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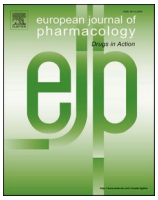

Involvement of interstitial cells of Cajal in nicotinic acetylcholine receptor-induced relaxation of the porcine lower esophageal sphincter

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ABSTRACT

The interstitial cells of Cajal (ICCs) play an important role in coordinated gastrointestinal motility. The present study aimed to elucidate whether or how ICCs are involved in the lower esophageal sphincter (LES) relaxation induced by stimulation of the nicotinic acetylcholine receptor. The application of 1,1-dimethyl-4-phenyl-piperazinium (DMPP; a nicotinic acetylcholine receptor agonist) induced a transient relaxation in the circular smooth muscle of the porcine LES. DMPP-induced relaxation was abolished by not only 1 μM tetrodotoxin but also the inhibition of ICC activity by pretreatment with 100 μM carbenoxolone (a gap junction inhibitor), pretreatment with 100 μM CaCCinh-A01 (an anoctamin-1 blocker acting as a calcium-activated chloride channel inhibitor), and pretreatment with Cl^--free solution. However, pretreatment with 100 μM N^{ω}-nitro-L-arginine methyl ester had little effect on DMPP-induced relaxation. Furthermore, DMPP-induced relaxation was inhibited by pretreatment with 1 mM suramin, a purinergic P2 receptor antagonist, but not by 1 μM VIP (6–28), a vasoactive intestinal peptide (VIP) receptor antagonist. Stimulation of the purinergic P2 receptor with adenosine triphosphate (ATP) induced relaxation, which was abolished by the inhibition of ICC activity by pretreatment with CaCCinh-A01. In conclusion, membrane hyperpolarization of the ICCs via the activation of anoctamin-1 plays a central role in DMPP-induced relaxation. ATP may be a neurotransmitter for inhibitory enteric neurons, which stimulate the ICCs. The ICCs act as the interface of neurotransmission of nicotinic acetylcholine receptor in order to induce LES relaxation.

1. Introduction

The coordinated esophageal motility function is indispensable for daily food intake. Major dysfunction of esophageal motility is collectively referred to as esophageal motility disorder, represented by achalasia. While not eating, the lower esophageal sphincter (LES) maintains a basal tone to prevent reflux of the gastric contents into the esophagus. High-resolution manometry has demonstrated that during eating, the LES relaxes at an appropriate time to allow food to pass to the stomach (Ihara et al., 2017). Although a high-resolution manometry study clearly demonstrated that LES relaxation was caused by the action of swallowing, the precise mechanism underlying LES regulation remains unclear.

In a similar manner to the gastrointestinal tract, coordinated esophageal motility is considered to be regulated by a close network among enteric neurons, interstitial cells of Cajal (ICCs) and smooth muscle cells, with the smooth muscle part responsible for the actual output of contractile and relaxant responses (Ward and Sanders, 2001). The action of swallowing has been shown to stimulate the afferent and efferent vagal nerves (Hornby and Abrahams, 2000). Indeed, stimulation of the nicotinic acetylcholine receptor is responsible for relaxation of the LES (Kadakia et al., 1996; Ruggieri et al., 2014). Accumulated evidence has shown that the relaxant responses to vagal stimulation are reduced by interfering with neurotransmitters, including nitric oxide

Abbreviations: LES, lower esophageal sphincter; ICCs, interstitial cells of Cajal; 137-NES, 137 mM normal extracellular solution; 118-KES, 118 mM K^+ extracellular solution; 40-KES, 40 mM K^+ extracellular solution; L-NAME, N^{ω}-nitro-L-arginine methyl ester; ATP, Adenosine triphosphate; DMPP, 1,1-dimethyl-4-phenyl-piperazinium; 4-DAMP, 4-Diphenylacetoxy-N-methylpiperidine methiodide; VIP, vasoactive intestinal peptide.

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(NO) (Kawahara et al., 1997; Paterson et al., 1992) and vasoactive intestinal polypeptide (VIP) (Rattan, 1986), in the gastrointestinal tract. However, the precise mechanisms whereby the activation of nicotinic acetylcholine receptors induces LES relaxation have not been fully elucidated. Furthermore, whether or how ICCs contribute to the LES relaxation induced by stimulation of the nicotinic acetylcholine receptor has never been determined.

