Korean J Physiol Pharmacol
Vol 14: 311−316, October, 2010
DOI: 10.4196/kjpp.2010.14.5.311

P2X and P2Y Receptors Mediate Contraction Induced by Electrical Field Stimulation in Feline Esophageal Smooth Muscle

Young Rae Cho, Hyeon Soon Jang, Won Kim, Sun Young Park, and Uy Dong Sohn

Department of Pharmacology, College of Pharmacy, Chung-Ang University, Seoul 156-756, Korea

It is well-known that electrical field stimulation (EFS)-induced contraction is mediated by a cholinergic mechanism and other neurotransmitters. NO, ATP, calcitonin gene-related peptide (CGRP), and substance P are released by EFS. To investigate the purinergic mechanism involved in the EFS-induced contraction, purinegic receptors antagonists were used. Suramine, a non-selective P2 receptor antagonist, reduced the contraction induced by EFS. NF023 (10^{-7}∼10^{-4} M), a selective P2X antagonist, inhibited the contraction evoked by EFS. Reactive blue (10^{-6}∼10^{-4} M), selective P2Y antagonist, also blocked the contraction in a dose-dependent manner. In addition, P2X agonist α,β-methylene 5'-adenosine triphosphate ($\alpha\beta$MeATP, 10^{-7}∼10^{-5} M) potentiated EFS-induced contraction in a dose-dependent manner. P2Y agonist adenosine 5'-[β-thio]diphosphate trilithium salt (ADPβS, 10^{-7}∼10^{-5} M) also potentiated EFS-induced contractions in a dose-dependent manner. Ecto-ATPase activator apyrase (5 and 10 U/ml) reduced EFS-induced contractions. Inversely, 6-N,*N*-diethyl-D-β,γ- dibromomethylene 5'-triphosphate triammonium (ARL 67156, 10^{-4} M) increased EFS-induced contraction. These data suggest that endogenous ATP plays a role in EFS-induced contractions which are mediated through both P2X-receptors and P2Y-receptors stimulation in cat esophageal smooth muscle.

Key Words: Electrical field stimulation, Smooth muscle, G protein, P2 receptor, ATP, Calcium

INTRODUCTION

Smooth muscle distributed in the gastrointestinal tract is under the control of the autonomic nervous system, and contraction or relaxation of the muscle plays an important physiological role in the motility of digestion. Recent physiological, pharmacological, and histochemical investigations indicate that neurotransmitters other than acetylcholine or noradrenaline are involved in peripheral autonomic neuro-effector transmission, and these neurotransmitters are generally termed non-adrenergic, non-cholinergic (NANC) neurotransmitters [1]. Putative NANC mediators include the peptides calcitonin gene-related peptide (CGRP) and substance P, and non-peptide mediators including nitric oxide (NO) and ATP [2]. We have investigated the components of EFS-induced responses and the intracellular factors that mediate electrically stimulated responses in feline distal esophageal smooth muscles. Muscle contractions were recorded using an isometric force transducer. Low-frequency EFS induced only off contraction but induced on contraction in the presence of N^G-nitro-L-arginine methyl ester, suggesting that NO acts as an inhibitory mediator in smooth muscle [3].

Extracellular purines (adenosine, ADP, and ATP) and pyrimidines (UDP and UTP) are important signaling molecules that mediate diverse biological effects via cell-surface receptors termed purine receptors. There are two main families of purine receptors, adenosine or P1 receptors, and P2 receptors, recognizing primarily ATP, ADP, UTP and UDP. Adenosine/P1 receptors have been further subdivided, according to convergent molecular, biochemical, and pharmacological evidence, into four subtypes, A_1, A_{2A}, A_{2B}, and A_3, all of which couple to G proteins. Based on differences in molecular structure and signal transduction mechanisms, P2 receptors divide naturally into two families of ligand-gated ion channels and G protein-coupled receptors termed P2X and P2Y receptors [4,5]. Recently, seven mammalian P2X receptors ($P2X_{1-7}$) and seven mammalian P2Y receptors ($P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$, $P2Y_{11}$ $P2Y_{12}$, $P2Y_{13}$) have been cloned, characterized, and accepted as valid members of the P2 receptor family [6].

