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MLCK and PKC Involvements via Gi and Rho A Protein in Contraction by the Electrical Field Stimulation in Feline Esophageal Smooth Muscle

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We have shown that myosin light chain kinase (MLCK) was required for the off-contraction in response to the electrical field stimulation (EFS) of feline esophageal smooth muscle. In this study, we investigated whether protein kinase C (PKC) may require the on-contraction in response to EFS using feline esophageal smooth muscle. The contractions were recorded using an isometric force transducer. On-contraction occurred in the presence of N^G-nitro-L-arginine methyl ester (L-NAME), suggesting that nitric oxide acts as an inhibitory mediator in smooth muscle. The excitatory composition of both contractions was cholinergic dependent which was blocked by tetrodotoxin or atropine. The on-contraction was abolished in Ca^{2+}-free buffer but reappeared in normal Ca^{2+}-containing buffer indicating that the contraction was Ca^{2+} dependent. 4-aminopyridine (4-AP), voltage-dependent K^+ channel blocker, significantly enhanced on-contraction. Aluminum fluoride (a G-protein activator) increased on-contraction. Pertussis toxin (a G_i inactivator) and C3 exoenzyme (a rhoA inactivator) significantly decreased on-contraction suggesting that Gi or rhoA protein may be related with Ca^{2+} and K^+ channel. ML-9, a MLCK inhibitor, significantly inhibited on-contraction, and chelerythrine (PKC inhibitor) affected on the contraction. These results suggest that endogenous cholinergic contractions activated directly by low-frequency EFS may be mediated by Ca^{2+}, and G proteins, such as Gi and rhoA, which resulted in the activation of MLCK, and PKC to produce the contraction in feline distal esophageal smooth muscle.

Key Words: Electrical field stimulation, Smooth muscle, Ca^{2+}, K^+, G protein, On contraction, Esophagus

INTRODUCTION

Nitric oxide (NO) is an important mediator of non-adrenergic, noncholinergic (NANC) inhibitory nerve effects in the human esophagus and lower esophageal sphincter (LES), and its influence appears to increase the distally functional gradient in the esophagus [1,2]. Acetylcholine (ACh) is an excitatory neurotransmitter that regulates esophageal motor function, although there are regional and species differences in the relative contributions made by cholinergic mechanisms to esophageal peristalsis [3-5], and stimulation of nerves in the esophagus induces NO-mediated hyperpolarization and relaxation [6,7].

The mechanical responses induced by EFS in esophageal smooth muscle are referred to as on- and off-contractions. Off-contraction is a response that occurs after the cessation of EFS, whereas on-contraction occurs from the initiation of the stimulus due to the abolishment of inhibitory mechanism [8-10]. The incidence of various patterns of responses by EFS in esophageal smooth muscle *in vitro*, which are composed of on- and off-contractions, differ at esophageal sites and are dependent on stimulation frequencies. These events result from the influence of the integration of intramural excitatory cholinergic and NANC inhibitory nitrergic nerves, which innervate esophageal smooth muscle [4,11]. In addition, ion currents generated via 4-AP-sensitive voltage-dependent K^+ channels in muscle might participate in EFS-induced responses [12]. In addition, EFS-induced those responses can be mediated by the activations Gi or Gs proteins, and that L-type Ca^{2+} channel may be activated by G-protein alpha subunits, indicating that K^+ and Ca^{2+} channel involve in the depolarization of esophageal smooth muscle [13]. Thus, studies are required to characterize the

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ABBREVIATIONS: MLCK, myosin light chain kinase; EFS, electrical field stimulation; L-NAME, N^G-nitro-L-arginine methyl ester; 4-AP, 4-aminopyridine; NO, nitric oxide; LES, lower esophageal sphincter; ACh, acetylcholine; PTX, pertussis toxin; AlF, aluminum fluoride.

physiological regulation of Ca^{2+} channel and to investigate the effects of other K^+ channels on EFS-induced on and off contractions.