ICCs have been shown to play a crucial role in the regulation of gastrointestinal motility via the interface for neurotransmission to control smooth muscle contractility (Ward et al., 2004). It was shown that NO-dependent inhibitory neurotransmission was attenuated (Burns et al., 1996; Ward et al., 1998), while the cholinergic response was abolished (Ward et al., 2000) in the LES of W/Wv mice lacking intramuscular ICCs (ICC-IMs). Furthermore, both the decreased expression of ICCs and loss of the ICC network have been observed in the LES of patients with achalasia (Goyal and Chaudhury, 2008; Nakajima et al., 2017). Thus, it is both physiologically and pathologically important to determine whether or not ICCs play any roles in the relaxation of the LES during swallowing.

The porcine esophagus is known to be structurally similar to that of humans in terms of anatomical asymmetry, neural tissue and neurotransmission and is commonly used in the study of esophageal motility (Brown and Timmermans, 2004; Pasricha et al., 1993). The present study therefore investigated the involvement of ICCs in the mechanisms of LES relaxation induced by stimulation of the nicotinic acetylcholine receptor using preparations of LES circular smooth muscle isolated from porcine esophagus. We demonstrated for the first time that ICCs play a crucial role in the LES relaxation induced by stimulation of nicotinic acetylcholine receptors.

2. Materials and methods

2.1. Tissue preparation

A section of porcine esophagus containing the esophagogastric junction was freshly obtained from a local slaughterhouse and immediately transported to our laboratory in normal extracellular solution containing 137.4 mM NaCl (137-NES). The specimen was incised longitudinally along the greater curvature of the stomach and fixed with pins on a rubber plate. After removal of the mucosal layer, LES smooth muscle sheets, including ICCs and the nerve plexus, were excised and cut into strips (2 × 5 mm) in a circular direction under a binocular microscope.

2.2. Measurement of force in LES circular smooth muscle strips

The LES circular smooth muscle strips were mounted vertically on a TB-612T force transducer (Nihon Koden, Tokyo, Japan) in a 5-ml organ bath containing NES at 37 °C and stretched to 1.3 times their resting length. During the equilibration period, strips were each stimulated at least 5 times with 118 mM K^+ extracellular solution (118-KES), every 10 min. After equilibration and a stable response were obtained, all experimental protocols were initiated. The degree of force development was expressed as the percentage of force, and unless otherwise specified, the force levels obtained at rest and at peak contraction induced by 100 μM carbachol at the beginning of the protocol were designated as 0% and 100%, respectively.

2.3. Solutions and drugs

The composition of 137-NES was (in mM) 137.4 NaCl, 5.9 KCl, 1.2 $CaCl_2$, 1.2 $MgCl_2$, 11.5 glucose and 11.6 HEPES at pH 7.3. 118-KES was prepared by replacing NaCl with equimolar KCl. The composition of extracellular Cl^--free solution was prepared by replacing Cl^- with equimolar Gluconate (Cl^--free-ES). All solutions used in the force measurement were equilibrated with room air. All chemicals, including 1,1-dimethyl-4-phenyl-piperazinium (DMPP), carbenoxolone, carbachol, tetrodotoxin, mecamylamine, hexamethonium, 4-Diphenylacetoxy-N-methylpiperidine methiodide (4-DAMP), adenosine 5′-triphosphate magnesium salt, glibenclamide, 4-aminopyridine, apamin and iberiotoxin, were obtained from Sigma (St. Louis, MO, USA) and were of reagent grade. N^{ω}-nitro-L-arginine methylester (L-NAME) and suramin sodium were obtained from Fujifilm Wako Pure Chemical (Osaka, Japan). L-760735, CaCCinh-A01, NF279 and VIP (6–28) were obtained from Tocris (Bristol, UK).

2.4. Data analyses

All data are expressed as the mean ± standard error of the mean (S.E.M.). Student's *t*-test was used to determine the statistical significance of differences between two groups. An analysis of variance followed by Dunnett's test was used to determine statistical significance between more than two groups. P values of <0.05 were considered to indicate statistical significance.

