of Chung-Ang University (No.14-0045).

Preparation of permeabilized smooth muscle cells
Cells were permeabilized, when required, to diffuse agents such as G protein antibodies and PLC isozyme antibodies, which do not diffuse across intact cell membrane. The process of preparation of permeabilized cells did not affect cell contraction (Cao et al., 2001; Horowitz et al., 1996; Murthy et al., 2003; Shim et al., 2002;Sohn et al., 1997). After completion of the enzymatic phase of the digestion process, the partly digested muscle tissue was washed with an enzyme-free cytosolic buffer of the following composition: 20 mM NaCl, 100 mM KCl, 5.0 mM $MgSO_4$; 0.96 mM NaH_2PO_4; 1.0 mM EGTA and 0.48 mM $CaCl_2$ and 2% bovine serum albumin. The cytosolic buffer was equilibrated with 95% O_2-5% CO_2 to maintain pH 7.2 at 31°C. The muscle cells were dispersed spontaneously in this medium. The cytosolic buffer contained 0.48 mM $CaCl_2$ and 1 mM EGTA, yielding 0.18 mM free Ca^{2+} (Fabiato and Fabiato, 1979).

After dispersion, the cells were permeabilized by incubation for 5 min in cytosolic buffer containing saponin (75 μg/ml). After exposure to saponin, the cell suspension was spun at 350 g, and the resulting pellet was washed with saponin-free modified cytosolic buffer that contained antimycin A (10 μM), ATP (1.5 mM) and an ATP-regenerating system that consisted of creatine phosphate (5 mM) and creatine phosphokinase (10 units/ml) (Bitar et al., 1986). The procedure was repeated twice to ensure complete removal of saponin. After the cells were washed free of saponin, they were resuspended in modified cytosolic buffer.

Measurement of contraction by scanning micrometry
Contraction of isolated muscle cells was measured by scanning micrometry (Murthy and Makhlouf, 1998; Sohn et al., 1993; 1995b). An aliquot of cell suspension containing 10^4 cells/ml was added to HEPES medium containing the test agents. The reaction was terminated by addition of acrolein (1% final concentration). The length of 30 to 40 muscle cells treated with a contractile agent was measured at random by scanning micrometry, with a phase contrast microscope (ULWCD 0.30 Olympus,Japan) and digital closed-circuit video camera (CCD color camera, Japan) connected to a Macintosh computer (Apple, USA) with a software program, NIH Image 1.57 (National Institutes of Health, USA). It was then compared with length of untreated cells. Contraction was expressed as the percentage decrease of mean cell length, as compared with control group. All measurements were done in the presence of adenosine A1 and A2 antagonists (1 mM DPCPX and 0.1 mM CGS-15943, respectively) (Murthy et al., 1995).

Protein assays
The protein concentration of supernatant was determined by the Bradford reagents, according to the instruction of the manufacturer (Bio-Rad Chemical Division, USA). The absorbance was measured spectrophotometrically at a wavelength of 595 nm.
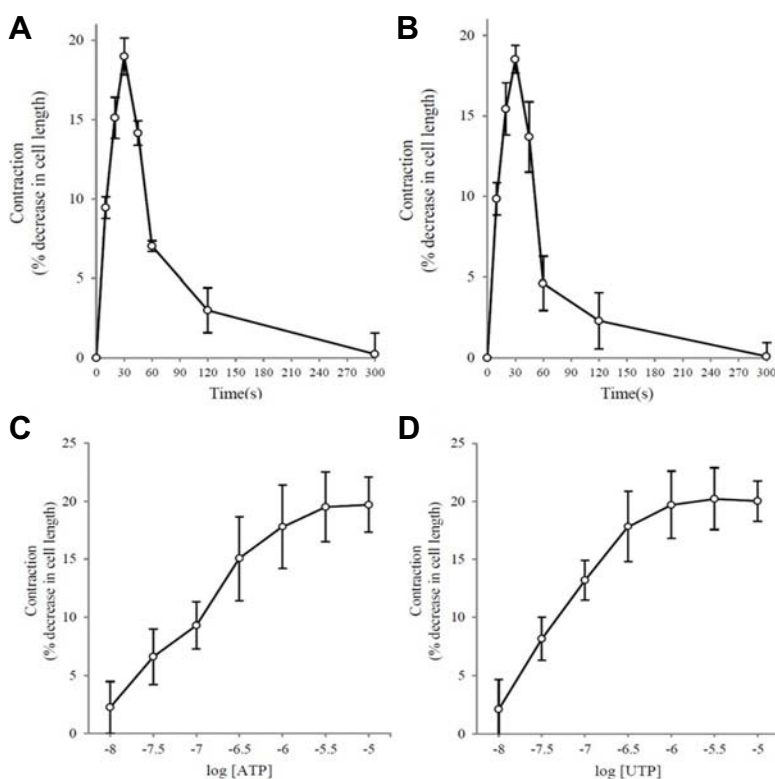
Fig. 1. Time course curves and concentration-response curves of contractile response induced by ATP and UTP in dispersed feline esophageal smooth muscle cells. (A) Time course curve of contraction in response to 1 μM ATP. The response was detected at 10, 20, 30, 45, 60, 120 and 300 s. (B) Time course curve of contraction in response to 1 μM UTP. (C) ATP-induced contraction of dispersed smooth muscle cells was measured at 30 s after treatment of ATP in different concentration (10^{-8}, $10^{-7.5}$, 10^{-7}, $10^{-6.5}$, 10^{-6}, $10^{-5.5}$, 10^{-5} M). (D) Concentration-response curve induced by UTP was measured at 30 s after ATP treatment at different concentrations (10^{-8} to 10^{-5} M). Muscle cell contraction was measured by scanning micrometry and expressed as percent decrease in cell length from control. Data are expressed as the mean $\pm$ S.E.M ($n = 5$).