In previous study and other experiments, myosin light chain kinase (MLCK) or protein kinase C (PKC) are involved in the EFS in some smooth muscles [14-17]. Nerve stimulation and treatments with exogenous transmitter substances involve different contraction mechanisms [18], thus factors released from unstimulated nerve endings not stimulated by EFS are not involved in the myogenic response. In contrast, factors released upon stimulation of nerve endings can modulate the amplitude of the myogenic response. The mechanism of exogenous ACh-induced contraction in feline esophageal smooth muscle is mediated by M_2 muscarinic receptors linked to G_{i3}-type G proteins, which activate PKC ε via diacylglycerol produced by phospholipase C and phospholipase D [19]. On the other hand, the intracellular factors that mediate the contractions induced by the direct activations of nerves in esophagus have not been studied yet.

Thus it was aimed to define the mechanism of EFS-induced responses and to identify the intracellular factors that mediate excitatory contraction during the EFS-induced responses in feline esophageal smooth muscle using external Ca^{2+}, inhibitor of MLCK, PKC inhibitor, G-protein inactivators, and K^+ channel blocker. We compared on-contraction with off-contraction [16] and the EFS-induced contraction was comparable to results obtained with the known mechanism of contraction induced by ACh.

METHODS

Solutions and drugs

All compounds were dissolved in H_2O, and it was confirmed that the vehicles of all compounds did not affect the basal states of smooth muscle strips. Solutions were prepared on the days of experiments. The doses of all compounds are reported in moles in organ baths. Tissues were maintained in normal Ca^{2+}-containing Krebs buffer of the following composition (mM): NaCl 116.6, $NaHCO_3$ 21.9, NaH_2PO_4 1.2, KCl 3.4, $CaCl_2$ 2.5, glucose 5.4, and $MgCl_2$ 1.2. The following were purchased from Sigma Chemical Co. (St. Louis, MO, USA); pertussis toxin (PTX), C3 exoenzyme, ML-9 [1-(5-chloronaphthalene-1-sulfonyl)-1H-hexahydro-1,4-diazepine hydrochloride], N^G-nitro-L-arginine methyl ester (L-NAME), 4-aminopyridine (4-AP), chelerythrine chloride and sodium fluoride. Tetrodotoxin citrate was purchased from Tocris Cookson Ltd. (Langford, UK). Atropine sulfate was purchased from Merck (Darmstadt, Germany). Aluminum fluoride (AlF) was prepared by mixing sodium fluoride and aluminum chloride at an appropriate concentration and stored in plastic bottles [20].

Preparation of isolated tissues and muscle strips

All cats were used approving the Institutional Animal Care and Use Committee at Chung Ang University in Seoul, Korea. The feline esophagus resembles that of man in terms of its anatomical structures, and is composed of proximal striated and distal smooth muscle. Adult cats of either sex weighing between 2.5 and 5 kg were used in this study. Animals were anesthetized with ketamine (50 mg/kg) and euthanized with a 25% urethane overdose (Aldrich, St. Louis, MO). In each case, the chest was opened and the entire esophagus, along with a cuff of stomach tissue, was isolated and transferred to a bath of normal Ca^{2+}-containing Krebs solution equilibrated with 95% O_2 and 5% CO_2 at 37°C and maintained at pH 7.4±0.05. After opening, the esophagus and the stomach (along the greater curvature), both were cleared of surrounding connective tissues, the LES region was defined, and all mucosa was removed by sharp dissection. Preparations of circular smooth muscle attached to longitudinal smooth muscle in the distal region (within 1~2 cm superior to the LES) were cut with razor blades held in a metal block. These preparations were then cut into 3 minor strips (2 mm wide and 10 mm long), which were tied with silk ligatures at both ends.