$P2X_1$ is the main P2X receptor subtype expressed in visceral and vascular smooth muscle [7], whereas $P2X_2$ and $P2X_3$ are the main receptor subtypes expressed in peripheral sensory ganglia [8,9]. Both $P2X_1$ and $P2X_3$ receptors have high affinity for ATP and α,β-meATP, and are rapidly desensitized [9,10]. $P2X_2$, $P2X_4$, and $P2X_6$ receptors are the predominant receptor subtypes expressed in the adult brain where they are present in various heteromeric combinations; these receptor subtypes exhibit lower affinity for

Received August 28, 2010, Revised October 7, 2010,
Accepted October 20, 2010

Corresponding to: Uy Dong Sohn, Department of Pharmacology, College of Pharmacy, Chung-Ang University, 221, Heukseok-dong, Dongjak-gu, Seoul 156-756, Korea. (Tel) 82-2-820-5614, (Fax) 82-2-826-8752, (E-mail) udsohn@cau.ac.kr

ABBREVIATIONS: EFS, electrical field stimulation; CGRP, calcitonin gene-related peptide; $\alpha\beta$MeATP, $\alpha\beta$-methylene 5'-adenosine triphosphate; a ADPβS, denosine 5'-[β-thio]diphosphate trilithium salt; NANC, non-adrenergic, non-cholinergic; NO, nitric oxide.

ATP, are insensitive to α,β-meATP, and are not readily desensitized [8,11,12]. Insensitivity to α,β-meATP restricts the usefulness of this analog as a radioligand for all but the $P2X_1$ and $P2X_3$ receptor subtypes [10].

P2Y receptors exhibit variable affinity for purine and pyrimidine nucleotides. $P2Y_1$ are purinoceptors and are adenine nucleotide-specific [10,13], whereas $P2Y_2$ receptors have equal affinity for adenosine and uridine nucleotide triphosphates (UTP≥ATP) [14,15]. $P2Y_4$ and $P2Y_6$ are pyrimindinoceptors; $P2Y_4$ is UTP selective whereas $P2Y_6$ is UDP selective [15,16]. The functional status of $P2Y_5$, which has low homology to other P2Y receptors, has not been resolved [16] while the $P2Y_7$ receptor has now been identified as the leukotriene B_4 receptor [17]. P2Y receptors are variously coupled to pertussis toxin-sensitive and -insensitive G proteins which activate or inhibit various effectors enzymes including phospholipase C-β (PLC-β), phospholipase D [18,19], phospholipase A_2 [20], and adenylyl cyclase [21,22].

Adenosine and ATP receptors have been suggested to play important roles in the modulation of motility in the gastrointestinal tract. Adenosine can directly activate receptors located on smooth muscle [23,24] or act prejunctionally, suppressing the release of excitatory neurotransmitters such as acetylcholine and substance P [25,26]. ATP is a neurotransmitter or co-transmitter in the central and peripheral nervous systems. In 1970, Burnstock and colleagues proposed that ATP was a transmitter involved in NANC nerve-mediated responses of smooth muscle in the gut [1]. Since then, evidence has accumulated in support of the hypothesis that ATP is a NANC transmitter in the enteric nerve system. In most species, ATP activates P2 receptors on smooth muscle to produce relaxation or contraction in the gastrointestinal tract [27].

In vitro studies of human esophageal strips using direct activation of enteric motor neurons by electrical field stimulation (EFS) have shown that lower esophageal smooth muscle relaxation is mainly caused by NO released from inhibitory enteric motor neurons, whereas esophageal contraction is mainly caused by acetylcholine released from excitatory enteric motor neurons acting on muscarinic receptors located in the smooth muscle [3, 28-30]. Except for acetylcholine, other neurotransmitters influence EFS-induced contraction in esophageal smooth muscle. In this study, we examined the role of P2X and P2Y-purinoceptors in EFS-induced contraction using cat esophageal smooth muscle.