3. Results

3.1. Effects of DMPP in porcine LES circular smooth muscle

Porcine LES circular smooth muscle strips spontaneously contract and maintain the basal tone due to the nature of sphincter smooth muscle. To evaluate the relaxation response of LES induced by stimulation of the nicotinic acetylcholine receptors, we examined the effects of DMPP (a nicotinic acetylcholine receptor selective agonist) on the basal tone. The application of DMPP at concentrations up to 1 μM had no effect on the basal tone. At 3 μM, DMPP started to induce transient relaxation, which became larger in a dose-dependent manner at concentrations of 3–30 μM (Fig. 1B). The maximal relaxation response was therefore obtained at 30 μM DMPP (Fig. 1A). Nevertheless, DMPP-induced relaxation was decreased at concentrations of >30 μM. The level of relaxation obtained at 100 μM (9.8% ± 3.9%, n = 5) was significantly smaller than that obtained at 30 μM (21.4% ± 4.0%, n = 8) (Fig. 1A and B). The relaxant response induced by 30 μM was selected to determine the mechanisms underlying DMPP-induced relaxation in this study.

Pretreatment with 1 μM tetrodotoxin, 10 μM mecamylamine (a selective nicotinic acetylcholine receptor antagonist) or 100 μM hexamethonium (another selective nicotinic acetylcholine receptor antagonist) had no effect on the basal tone. The DMPP (30 μM)-induced relaxation was completely inhibited with 1 μM tetrodotoxin (1.3% ± 0.9%, n = 4) (Fig. 1C and E), 10 μM mecamylamine (1.1% ± 0.4%, n = 5) (Fig. 1D and E) or 100 μM hexamethonium (0.4% ± 0.4%, n = 4). Pretreatment with 100 nM 4-DAMP (a M3 muscarinic receptor antagonist) and 10 μM L-76035 (a neurokinin-1 receptor antagonist) had no effect on the basal tone or on the DMPP (30 μM)-induced maximal relaxation. The extent of DMPP (30 μM)-induced maximal relaxation in the presence of 100 nM 4-DAMP (16.9% ± 3.9%, n = 5) or 10 μM L-76035 (19.6% ± 11.8%, n = 5) was not significantly different from that in the absence of 4-DAMP or L-76035 (21.4% ± 4.0%, n = 8) (Fig. 1F–H). However, the recovery time from maximum relaxation to the baseline in the presence of 100 nM 4-DAMP (324.6 ± 74.6 s, n = 5) was significantly longer than that in the absence of 4-DAMP (131.6 ± 14.8 s, n = 8).

3.2. The involvement of nitric oxide and membrane hyperpolarization in DMPP-induced relaxation in porcine LES circular smooth muscle

Next, we examined whether or not NO and/or membrane hyperpolarization were involved in DMPP-induced relaxation. Pretreatment with 100 μM L-NAME had no significant effects on the basal tone and tended to suppress DMPP-induced relaxation (16.2% ± 4.9%, n = 5; Fig. 2A and C); however, this did not reach statistical significance. In contrast, DMPP-induced relaxation mostly disappeared during pre-