Western blot analysis

Dispersed muscle cells isolated from the esophagus were resuspended in DMEM, containing penicillin (100 unit/ml), streptomycin (0.1 mg/ml), amphotericin-b (0.25 μg/ml) and 10% fetal bovine serum (DMEM). The muscle cells were plated at a concentration of 10^4 cells/ml and incubated at 37°C in a CO_2 incubator. DMEM-10 medium was replaced every 3 days for 2-3 weeks until confluence was attained. All experiments were done on cells in the second passage (Murthy et al., 2003).

Phosphorylated MLC_{20} was determined by immunoblot analysis using a phospho-specific antibody (Huang et al., 2005; Murthy et al., 2003). Previously frozen samples of dispersed muscle cells were homogenized in a buffer containing 20 mM Tris-HCl (pH 7.4), 0.5 mM EDTA, 0.5 mM EGTA, 1% (w/v) Triton X-100, 0.01% (w/v) SDS, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 1 mM PMSF, phosphatase inhibitor cocktail-3 10 μl/ml and β-mercaptoethanol 0.7 μg/ml. Samples of the homogenates were then centrifuged for 10 min at 4°C, and the supernatants collected. Equal amounts of proteins from each sample were resolved on an SDS- polyacrylamide gel by electrophoresis. Prestained molecular mass marker in loading buffer: 25 mM Tris (pH 8.3), 192 mM glycine and 0.1% SDS, was also run in an adjacent lane to permit molecular mass determination using a power supply (Power Pac 1000, Bio-Rad, USA). The separated proteins were transferred to 0.45 μm nitrocellulose membrane in transfer buffer: 25 mM Tris (pH 8.3), 192 mM glycine and 20% (v/v) methanol, using a power supply (Power Pac 1000, Bio-Rad, USA). To confirm uniformity of gel loading, blots were stained with Ponceau S. After confirmation, the membrane was washed with TBS, and then the membrane was incubated in TBS buffer containing 3% BSA and 0.05% Tween20 (TBST) for 3 h at room temperature to block nonspecific binding. After washing thrice for 15 min with TBS, the membrane was incubated with antibodies (1:1,000 dilution) to

$pMLC_{20}$ (Ser19/Thr18) in a TBST solution containing 3% BSA at 4°C overnight. Then the membrane was washed twice for 5 min using TBST and incubated with horseradish peroxidase-conjugated secondary antibody (1:5,000 dilution) for 1 h at room temperature. The immunoreactive bands, detected by enhanced chemiluminescence agents (ECL, Perkin Elmer, USA), were developed by X-ray film developer and fixer. Developed films from ECL were scanned and analyzed densitometrically using Scion Image. Phosphorylation of MLC_{20} was calculated as the ratio of phosphorylated MLC_{20} to total MLC_{20} (Cao et al., 2001; Ijzeret al., 2009; Nam et al., 2013). All measurements were done in the presence of adenosine A1 and A2 antagonists (1 mM DPCPX and 0.1 mM CGS-15943, respectively) (Murthyet al., 1995).