METHODS

Animals

Adult cats of either sex weighing between 2.5 and 4 kg were used in this study. The cats were anesthetized with ketamine (50 mg/ml/kg) and killed by bleeding. The chest and abdomen were opened with a mid-line incision exposing the esophagus and stomach. The esophagus and stomach were removed together and pinned onto a wax block in their *in vivo* dimensions and orientation. The esophagus and stomach were opened along the lesser curvature. The location of the squamo-columnar junction was identified and the mucosa was peeled away. The high-pressure zone is characterized by a visible thickening of the circular muscle layer in correspondence of the squamo-columnar junction, and is immediately proximal to the sling fibers of the stomach. We have previously shown that a 5～8 mm band of tissue coinciding with the thickened area constitutes the LES. Esophageal body was selected from a region extending from 1 to 3 cm above the LES. This constituted the most distal portion of the esophagus and comprised ～15% of the length of the whole esophagus.

Tissue bath studies

Transversely oriented muscle strips measuring 2 mm wide and 7 mm long were taken from esophageal body. Such preparations were then cut into 4～5 minor strips and silk-ligatures were tied at both ends. The muscle strips were mounted in separated 1 ml muscle chambers. One wire was fixed and the other attached to a force transducer (FT03 Grass Instruments Co., Quincy, MA, U.S.A.). Changes in isometric force were recorded on a polygraph (Grass model 79).

The strips were initially stretched to 2 g to bring them to near conditions of optimal force development and were equilibrated for 2 hr while being perfused continuously with oxygenated Krebs. During this time the tension in the muscle strips decreased rapidly and stabilized at less than 0.5 g. The solution was equilibrated with a gas mixture containing 95% O_2 and 5% CO_2 at pH 7.4 and 37°C.

Electrical field stimulation

The strips were stimulated with pulse trains of 80 V in amplitude and 10 s in duration, with a pulse duration of 1 ms at a frequency of 4 Hz using a stimulator (Model S 88; Grass Instruments) through platinum wire electrodes placed longitudinally on either side of the strips. After a stable resting tone of muscle strips was obtained, frequency-response relationships (4 Hz) were constructed, and the rings were washed three times and allowed to equilibrate for 30 min after EFS to permit the strips to recover completely from the contractile responses to EFS.

Assessment of drug responses

The control responses of EFS on the resting tension of circular smooth muscle were investigated. To blocking adrenergic response, all muscle strips are pretreated with guanethidine (1 μM) before 15 min every experiments. To determine whether P2 purinoceptors were involved in EFS-induced contraction, strips were pretreated with suramine (a nonselective P2 receptor antagonist) for 10 min at each concentration (10^{-6}～10^{-4} M). To assess the effects of P2X purinoceptor on the contraction of circular smooth muscle, the contraction amplitude was measured in muscle strips which were exposed to α,β-methylene ATP (α,β-meATP, P2X agonist) and 8,8'-[Carbonylbis(imino-3, 1-phenylenecarbonyl-imino)]-bis-(1,3,5-naphthalenetrisulphonate; NF023; a P2X antagonist). In addition, to determine whether P2Y purinoceptors were involved in the response to EFS, strips were pretreated with adenosine 5'-O-(2-thiodiphosphate; ADPβS; a P2Y agonist) and Reactive blue (a P2Y antagonist). To investigate the effect of ecto-ATPase on the EFS-induced contraction, strips were pretreated with apyrase (exogenous ecto-ATPase, 5 and 10 U/ml) for 10 min and 6-N,N-diethyl-D-β,γ-dibromomethylene 5'-triphosphate triammonium (ARL 67156, an ecto-ATPase inhibitor) for 15 min.

Solutions and drugs

Tissues were maintained in Krebs buffer of the following composition [19]: NaCl 116.6 mM, $NaHCO_3$ 21.9 mM, NaH_2PO_4 1.2 mM, KCl 3.4 mM, $CaCl_2$ 2.5 mM, glucose 5.4 mM and $MgCl_2$ 1.2 Mm. α,β-meATP, ADPβS, NF023, ARL 67156, apyrase, suramine, and guanethidine; all reagents were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A). Reactive blue was purchased from Tocris Cookson Ltd. (Langford, UK). The solutions were prepared on the day of experimentation. Doses of all compounds were given in molar concentrations and are referred to their final concentration in the organ bath.