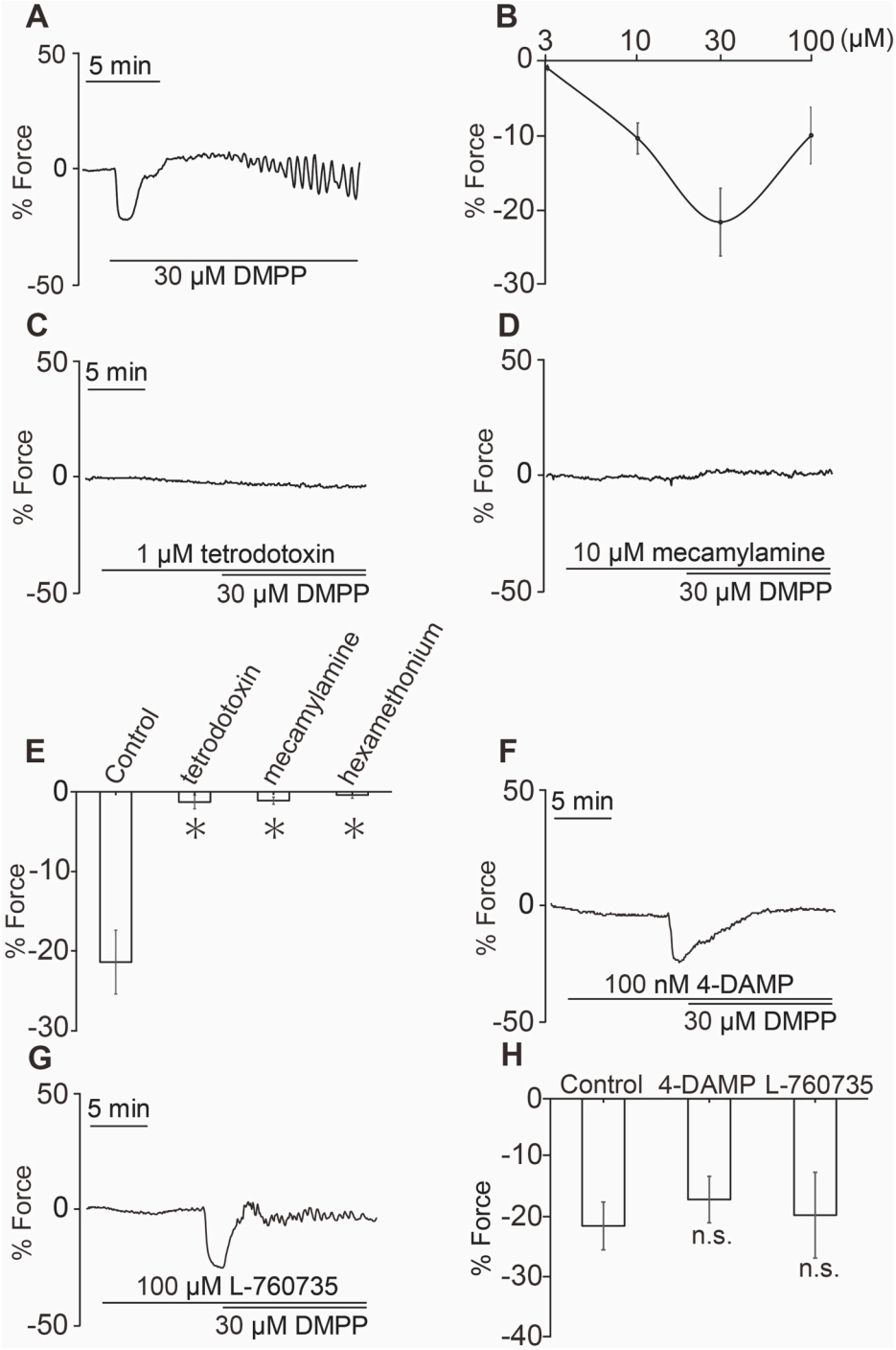

Fig. 1. Relaxant responses induced by DMPP in the porcine LES. A. A representative recording of 30 μM DMPP-induced relaxation in the circular smooth muscle strips of the porcine LES. B. Concentration-dependent response curves of relaxation induced by DMPP (3–100 μM) (n = 8). C, D. Representative recordings of the 30 μM DMPP-induced relaxation in the presence of 1 μM tetrodotoxin (C) and 10 μM mecamylamine (D). E. The cumulative data of tetrodotoxin (n = 4), mecamylamine (n = 5) and hexamethonium (n = 4) are shown. F, G. Representative recordings of the 30 μM DMPP-induced relaxation in the presence of 100 nM 4-DAMP, a M3 muscarinic receptor antagonist (F), and 10 μM L-760735, a neurokinin-1 antagonist (G). H. The cumulative results of 4-DAMP (n = 5) and L-760735 (n = 5). Force is expressed as the percentage, with the levels of force obtained at rest in 137-NES and at peak contraction induced by 100 μM carbachol set at 0% and 100%, respectively. The data are shown as the mean ± S.E.M. *P < 0.05 vs. control. DMPP, 1,1-dimethyl-4-phenyl-piperazinium; 4-DAMP, 4-Diphenylacetoxy-N-methylpiperidine methiodide.

contraction with 40-KES, which induced a sustained contraction by membrane depolarization (4.2% ± 0.6%, n = 4; Fig. 2B and C). Since the contribution of NO was relatively small in comparison to membrane hyperpolarization, we assessed whether or not nitrergic neurons existed in the porcine LES circular smooth muscle by examining the effects of L-NAME on the 118-KES-induced contraction, which can stimulate myenteric neurons. Stimulation with 118-KES reproducibly induced an initial slight relaxation followed by transient contraction, where the force level returned to the pre-stimulation level. Pretreatment with 100 μM L-NAME abolished the initial relaxation and augmented the contraction to the peak level (163.5% ± 12.8%, n = 4); furthermore, it induced subsequent sustained contraction (770.1% ± 246.7%, n = 4), assuming the 118-KES-induced contraction in the absence of 100 μM L-NAME to be 100%, indicating that the porcine LES possesses an NO production system when required.