Measurements of muscle contraction in vitro

The esophageal smooth muscle strips, prepared as described above, were mounted in separate 1-ml volume muscle chambers as follows. One end of each strip was attached to a force-displacement transducer (FT03 Grass Instruments Co., Quincy, MA, USA), and the other end was fixed to the bottom of the muscle chamber to allow isometric tension to be continuously monitored. Changes in isometric force were recorded on a writing-ink pen polygraph (Grass model 79, Grass Instruments Co., Quincy, MA, USA). The muscle strips were initially stretched at a tension of 2 g to condition them for optimal force development, and were then equilibrated for 1 hr while being circulated continuously with oxygenated normal Ca^{2+}-containing Krebs buffer. During this time, strip tension decreased rapidly and stabilized at less than 0.5 g. The buffer solution was equilibrated and maintained in a 95% O_2 and 5% CO_2 environment at pH 7.4 and 37°C throughout the study.

Electrical field stimulation (EFS)

The muscle strips were stimulated with pulse trains of 80 V in amplitude and 10 seconds in duration, with a pulse duration of 1 millisecond at a frequency of 4 Hz using a stimulator (model S88; Grass Instruments) through platinum wire electrodes placed longitudinally on either side of the muscle strips. After a stable resting state was obtained, the frequency-response relationship (4 Hz) was constructed and the strips were washed three times and allowed to equilibrate for 1 hr after EFS to permit complete recovery before drug application. We chose a frequency of 4 Hz based on preliminary tests, because muscle contraction in response to this stimulus parameter was tetrodotoxin-sensitive and atropine-sensitive and of proper amplitude to visualize a change of contraction [16].

In this study, the contraction changed off-contraction into on-contraction, which contraction occurred during EFS in the presence of 100 μM L-NAME (NOS inhibitor). The effect of extracellular Ca^{2+} concentration on EFS-induced contraction was tested by incubating strips for 30 min with Ca^{2+}-free Krebs buffer (Ca^{2+} and 2 mM of EGTA), normal Ca^{2+}-containing Krebs buffer, or 10 mM Ca^{2+}-containing Krebs buffer. The concentrations of normal Ca^{2+} used in this study were 2.5 mM, which commonly used in physiological experiments, and 10 mM. The effect of K^+ current on EFS-induced contraction was tested using 1~2 mM 4-AP (a K^+ channel blocker). In other experiments, the roles of intracellular mediators in the contractile apparatus of EFS-induced contraction were tested using 10, 30, or 100 μM

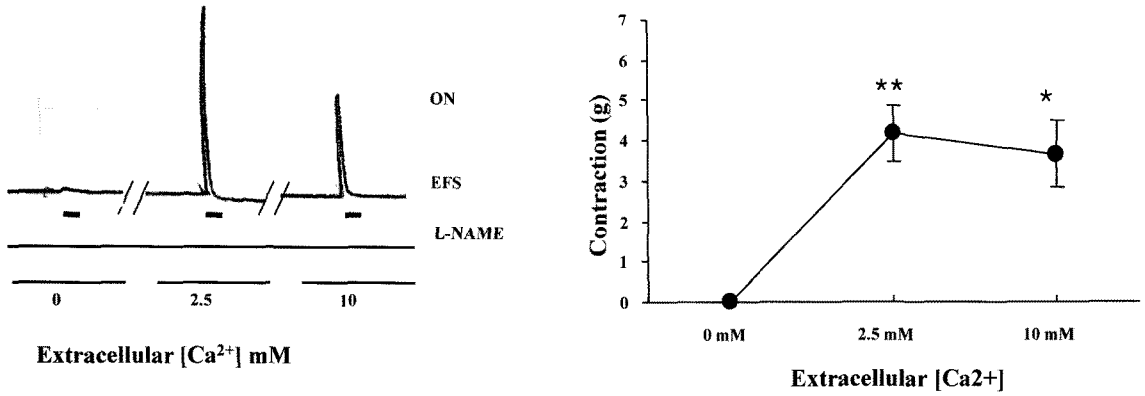

Fig. 1. Effect of external [Ca^{2+}] on the on-contraction in the presence of L-NAME by EFS. (A) Representative trace of the effects of various external [Ca^{2+}] on the on-contraction in the presence of 100 μM L-NAME (left). (B) The changes in amplitudes of the contraction affected by various external [Ca^{2+}] were expressed as gram (g) (right). Results are expressed as means±S.E. of 4 experiments. *$p<0.05$, **$p<0.01$ compared with 0 mM external [Ca^{2+}] for on-contraction in the presence of 100 μM L-NAME (ANOVA).