Data analysis

The amplitude of the contraction induced by electrical field stimulation was expressed as gram (g). Data are expressed as means±S.E.M. of different experiments. Student's *t*-test for grouped data was used to determine statistical significance with $p<0.05$.

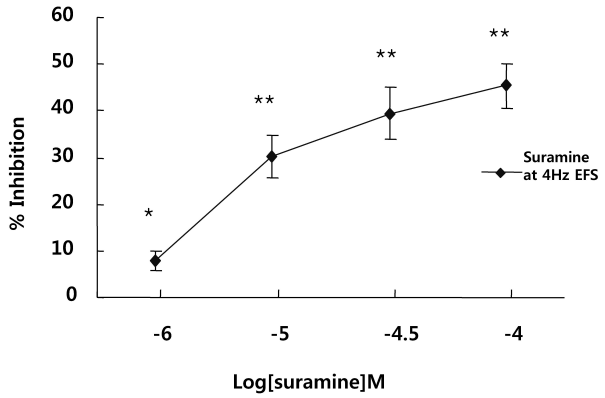

Fig. 1. Effect of the P2 receptor antagonist on the response of EFS-induced contraction. Suramine (a P2 receptor antagonist) inhibited EFS-induced contraction in a concentration-dependent manner, suggesting that P2 receptors participate in muscle contraction in response to EFS. Values are expressed as means± S.E.M. *$p<0.05$, **$p<0.01$ versus the control.

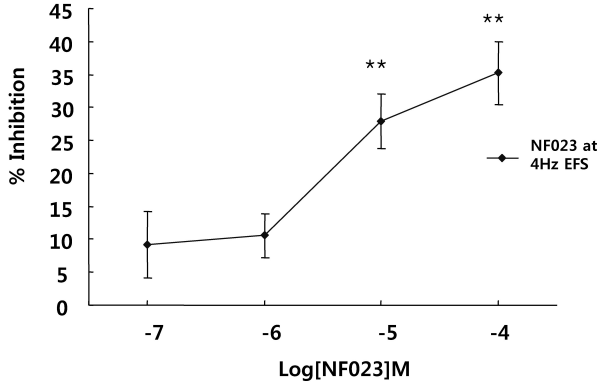

Fig. 2. Effect of the P2X receptor antagonist on the response of EFS-induced contraction. NF023 (a P2X receptor antagonist) inhibited EFS-induced contraction in a concentration-dependent manner, suggesting that P2X receptors participate in muscle contraction in response to EFS. Values are expressed as means± S.E.M. **$p<0.01$ versus the control.

RESULTS

Effects of P2-receptor antagonist on EFS-induced contraction

To test whether the P2 receptor may mediate EFS-induced contraction, muscle strips were pretreated to suramine (a nonselective P2 receptor antagonist, $10^{-6}\sim10^{-4}$ M) for 10 min before electrical stimulation. Suramine decreased the amplitude of EFS-induced contractions in a concentration-dependent manner. Contraction was inhibited by 45% at 10^{-4} M (n=5, $p<0.01$) (Fig. 1).

Effect of P2X antagonist on EFS-induced contraction

To test the role of the P2X receptor in EFS-induced contraction, muscle strips were pretreated with NF 023, a P2X receptors antagonist, for 10 min before electrical stimulation. NF 023 decreased contraction in a concentration-dependent manner. Contraction was inhibited by 35% at 10^{-4} M (n=5, $p<0.01$) (Fig. 2).

Effects of α,β-meATP on EFS-induced contraction

To test whether the P2X-receptor was involved in EFS-induced muscle contraction, muscle strips were pretreated with α,β-meATP (a P2X agonist of mainly $P2X_1$, $10^{-7}\sim10^{-5}$ M) for 5 min before electrical stimulation. α,β-meATP potentiated EFS-induced contraction in a concentration-dependent manner in the range $10^{-7}\sim10^{-5}$ M. Contraction was increased by 156% at 10^{-5} M (n=5, $p<0.01$) (Fig. 3).