Next, we investigated whether or not any potassium channels were involved in DMPP-induced membrane hyperpolarization. The application of 10 μM glibenclamide (an ATP-sensitive K^+ channel blocker), 0.5 mM 4-aminopyridine (a voltage-gated K^+ channel blocker), 1 μM apamin (a small conductance Ca^{2+}-activated K^+ channel blocker) or 100 nM iberiotoxin (a large conductance Ca^{2+}-activated K^+ channel blocker, n = 4) had no significant effects on DMPP-induced relaxation (Fig. 2D), indicating that no K^+ channels were involved in this response.

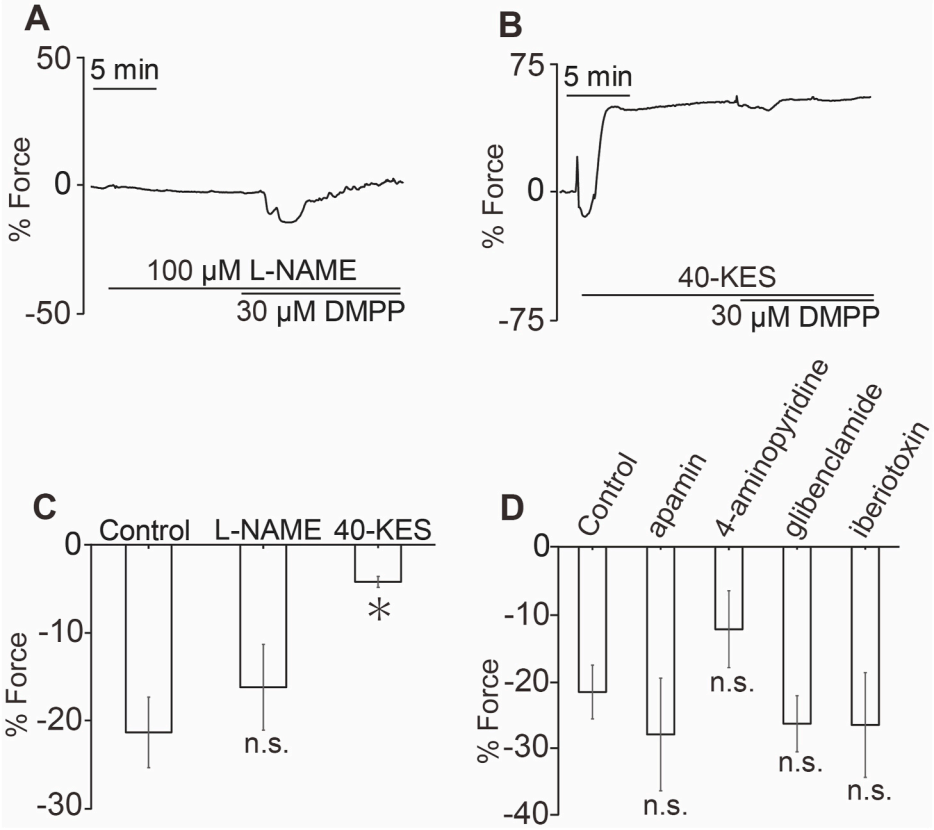

Fig. 2. Effects of L-NAME, 40-KES depolarization and several K^+ channel blockers on DMPP-induced relaxation in the porcine LES. A, B. Representative recordings of 30 μM DMPP-induced relaxation in the presence of 100 μM N^{ω}-nitro-L-arginine methyl ester (L-NAME) (A) and 40-KES (B). C. The cumulative data on the effects of L-NAME (n = 5) and 40-KES (n = 4) on DMPP-induced relaxation. D. The cumulative data on the effects of K^+ channel blockers, including 10 μM glibenclamide (n = 4), 0.5 μM 4-aminopyridine (n = 5), 1 μM apamin (n = 4) and 100 nM iberiotoxin (n = 4), on 30 μM DMPP-induced relaxation. The data are shown as the mean ± S.E.M. *$P < 0.05$ vs. control. DMPP, 1,1-dimethyl-4-phenyl-piperazinium; L-NAME, Nω-nitro-L-arginine methyl ester; 40-KES, 40 mM K+ extracellular solution.