Analysis of data

The results were expressed as mean + or $\pm$ S.E.M. of n experiments. P values were determined by one-way ANOVA with post-hoc Tukey HSD (Honest Significant Differences) using GraphPad PRISM (GraphPad Software, USA). Each experiment was done on cells obtained from different animals. Values were considered statistically significant when P value < 0.05.

RESULTS

Contraction induced by ATP and UTP in dispersed smooth muscle cells

Dispersed feline esophageal smooth muscle cells were treated for 10, 20, 30, 45, 60, 120, and 300 s with 1 μM ATP or UTP. Exposure of dispersed smooth muscle cells to 1 μM ATP caused immediate contraction that significantly increased until 30 s. Maximal contraction was attained at 30 s followed by a decline to lower levels. The contraction almost disappeared after 5 min (Fig. 1A).
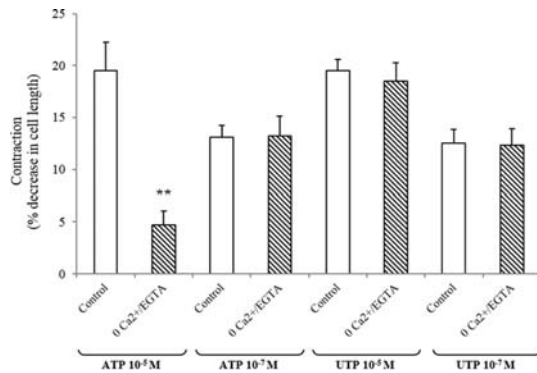
Fig. 2. Inhibition of ATP- and UTP-induced contraction in dispersed feline esophageal smooth muscle cells in the absence of Ca^{2+} from the medium (0 Ca^{2+}/ 2 mM EGTA). Dispersed smooth muscle cells were preincubated in Ca^{2+}-free medium and then treated with 10 μM ATP, 0.1 μM ATP, 10 μM UTP, and 0.1 μM UTP for 30 s. Muscle contraction was measured by scanning micrometry. Data are expressed as means + S.E.M (n = 4). **$P < 0.01$ versus control.

The time course was identical to that observed with 1 μM UTP (Fig. 1B). The maximal response at 30 s was used in concentration-response curves. Contraction of dispersed feline smooth muscle cells were measured at 30 s after treatment of $10^{-8}, 10^{-7.5}, 10^{-7}, 10^{-6.5}, 10^{-6}, 10^{-5.5}, 10^{-5}$ M ATP or UTP respectively. Treatment of ATP caused concentration-dependent contraction of dispersed feline esophageal smooth muscle cells. Each concentration of ATP was treated for a 30 s duration. The maximal peak was attained by 10 μM concentration of ATP (Fig. 1C). The concentration-response curve showed the same pattern to that observed with treatment of UTP (Fig. 1D).

In calcium-free medium, contraction induced by 10^{-5} M ATP was inhibited but contraction induced by ATP 10^{-7} M or UTP

was not inhibited. This result supported that contraction induced by 10^{-7} M ATP or UTP was activated by intracellular IP_3 pathway although contraction induced by 10^{-5} M ATP depends on extracellular calcium ion (Fig. 2).

The signaling of contraction induced by ATP was altered depending on its concentration
P2X receptors are ligand-gated ionotropic channel family, especially Ca^{2+} channel, and P2Y receptors are involved in pertussis toxin-sensitive and -insensitive G proteins thatregulate diverse enzymes (Akbar et al., 1996; Chang et al., 1995; Cowen et al., 1990; Dubyak and el-Moatassim, 1993; Harden et al., 1995; Lazarowski and Harden, 1994).
Dispersed smooth muscle cells were pretreated with Ca^{2+} channel blocker nifedipine 1 μM for 10 min or with pertussis toxin PTX 400 ng/ml and GDPβS 10 μM for 1 h respectively, and then treated with 10 μM or 0.1 μM ATP and UTP for 30 s. Contraction induced by 10 μM ATP were abolished by only the Ca^{2+} channel blocker, nifedipine but were not affected by pretreatment of dispersed cells with PTX, and GDPβS (Fig. 3A). In contrast, contraction induced by 0.1 μM ATP wasinhibited by PTX or GDPβS but not affected by nifedipine (Fig. 3B). Contractions induced by 10 μM and 0.1 μM UTP were abolished by PTX, and GDPβS but were not affected by pretreatment of dispersed cells with nifedipine (Figs. 3C and 3D). The signaling of contraction induced by ATP was concentration-dependent. Higher concentration of ATP mediated contraction via P2X receptors. In contrast, lower concentration of ATP mediated contraction via P2Y receptors in accordance with UTP-induced contraction.