ML-9 (a MLCK inhibitor, which was pretreated for 20 min), 1, or 10 μM chelerythrine (a protein kinase C inhibitor, also pretreated for 20 min), 2 or 10 mM AlF (a direct G-protein activator, pretreated for 30 min), 800 ng/ml PTX (a G_i inactivator), and 300 ng/ml C3 exoenzyme (a rhoA inactivator). Muscle strips were treated with the above toxins for 3 hr prior to EFS. Muscle strips were equilibrated for 1 hr after washing them 5 times with normal Ca^{2+}-containing Krebs buffer between experiments. Each experiment was conducted on at least 3 separate occasions.

Data analysis

The amplitudes of contraction induced by EFS are expressed as grams (g) or percentages of the control. Statistical comparisons were made using the two-tailed paired Student's *t*-test for paired observations or using one-way analysis of variance (ANOVA). Data are presented as mean±SE of different experiments. Differences were considered statistically significant when p values were <0.05. Traces are representative of at least triplicate experiments from three cats using six or more muscle preparations (6~12 strips).

RESULTS

We have been shown that the strips stimulated with various frequencies (1~15 Hz), the off-contraction reached maximal contractile amplitude at 4 Hz, thus this experiments were used 4 Hz frequency as a stimulus parameter in this study. On-contraction at 4 Hz in the presence of L-NAME occurred at the initiation of the stimulus and then lasted to the endpoint of the off contraction. Additions of various concentrations of L-NAME were found to cause no proportional change in latency [16,21].

Effect of external [Ca^{2+}]

We investigated whether external [Ca^{2+}] plays a role in the on-contraction by EFS. The concentration of Ca^{2+} in the bath solution of muscle chamber was prepared to 0~10 mM, and then muscle strips were stimulated by EFS. As shown in Fig. 1, on-contraction was completely abolished in 0 mM [Ca^{2+}] of bath solution and peaked at 2.5 mM [Ca^{2+}]. At 10 mM [Ca^{2+}] the on-contraction was declined (Fig. 1). The data suggest that influx of external Ca^{2+} via Ca^{2+} channel located on membrane plays a role in occurrence of excitatory response of on-contraction by EFS.

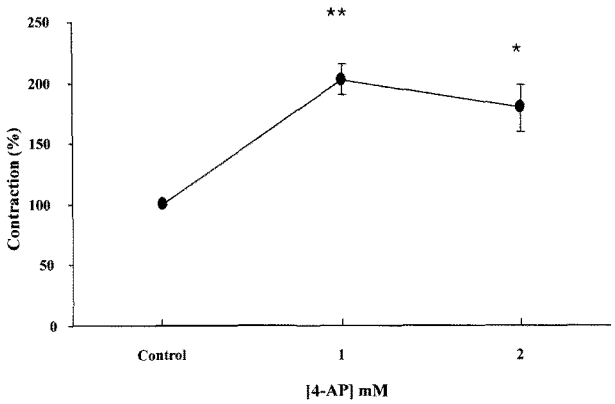

Fig. 2. Effect of 4-AP on the on-contraction in the presence of L-NAME by EFS. The changes in amplitudes of the on-contractions affected by 4-AP were expressed as % of control. The contraction was significantly augmented by 4-AP. Results are expressed as means±S.E. of 6 experiments *$p<0.05$, **$p<0.01$ compared with control of on-contraction in the presence of L-NAME (ANOVA).

Effect of 4-AP

To test whether K^+ channel plays a role in EFS-induced on-contraction in the presence of L-NAME, muscle strips were incubated with 4-AP, which is voltage-dependent K^+ channel blocker. Application of 4-AP in on-contraction in the presence of L-NAME significantly increased contractile amplitude (Fig. 2). 4-AP alone had no effect.