3.3. Involvement of adenosine triphosphate (ATP) in DMPP-induced relaxation in porcine LES circular smooth muscle

We investigated whether or not any neurotransmitters other than NO, such as VIP and/or ATP, were involved in DMPP-induced relaxation as candidate agents causing membrane hyperpolarization. Pretreatment with 1 μM VIP (6–28) (a VIP receptor antagonist) increased the basal tone (7.9% ± 2.7%, n = 6). However, it had no effects on DMPP-induced relaxation. The extent of relaxation in the presence of VIP (6–28) (18.0% ± 7.3%, n = 6) was not significantly different from that in its absence (21.4% ± 4.0%, n = 8) (Fig. 3A and C). However, pretreatment with P2 receptor antagonists 1 mM suramin and 10 μM NF279 significantly suppressed DMPP-induced relaxation. The extent of relaxation in the presence of 1 mM suramin (3.7% ± 1.3%, n = 6) was significantly lower than that in its absence (21.4% ± 4.0%, n = 8) (Fig. 3B and C), and the extent of relaxation in the presence of 10 μM NF279 (10.9% ± 3.1%, n = 5; Fig. 3D and E) was significantly lower than that in its absence (21.4% ± 4.0%, n = 8), indicating that purinergic P2 receptors (P2 receptors) contributed to DMPP-induced relaxation.

3.4. Contribution of ICCs to DMPP-induced relaxation in porcine LES circular smooth muscle

Next, we investigated whether or not ICCs played any roles in DMPP-induced relaxation. Pretreatment with either 100 μM carbenoxolone (an inhibitor of gap junctions between ICCs and smooth muscle) or 100 μM CaCCinh-A01 (an anoctamin-1 blocker acting as a calcium-activated chloride channel inhibitor) suppressed the basal tone (11.8% ± 2.8%, n = 8 and 10.2% ± 2.4%, n = 5, respectively) and abolished DMPP-induced relaxation (Fig. 4A, B and C). Neither carbenoxolone nor CaCCinh-A01 affected the contraction induced by carbachol, which acts directly on the smooth muscle. The extent of carbachol-induced contraction in the presence of 100 μM carbenoxolone (114.9% ± 24.4%, n = 4) or 100 μM CaCCinh-A01 (94.6% ± 12.5%, n = 5) was not significantly different from that in its absence (102.1% ± 11.0%, n = 6).

To further assess whether or not the Cl^- channel, which plays a crucial role in the ICC function, was involved in DMPP-induced relaxation, we examined the effects of Cl^--free-ES on DMPP-induced relaxation. Cl^--free-ES had an effect on the contraction induced by carbachol and suppressed the basal tone (14.9% ± 3.4%, n = 6). The DMPP-induced relaxation was almost abolished when 137-NES was changed to Cl^--free-ES (3.0% ± 1.1%, n = 6; Fig. 4D and E).

Finally, we explored the link between ATP/P2 receptor signaling and the ICC function by examining the response to ATP in the presence or absence of CaCCinh-A01 or carbenoxolone. As expected, the application of 1 mM ATP induced apparent relaxation in LES circular smooth muscle. This relaxation was not suppressed by pretreatment with 100 μM ARL 67156 (an inhibitor of NTPDases) but was inhibited by pretreatment with 100 μM CaCCinh-A01 or 100 μM carbenoxolone. The extent of ATP-induced relaxation was 16.3% ± 3.5% (n = 6) (Fig. 5A) without any pretreatment, and it decreased to 6.4% ± 1.9% (n = 6) and 4.8% ± 0.8% (n = 5) in the presence of CaCCinh-A01 and carbenoxolone, respectively (Fig. 5B, C and D). In contrast, however, ATP-induced relaxation was not suppressed by posttreatment with CaCCinh-A01 (Fig. 5E). There was no significant difference in the extent of ATP-induced relaxation in the presence (22.0% ± 5.7%, n = 3) or absence (18.4% ± 4.7%, n = 3) of 100 μM CaCCinh-A01.