Identification of the G protein subtypes related to ATP- and UTP-induced contraction
The above experiment revealed that preferential signaling of ATP- and UTP-induced contraction was mediated by P2Y receptors. A previous study showed that $G\alpha i_1$, $G\alpha i_2$, $G\alpha i_3$, $G\beta$ (40 kDa), $G\alpha o$ (40 kDa), $G\alpha q$ (42 kDa), and $G\alpha s$ (46 kDa) proteins are expressedin cat smooth muscle cells (Yang et al., 2000).
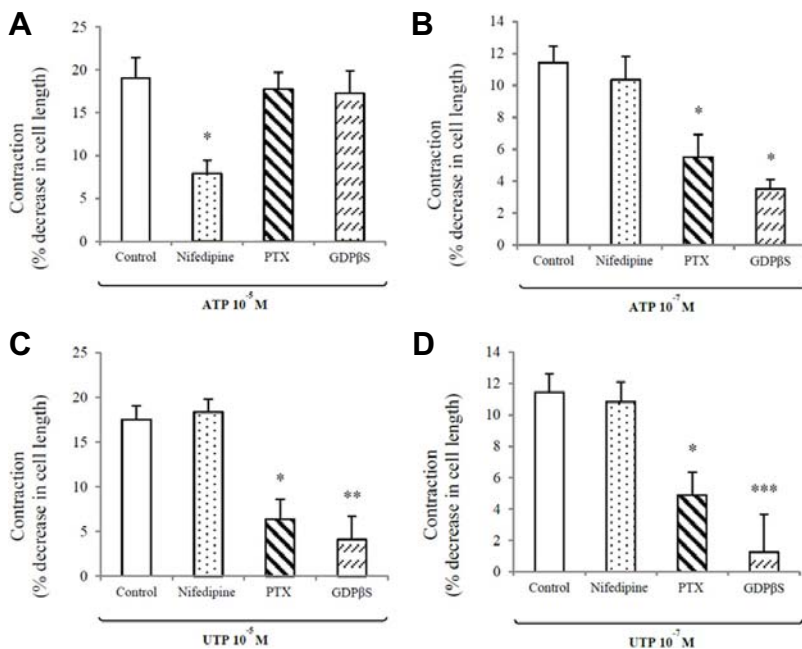

Fig. 3. Inhibition of ATP- and UTP-induced contraction in dispersed feline esophageal smooth muscle cells by nifedipine, pertussis toxin (PTX) and guanosine-5′-(β-thio)-diphosphate (GDPβS). Dispersed smooth muscle cells were preincubated with nifedipine (1 μM) for 10 min, PTX (400 ng/ml) and GDPβS (10 μM) for 1 h respectively, and then treated with A) 10 μM ATP, B) 0.1 μM ATP, C) 10 μM UTP, and D) 0.1 μM UTP for 30 s. Muscle contraction was measured by scanning micrometry. Data are expressed as the mean + S.E.M (n = 5). *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ versus control.
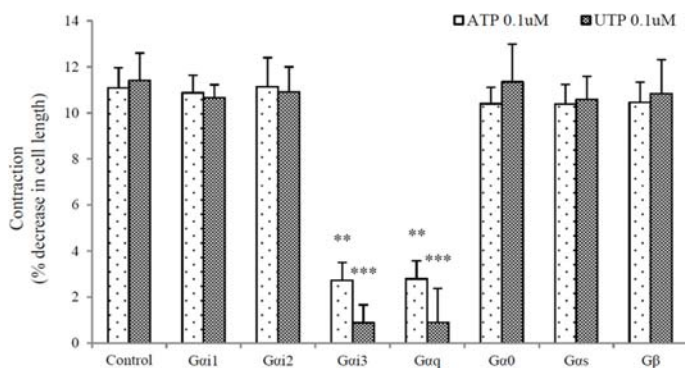
Fig. 4. Inhibition of ATP- and UTP- induced contraction in permeabilized feline esophageal smooth muscle cells by antibodies to G protein isoforms. Permeabilized cells were preincubated with antibodies to Gαi1, Gαi2, Gαi3, Gαq, Gαo, Gαs, and Gβ (1:200) for 1 h respectively, and then treated with 0.1 μM ATP or 0.1 αM UTP for 30 s. Muscle contraction was measured by scanning micrometry. Data are expressed as the mean + S.E.M (n = 4). **$P < 0.01$, ***$P < 0.001$ versus control.
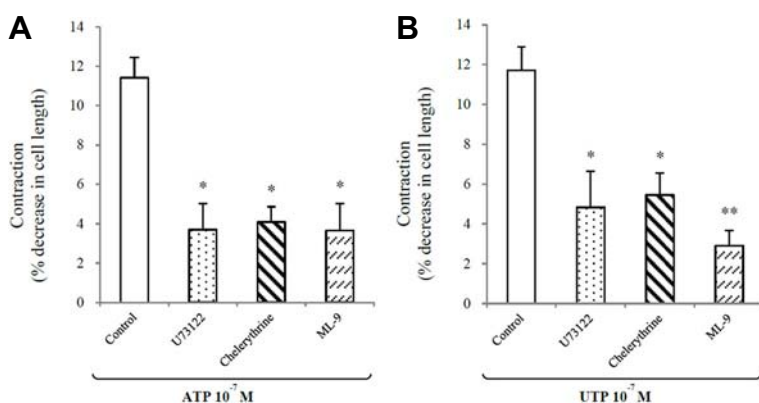