Effect of G protein activation

To test the effect of G protein activation in on-contraction in the presence of L-NAME by EFS, various concentration of AlF, nonspecific G protein activator, was added in muscle

chamber bath for 10 min and 30 min before EFS. AlF treatment enhanced the contraction in a concentration-dependent manner, and AlF itself had no effect (Fig. 3).

Effect of Gi or rhoA protein inactivator

Then, we investigated whether activation of Gi protein or rhoA protein mediates the on-contraction in the presence of L-NAME by EFS, PTX or/and C3 exoenzyme was added in the muscle bath solution. PTX or C3 exozyme itself had no effect. The contraction was suppressed by not only PTX but also C3 exoenzyme. Moreover, when PTX and C3 exoenzyme were simultaneously pretreated, the contraction was additively inhibited more than PTX or C3 exoenzyme alone, respectively (Fig. 4A). In addition, Y27632, inhibitor of rho kinase which is a downstream effector of rhoA protein, inhibited the contraction in a concentration-dependent manner (Fig. 4B).

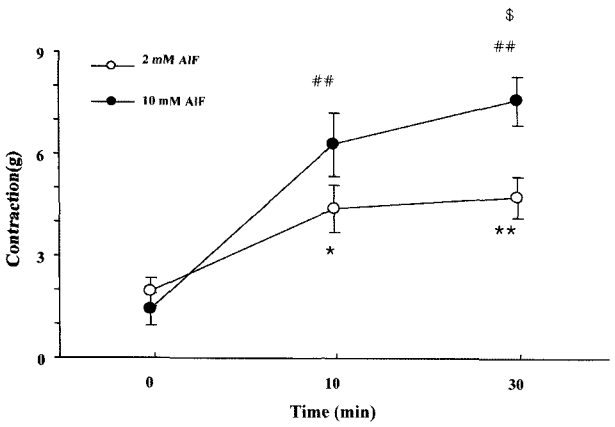

Fig. 3. Effect of AlF the on-contraction in the presence of L-NAME. Muscle strips were incubated with AlF for 10 min or 30 min in various concentrations and then changes of EFS-induced contractile response in presence of L-NAME were measured. AlF increased the on-contraction in the presence of L-NAME in a concentration-dependent manner. Results are expressed as means±S.E. of 5 experiments. *$p<0.05$, **$p<0.01$, compared with control of on-contraction in the presence of L-NAME for set of 2 mM AlF , $^{\#\#}p<0.01$ compared with control of on-contraction in the presence of L-NAME for set of 10 mM AlF(ANOVA), $^{\$}p<0.05$ compared with on-contraction at 30 min pretreatment with 2 mM of AlF (Student *t*-test).

Effect of ML-9 or chelerythrine

To test whether MLCK mediates EFS-induced on-contraction, ML-9 was added to muscle baths at various concentrations (10, 30, or 100 μM) prior to EFS stimulation, and applied ML-9 (MLCK inhibitor) and then decreased the amplitude of contraction significantly in a concentration-dependent manner (Fig. 5A and 5B). To test that PKC (Fig. 5C) may involve the contraction, chelerythrine (PKC inhibitor) was treated in the muscle bath. The on-contraction in the presence of L-NAME by EFS was inhibited by increasing concentration of ML-9 (10, 30 or 100 μM) or chelerythrine (1 or 10 μM).

DISCUSSION

In the present study, direct EFS of esophageal smooth muscle strips produced only on-contraction in the presence of L-NAME. The on-contraction occurred with stimulus start and then lasted to off-contraction finish. These results are consistent with view that EFS stimulates NO release and the inhibitory effect of released NO causes the mechanical quiescent response during EFS in terms of off-contraction [16]. In other study, released NO was determined that from myenteric neurons controls both the amplitude and the timing of off response in distal esophageal smooth muscle [21]. We also found that EFS activates excitatory cholinergic nerves and releases NO and subsequently induces off-contraction or on-contraction in the absence or presence of L-NAME. On the other hand, the higher amplitude of on-contraction than that of off-contraction in the presence of L-NAME may have been due to a blockade of