Effects of P2Y antagonist on EFS-induced contraction

To test the role of the P2Y receptor in EFS-induced contraction, muscle strips were pretreated with Reactive blue for 10 min. Reactive blue, a P2Y receptor antagonist, decreased contraction in a concentration-dependent manner.

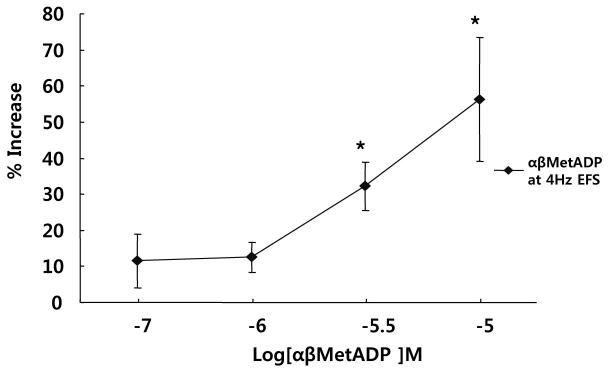

Fig. 3. Effect of the P2X receptor agonist on the response of EFS-induced contraction. α,β-meATP, P2X receptor agonist, potentiated EFS-induced contractions, suggesting that EFS-induced contraction involves P2X receptors, mainly $P2X_1$. Values are expressed as means±S.E.M. *$p<0.05$ versus the control.

Contraction was inhibited by 57% at 10^{-4} M (n=4, $p<0.01$) (Fig. 4).

Effects of P2Y receptor agonist on EFS-induced contraction

To test whether the P2Y receptor was involved in the contractions, muscle strips were exposed to ADPβS (10^{-8}~10^{-5} M) for 5 min before electrical stimulation. ADPβS potentiated EFS-induced contraction in a concentration-dependent manner in the range of 10^{-8}~10^{-5} M. Contraction was increased by 154% at 10^{-5} M (n=4, $p<0.01$) (Fig. 5).

Effects of apyrase on EFS-induced contraction

To investigate the endogenous ATP release from nerve terminals via EFS, muscle strips were pretreated with the ecto-ATPase activator apyrase for 20 min. Apyrase (5 and 10 U/ml) reduced EFS-induced contraction. Contraction was decreased by 17% at 10 U/ml (n=4, $p<0.05$) (Fig. 6). 1 Unit means 1.0 μmol of inorganic phosphate liberated from ATP or ADP per min at pH 6.5 at 30°C.

Effects of ARL 67156 on EFS-induced contraction

To confirm endogenous ATP release from nerve terminals via EFS, the ecto-ATPase inhibitor ARL 67156 was used. Muscle strips were pretreated with ARL 67156 (10^{-6}~10^{-4} M) for 10 min before EFS induction. ARL 67156 increased EFS-induced contraction by 118% at 10^{-4} M (n=4, $p<0.05$) (Fig. 7).

DISCUSSION

In humans and cats, atropine blocks peristalsis in the distal esophagus, indicating a prominent role for muscarinic cholinergic excitation and contraction of esophageal smooth muscle [28]. The activation of muscarinic receptors controls contraction of smooth muscle in part by regulating the concentration of cytosolic free Ca^{2+} [31]. By EFS, ace-