Fig. 5. Inhibition of ATP- and UTP-induced contraction in dispersed feline esophageal smooth muscle cells by PLC inhibitor (U73122), PKC inhibitor (chelerythrine), MLC kinase inhibitor (ML-9). Dispersed smooth muscle cells were preincubated with U73122 (1 μM) and ML-9 (10 μM) for 10 min and chelerythrine (10 μM) for 1 min, respectively, and then treated with (A) 0.1 μM ATP and (B) 0.1 μM UTP for 30 s. Muscle contraction was measured by scanning micrometry. Data are expressed as the mean + S.E.M (n = 5). *$P < 0.05$, **$P < 0.01$ versus control.

The subtypes of G proteins activated by ATP and UTP in smooth muscle were identified by contractile blockade with G protein-specific antibodies. Permeabilized feline esophageal smooth muscle cells were preincubated with specific antibodies to Gαi1, Gαi2, Gαi3, Gαq, Gαo, Gαs, and Gβ for 1 h respectively, and then treated with 0.1 μM ATP or 0.1 μM UTP for 30 s. Contraction induced by 0.1 μM ATP was partially abolished by Gαi3 and Gβ. Treatment of 0.1 μM UTP also produced the same results as treatment of 0.1 μM ATP (Fig. 4).

The signaling pathway of contraction activated by ATP and UTP
P2Y receptors involve diverse enzymes including phospholipase C (Akbar et al.,1996; Dubyak and el-Moatassim, 1993; Harden et al., 1995; Lazarowski and Harden, 1994), and protein kinase C (van der Weyden et al., 2000), which can induce activation of MLC kinase and inhibition of MLC phosphatase (Ikebe et al., 1987). Dispersed smooth muscle cells were pretreated with PLC inhibitor (U73122) 1 μM, PKC inhibitor (chelerythrine) 10 μM, or MLC kinase inhibitor (ML-9) 10 μM for 10 min, respectively, and then treated with 0.1 μM ATP or UTP for 30 s. Contraction induced by 0.1 μM ATP and UTP were partially abolished by U73122, chelerythrine, and ML-9 (Figs. 5A and 5B). The results suggested that the contraction mediated by P2Y receptors involved PLC, PKC, and MLC kinase.

Identification of the PLC isozymes involved in ATP- and UTP- induced contraction
It was found that contraction mediated by P2Y receptors was related to phosphoslipase C. Previous study in our laboratory demonstrated the presence of immunoreactive bands of 150

kDa with PLCβ1 and PLCβ3 antibodies, and 145 kDa band with PLCγ1 antibody in dispersed feline smooth muscle cells (Yang et al., 2000). The inhibitory effect of PLC isoform specific antibodies to ATP- and UTP-induced contraction was examined to determine which PLC isoforms mediated contraction. After preincubation of permeabilized smooth muscle cells for 1 h with PLC isozymes specific antibodies (1:200), dispersed cells were treated with 0.1 μM ATP and UTP for 30 s. PLC β1 or PLC β3 specific antibodies inhibited ATP-induced contraction. On the contrary, PLC γ1 specific antibody did not have a significant inhibitory effect on contraction (Fig. 6A). UTP-induced contraction was also decreased by pretreatment with PLC β1 or PLC β3 specific antibodies (Fig. 6B).