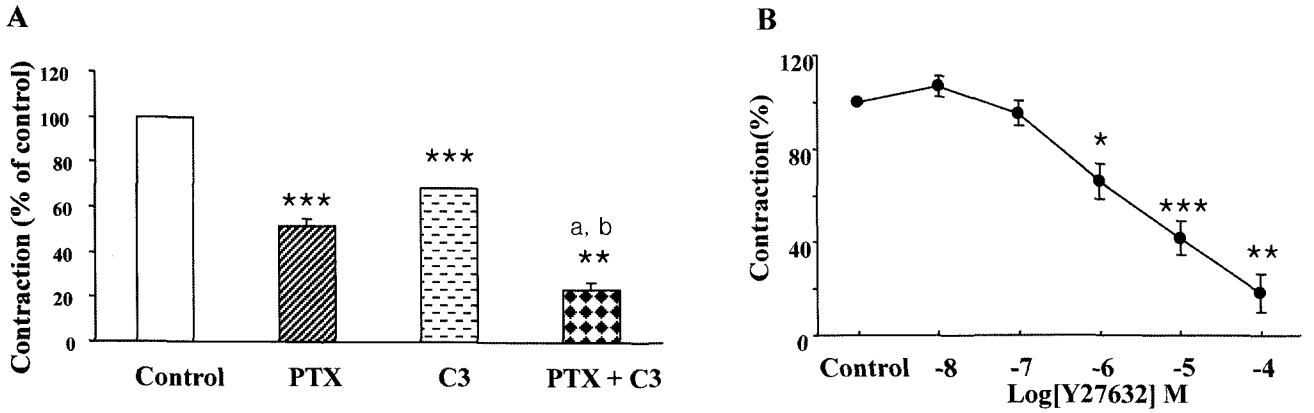

Fig. 4. Effect of PTX, C3 exoenzyme and Y27632 on the on-contraction in the presence of L-NAME. (A) Muscle strips were pretreated with PTX or C3 exoenzyme for 3 hr, and then the contraction was measured. PTX significantly inhibited the contraction; in particular, co-treatment with PTX and C3 toxin additively inhibited the contraction. (B) Muscle strips were incubated with various concentration of Y27632 for 30 min before EFS, and then the contraction was measured Results are expressed as means±S.E. of 3 experiments. *$p<0.05$, **$p<0.01$, ***$p<0.001$ compared with control of the on-contraction in the presence of L-NAME (ANOVA), $^{a}p<0.01$ compared with PTX alone, $^{b}p<0.01$ compared with C3 exoenzyme alone (ANOVA).