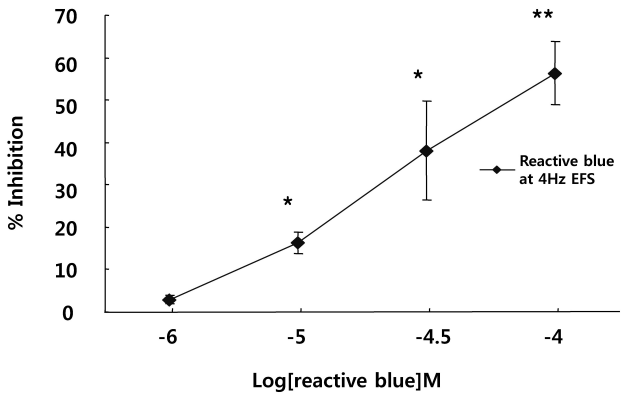

Fig. 4. Effect of the P2Y receptor antagonist on the response of EFS-induced contraction. Reactive blue (a P2Y receptor antagonist) inhibited EFS-induced contraction in a concentration-dependent manner, suggesting that P2Y receptors participate in muscle contraction in response to EFS. Values are expressed as means±S.E.M. *$p<0.05$, **$p<0.01$ versus the control.

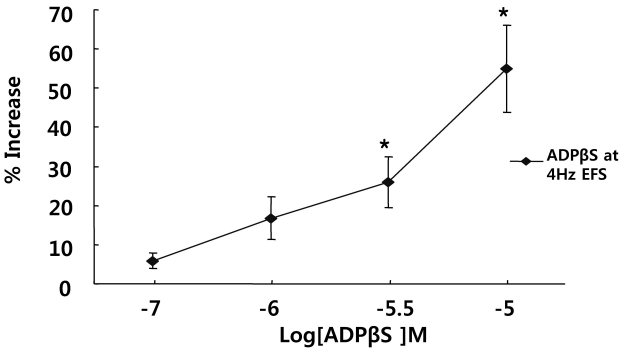

Fig. 5. Effect of the P2Y receptor agonist on the response of EFS-induced contraction. ADPβS, a P2Y receptor agonist, potentiated EFS-induced contractions, suggesting EFS-induced contraction involves P2Y receptors, mainly P2Y1. Values are expressed as means±S.E.M. *$p<0.05$ versus the control.

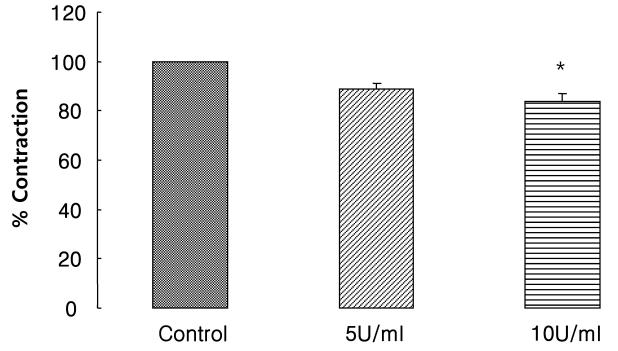

Fig. 6. Effect of apyrase on the response of EFS-induced contraction. Apyrase (an ecto-ATPase activator, n=4) inhibited EFS-induced contractions, suggesting endogenous ATP is released upon EFS. Values are expressed as means±S.E.M. *$p<0.05$ versus the control.

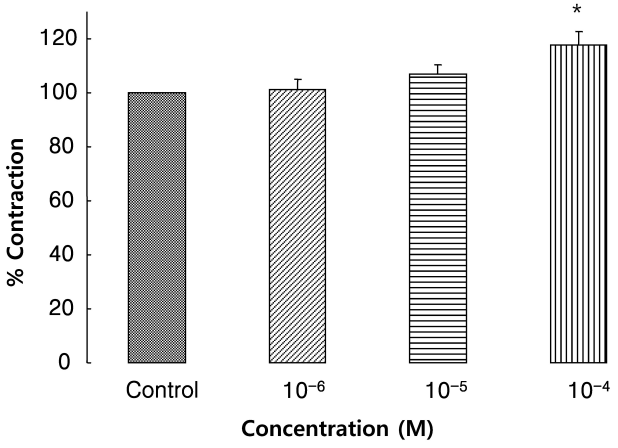

Fig. 7. Effect of ARL67156 on the response of EFS-induced contraction. ARL 67156 (an ecto-ATPase inhibitor, n=4) increased EFS-induced contractions, suggesting endogenous ATP is released upon EFS. Values are expressed as means±S.E.M. *$p<0.05$ versus the control.

tylcholine is released and mediated via M2 muscarinic receptors. M2 muscarinic receptors are linked mostly to G_{i3} because Ach-induced contraction of permeable cells is inhibited by antibodies against the α-subunit of G13 [32]. In the muscle response to EFS, except acethycholine, purinergic mechanisms are involved in esophageal contraction.