ATP- and UTP-induced phosphorylation of MLC₂₀
The primary mechanism of smooth muscle contraction is phosphorylation of the 20 kDa myosin light chain (MLC$_{20}$). (Ikebe et al., 1987; Puetz et al., 2009; Webb, 2003). To verify that phosphorylation of MLC$_{20}$ is related in esophageal contraction, Western blot analysis was performed to measure phosphorylated MLC$_{20}$ (pMLC$_{20}$) and total MLC$_{20}$ (tMLC$_{20}$) using specific antibodies to pMLC$_{20}$ and tMLC$_{20}$, respectively. Phosphorylation of pMLC$_{20}$ was significantly increased in cultured cells treated with ATP and UTP, as compared to control (Fig. 7). The results suggested that both ATP and UTP mediate contraction of feline esophageal smooth muscle via phosphorylation of MLC$_{20}$.

DISCUSSION

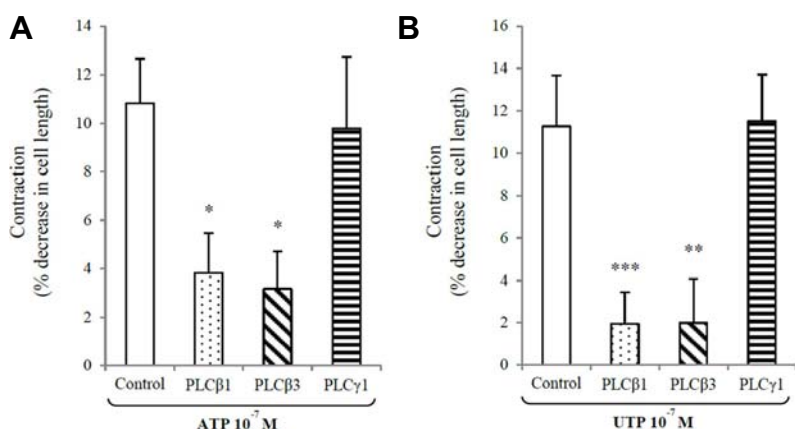A variety of signaling mechanisms are involved in contraction or

Fig. 6. Inhibition of ATP- and UTP- induced contraction in permeabilized feline esophageal smooth muscle cells by antibodies to PLC isoforms. Permeabilized cells were preincubated with antibodies to PLCβ1, PLCβ3 and PLCγ1 (1:200) for 1 h, respectively, and then treated with (A) 0.1 μM ATP or (B) 0.1 μM UTP for 30 s. Muscle contraction was measured by scanning micrometry. Data are expressed as means + S.E.M (n = 4). *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ versus control.
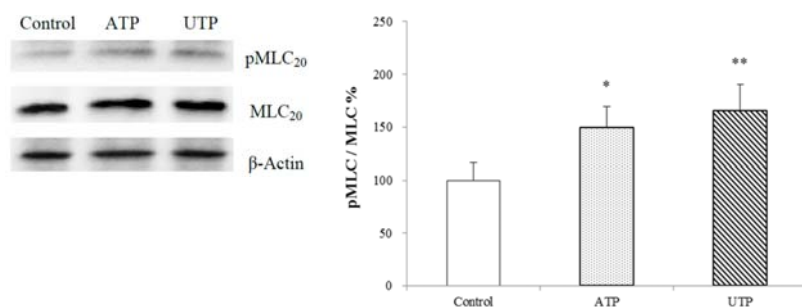

Fig. 7. ATP- and UTP- induced Contraction was mediated by MLC$_{20}$ phosphorylation.Experiments were done on primary cultures of feline smooth muscle cells. Cultured cells were incubated with 0.1 μM ATP or 0.1 μM UTP, respectively. The 20 kDa-myosin light chain phosphorylation was confirmed by Western blot analysis using phosphorylated MLC$_{20}$ (Ser19/ Thr18) antibody and MLC$_{20}$ antibodies. Data were expressed as percent increase + S.E.M compared with control (n = 6). *$P < 0.05$, **$P < 0.01$ versus control.

relaxation of esophageal smooth muscle. However, the detailed signaling mechanism of contraction induced by P2 receptors in esophageal smooth muscle has not been studied. It was reported that the NANC neurotransmitters play a key role in the diseased condition of GI tract (Lefebvre, 1993; Matsuda and Miller, 2010). Investigation of the NANC nucleotide ATP- and UTP-induced signaling mechanism of esophageal smooth muscle contraction contributes to understanding pathophysiology of esophageal diseases.