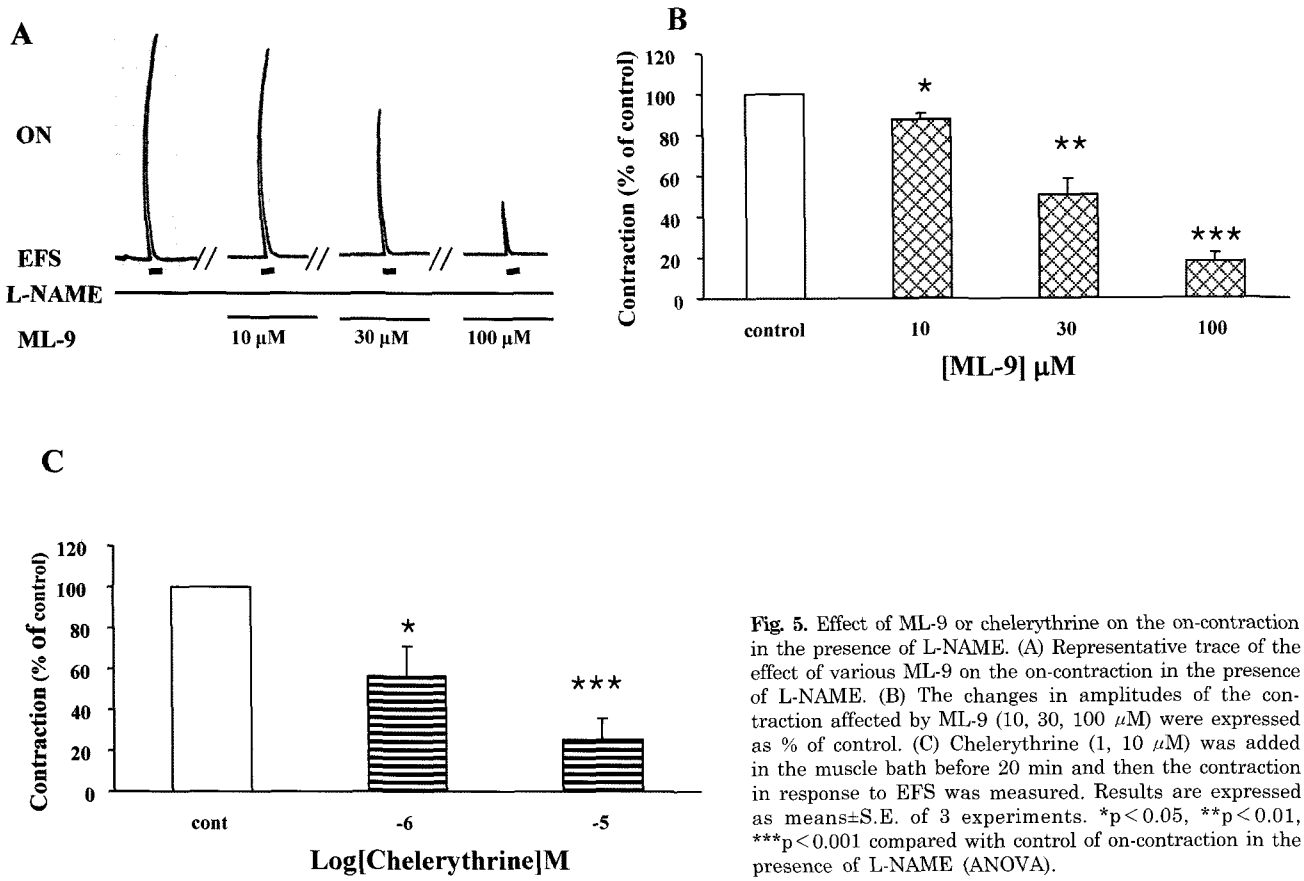

Fig. 5. Effect of ML-9 or chelerythrine on the on-contraction in the presence of L-NAME. (A) Representative trace of the effect of various ML-9 on the on-contraction in the presence of L-NAME. (B) The changes in amplitudes of the contraction affected by ML-9 (10, 30, 100 μM) were expressed as % of control. (C) Chelerythrine (1, 10 μM) was added in the muscle bath before 20 min and then the contraction in response to EFS was measured. Results are expressed as means±S.E. of 3 experiments. *$p<0.05$, **$p<0.01$, ***$p<0.001$ compared with control of on-contraction in the presence of L-NAME (ANOVA).

the NO-mediated inhibitory effect activated by EFS. Moreover, it has been reported that the NO-mediated pathways inhibits acetylcholine release from cholinergic nerve endings in rabbit bladder [21]. Rae et al. [22] suggested that the inhibitory actions of NO on cholinergic transmission appear to be post- rather than pre-junctional in canine colonic circular smooth muscle.