This study shows that cat esophageal smooth muscle involved P2X and P2Y receptors, and endogenous ATP released by EFS or ATP analogue in cat esophagus caused smooth muscle contraction mediated via P2X and P2Y receptors. EFS-induced contraction was frequency-dependent and tetrodotoxin-sensitive. Contractile response induced by EFS was observed in the presence of guanethidine (10^{-6} M), while EFS-induced contraction was inhibited by the non-selective P2 receptor antagonist suramine. These data suggest that EFS stimulates the release of ATP from nerve terminals, probably sympathetic nerve terminals. This result seems to be supported by the finding that the ecto-ATPase activator apyrase reduced EFS-induced contraction. Furthermore, the ecto-ATPase inhibitor ARL 67156 increased EFS-induced contraction.

There is evidence that mammals have purinoceptors in the gastrointestinal tract. The mouse stomach fundus, duodenum, ileum, and colon have purinoceptors. The predominant effect of stimulation of these receptors is relaxation with the exception of colon longitudinal muscle which also possesses a contractile P2 receptor [33]. In the rabbit, gastric fundus co-expresses P2X and P2Y receptors in dispersed smooth muscle cells. In the rabbit dispersed gastric smooth cell, ATP preferentially activates P2Y receptors to elicit Ca^{2+} mobilization and muscle contraction [34]. In the guinea-pig, gastric antral circular muscles have P2 receptors which cause contraction via P2Y by ATP and its analogues [35].

Our data suggest that in cat esophageal smooth muscle, the purinergic mechanism involves P2X receptors. α,β-metATP potentiated EFS-induced contraction in a concentration-dependent manner. α,β-metATP was the most useful agonist acting on P2X receptors (specifically at $P2X_1$, $P2X_3$) and generally inactivated P2Y receptors. Also, the P2X antagonist NF 023 inhibited EFS-induced contractions in a concentration-dependent manner. NF 023 is a suramin-based compound which is moderately selective as an antagonist of P2X receptors. NF 023 is about 30-fold selective for $P2X_1$-like receptors in the rat deferens versus P2Y1-like receptors in the guinea-pig taenia coli [36].

ADPβS, a potent P2Y receptor agonist, potentiated EFS-induced contractions in a concentration-dependent manner. Reactive blue, selective P2Y antagonist, inhibited EFS-induced contraction in a concentration-dependent manner. These data suggest that cat esophageal smooth muscle has P2Y receptors and the predominant effect of stimulation is contraction.

Generally, P2X receptors mediate the rapid non-selective passage of cation (Na^+, K^+, Ca^{2+}) across the cell membrane resulting in an increase in intracellular Ca^{2+} and depolarization [37]. P2X receptors selectively activated by α,β-metATP mediate Ca^{2+} influx via dihydropyridine-sensitive, voltage-gated Ca^{2+} channels; the Ca^{2+} channels are activated by depolarization of the plasma membrane that result from the opening of ligand-gated cation P2X receptors/channel [12]. P2Y receptors, which promote phospholipase C-catalyzed generation of inositol phosphate and subsequent mobilization of intracellular calcium, are receptors for endogenous ADP and ATP, with ADP being the most potent natural agonist [38].

In summary, these studies suggested that P2 purinoceptors are involved in EFS-induced contraction. Upon EFS, endogenous ATP or ATP-related purine analogues are released which stimulate both P2X receptors and P2Y receptors. However, the subtype of the receptors that mainly mediate contraction is not known, because there are presently few selective agonists and antagonists that clearly discriminate between families of P2X and P2Y receptors, or between subtypes of receptors within each family. In addition, immunohistochemical and biochemical assays are needed to confirm that P2X and P2Y receptors exist.

ACKNOWLEDGEMENTS

This research was supported by Chung-Ang University Research Scholarship Grants in 2009-2010.

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