In the present study, we found that contraction induced by 10 μM ATP was mediated by P2X receptors that can induce influx of Ca^{2+} from extracellular space. In contrast, contraction induced by 0.1 μM ATP was triggered by P2Y receptors linked to PTX-sensitive Gαi$_3$ and PTX-insensitve Gα$_q$ proteins, which activate PLCβ$_1$ & PLCβ$_3$. This result was in concordance with the hypothesis that P2Y receptors preferentially induce esophageal smooth muscle contraction in response to ATP.

These conclusions derive from the following findings:

ATP preferentially activated P2Y receptors to elicit smooth muscle contraction

P2 receptors have been classified into 2classes comprising P2X and P2Y receptors (Fredholm et al., 1994; 1997). P2X receptors are ligand-gated ionotropic channel family members that can induce transfer of calcium ion from extracellular space to cytoplasm (Fredholm et al., 1994; 1997). P2Y receptors are G-protein coupled receptor (GPCR) family that activate phospholipase C (PLC) (Fredholm et al., 1994; 1997; Lee et al., 2000). Previous study demonstrated that the co-existence of ligand-gated P2X and G protein-coupled P2Y receptors in freshly dispersed gastric smooth muscle cells and that ATP

preferentially activates P2Y receptors to elicit Ca^{2+} mobilization and muscle contraction (Murthy and Makhlouf, 1998). Contraction induced by 10 μM ATP was abolished by only the Ca^{2+} channel blocker, nifedipinethat can inhibit P2X receptor (Murthy and Makhlouf, 1998). P2Y receptors trigger release of Ca^{2+} from endoplasmic reticulum that leads to increase of calcium concentration. From our findings intracellular calcium concentration plays a key role in smooth muscle contraction induced by P2 receptors. It seems that contraction was not inhibited by Gαi antibody because increase of calcium concentration induced by P2Y receptors is negligible compared to that of P2X receptors when treated with high concentration of ATP. In contrast, contraction induced by 0.1 μM ATP, 10 μM UTP and 0.1 μM UTP wasinhibited by PTX or GDPβS.

The ATP-induced contraction signaling was concentration-dependent. Response to UTP and lower concentration of ATP was mediated by PTX-sensitive G protein and PTX-insensitive G protein but contraction induced by higher concentration of ATP occurred via ligand-gated calcium channel. Therefore, these results suggested that the purine nucleotide, ATP released from nerves as extracellular transmitters preferentially activate P2Y receptors to elicit smooth muscle contraction.

P2Y receptors were linked to Gαi$_3$ and Gαq proteins activating PLCβ$_1$ and PLCβ$_3$

G proteins, also known as guanine nucleotide-binding proteins, are a family of proteins involved in transmitting signals from a variety of intracellular signaling such as activation of PLCβ that can cleave PIP$_2$ into IP$_3$ and DAG (Gilman, 1987; Lechleiter et al., 1990; Somlyo and Somlyo, 1994). Contraction mediated by P2Y receptors was decreased by Gαi$_3$ and Gαq specific anti-
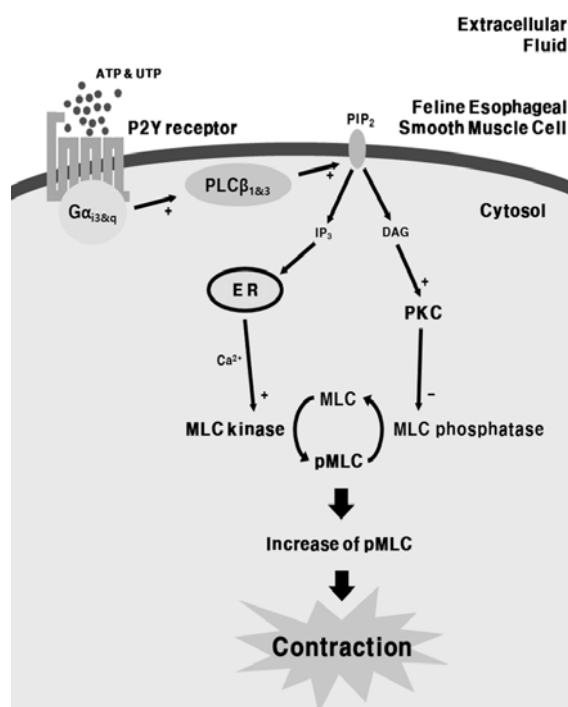
Fig. 8. Diagram of the preferential signaling mechanism mediated by ATP and UTP in feline esophageal smooth muscle cells. ATP-induced contraction of feline esophageal smooth muscle cells was preferentially mediated by P2Y receptors coupled to $G\alpha_{i3}$ and $G\alpha_q$ proteins, which activate $PLC\beta_1$ and $PLC\beta_3$. Subsequent, increased intracellular Ca^{2+} by IP_3 and activated PKC by DAG triggered stimulation of MLC kinase and inhibition of MLC phosphatase, respectively. Finally, increased $pMLC_{20}$ generated smooth muscle contraction of the feline esophagus.