In this experiments, EFS-induced muscle strip on-contractions were abolished by incubation in Ca^{2+}-free Krebs buffer, whereas contractions showed in normal Ca^{2+}-containing Krebs buffer, indicating that Ca^{2+} appears to play a key role, like off-contraction, which links the arrival of an action potential at a synaptic terminal and nerve-evoked neurotransmitter release at neuromuscular junctions. Furthermore, Ca^{2+} influx via voltage-dependent Ca^{2+} channels is required for ACh-induced contraction in esophagus [23-25]. Therefore, our results also indicate that maintenance of a normal concentration of Ca^{2+} in extracellular fluid may be necessary for transmitter release upon nerve activation by EFS and for the triggering of an excitation-contraction coupling mechanism in esophageal smooth muscle [26]. MLCK, another intracellular mediator that is activated in an intracellular Ca^{2+}/calmodulin-dependent manner, plays an important role in force development in smooth muscle [27]. According to our results, ML-9 significantly inhibited EFS-induced off-contraction in a concentration-dependent manner. Furthermore, chelerythrine (a selective and cell permeable protein kinase C inhibitor) significantly inhibited this contraction. These results suggest that endogenous cholinergic contraction activated by EFS may be mediated by the activation of MLCK and PKC in feline esophageal smooth muscle. Myosin light chain kinase (MLCK) or protein kinase C (PKC) are involved in the EFS in some smooth muscles [14-17]. Nerve stimulation and treatments with exogenous transmitter substances involve different contraction mechanisms [18], thus factors released from unstimulated adrenergic nerve endings not stimulated by EFS are not involved in the myogenic response. In contrast, factors released upon stimulation of nerve endings can modulate the amplitude of the myogenic response. These results supported our previous finding that ACh-induced contraction was mediated via phospholipases , but the contraction EFS was not [16].

AlF ions, which were used in this study, directly activate G-protein because of their structural resemblance to phosphate ions. Thus, they bind adjacent to guanosine diphosphate, mimic the γ phosphate group of guanosine triphosphate, and activate G-protein [28,29]. In the present study, AlF enhanced EFS-induced contractile amplitude in a concentration-dependent manner, and PTX or C3 exoenzyme significantly inhibited contractile amplitude compared with control group, which suggests that G_i or rhoA may regulate excitatory-evoked cholinergic contractions by EFS. Furthermore, G_i and rhoA might participate in cholinergic contraction via other pathways, because co-treatment with PTX and C3 exoenzyme showed an additive inhibitory effect on EFS-induced contraction compared with PTX or C3 exoenzyme alone, respectively. This result concurs with our

previous findings [30]. Above all, the observation that MLCK mediated endogenous cholinergic on-contraction by EFS is not comparable with the mechanism of exogenous ACh-induced contraction in feline esophagus. This could be because of different activation transmissions by EFS due to the existence of interstitial cells of Cajal in neuromuscular structures as opposed to exogenous ACh-induced transmission activation in the esophagus, or it could be due to the effects of other smaller undetected mediators released by EFS [31,32].

Studies on outward currents suggest that potassium channels can participate in the regulation of membrane potential and the excitability of smooth muscle [12]. In particular, the 4-AP-induced inhibition of K^+ channels depolarizes pulmonary arterial smooth muscle cells and then causes vasoconstriction [33]. In addition, NO reportedly plays a role as an activator of K_v channels, which results in hyperpolarization and induces vasodilation in pulmonary arteries and in the opossum esophagus [21,34]. In the present study, although 4-AP did not significantly enhance EFS-induced off-contraction, this contraction was found to increase in a concentration-dependent manner. Mechanistically, this could be explained by an NO-related inhibitory pathway, unaffected by 4-AP or another inhibitory pathway activated by EFS, overwhelming the effect of a 4-AP-sensitive K^+ channel (including K_v) blockade. NO has been proposed to cause the hyperpolarization of esophageal smooth muscle by suppressing Ca^{2+}-stimulated Cl^--permeable conductance [35], and Crist et al. [36] suggested that inhibitory junction potentials observed in esophageal smooth muscle in response to single pulse stimulation are due to decreases in Cl^- conductance.

From these findings, we conclude that endogenous excitatory cholinergic contractions activated directly by low-frequency EFS may be mediated by Ca^{2+} influx and the inhibition of K^+ channel opening, consequently actvates Gi and rhoA, which resulted in the the activation of MLCK, and PKC. Moreover, contractions induced by activations of MLCK involved in endogenous cholinergic contractions differed from those of exogenous added ACh.

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