bodies and by $PLC\beta_1$ and $PLC\beta_3$ antibodies. Thus, P2Y receptors are involved in $G\alpha_{i3}$ and $G\alpha_q$ proteins triggering activation of $PLC\beta_1$ and $PLC\beta_3$.

Contraction mediated by P2Y receptors was involved in IP_3-dependent and PKC-dependent pathway

PLC catalysesthegeneration ofinositol triphosphate (IP_3) and diacylglycerol (DAG). IP_3 then binds to IP_3 receptors, particularly Ca^{2+} channels in the smooth endoplasmic reticulum (ER). This induces increased cytoplasmic concentration of Ca^{2+}, causing a cascade of intracellular changes and the activation of calmodulin and MLC kinase (MLCK)-dependent phosphorylation of MLC_{20} (Billah and Anthes, 1990; Sohn et al., 1993; 1995a; Somlyo and Somlyo, 1994). DAG functions as a membrane bound second messenger signaling lipid. In addition, calcium and DAG together activate protein kinase C (PKC), which phosphorylates other molecules, leading to altered cellular activity such as regulating myosin light chain phosphatase (MLCP) that can dephosphorylate pMLC (Puetz et al., 2009).

Contraction in response to 0.1 μM ATP, 0.1 μM UTP, and 10 μM UTP, but not 10 μM ATP,was significantly inhibited by PLC inhibitor U73122, PKC inhibitor chelerythrine, and MLC kinase inhibitor ML-9. The results showed that P2Y receptors mediate esophageal smooth muscle contraction through the IP_3-dependent and PKC-dependent pathways.

P2Y receptors triggered contraction by MLC_{20} phosphorylation

The primary mechanism of smooth muscle contraction is phosphorylation of the 20 kDa myosin light chain (MLC_{20}) by a MLC_{20} kinase that is activated by Ca-calmodulin (Ikebeet al., 1987). Relaxation, then, is primarily the result of dephosphorylation of MLC_{20} by MLC phosphatases (Bialojan et al., 1987; Haeberle et al., 1985). Phosphorylation of MLC_{20} was increased by activating P2Y receptors. Our results demonstrated that P2Y receptors modulate smooth muscle contraction through myosin light chain phosphorylation, caused by the IP_3-dependent and PKC-dependent pathways.

In conclusion, ATP- and UTP-induced contraction of feline esophageal smooth muscle cells was preferentially mediated by P2Y receptors coupled to $G\alpha_{i3}$ and $G\alpha_q$ proteins, which activate $PLC\beta_1$ and $PLC\beta_3$. Subsequently, increased intracellular Ca^{2+} by IP_3 and activated PKC by DAG triggered stimulation of MLC kinase and inhibition of MLC phosphatase, respectively. Finally, increased $pMLC_{20}$ generated smooth muscle contraction of the feline esophagus (Fig. 8).

In time course ATP- and UTP-induced contraction curves, the contraction completely disappeared 300 s after ATP and UTP treatment. This pattern was different from other neurotransmitters that induce sustained contraction. It can be hypothesized that P2 receptor activation is associated with lower esophageal sphincter (LES) relaxation induced by GERD or other esophageal diseases. Further research is required to investigate P2 receptor induced-signaling of contraction in esophageal smooth muscle under pathophysiologicalor diseased conditions.

In summary (Fig. 8), esophageal contraction induced by ATP and UTP was preferentially mediated by P2Y receptors coupled to $G\alpha_{i3}$ and $G\alpha_q$ proteins, which activate $PLC\beta_1$ and $PLC\beta_3$. Subsequently, increased intracellular Ca^{2+} and activated PKC triggered stimulation of MLC kinase and inhibition of MLC phosphatase,and increased $pMLC_{20}$ generated esophageal contraction.

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