4. Discussion

Although several studies have been conducted to determine the mechanisms underlying the LES relaxation induced by stimulation of the nicotinic acetylcholine receptor, with a focus on the neurotransmitters produced by vagal nerves (Farré et al., 2007; González et al., 2004; Lecea et al., 2009; Rattan and Goyal, 1975), whether or how ICCs are associated with this LES relaxation has never been determined. The

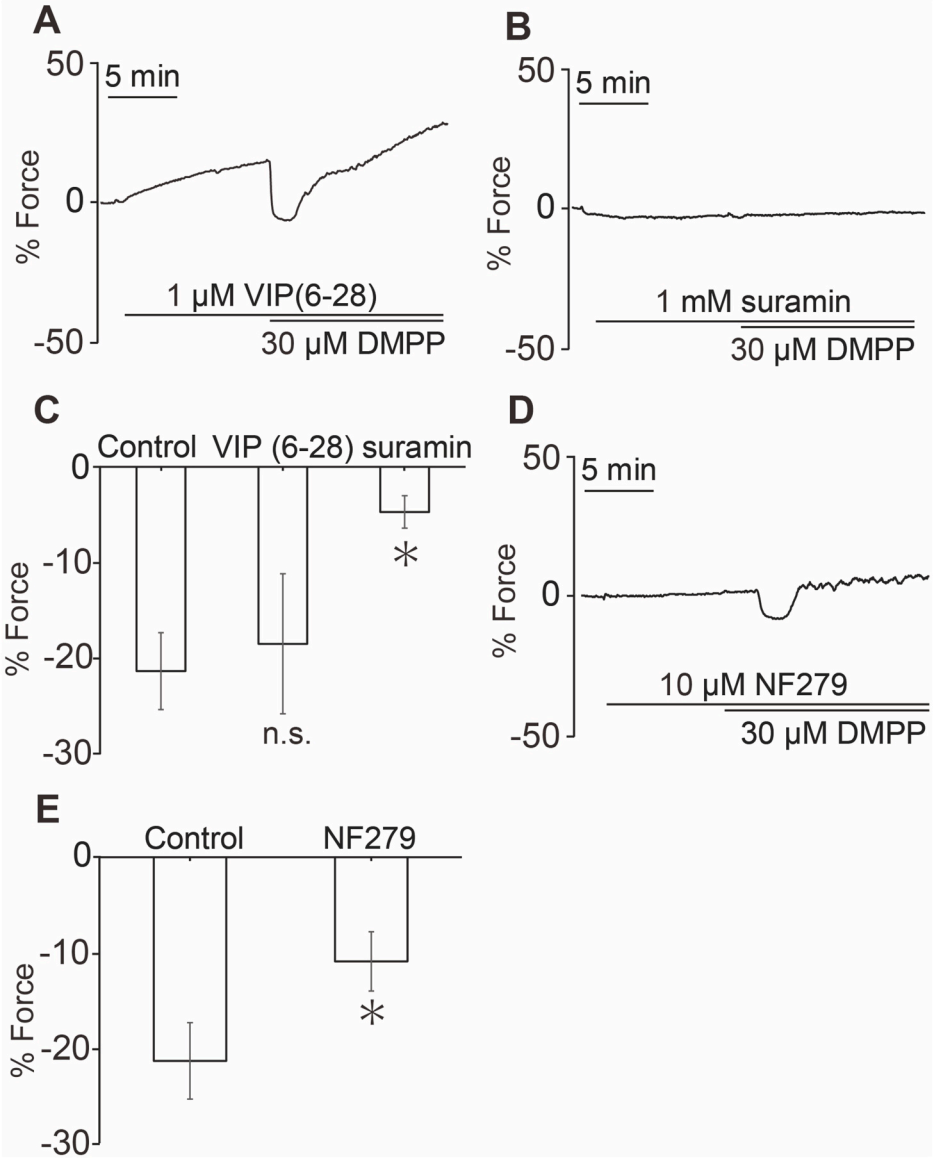

Fig. 3. Effects of a VIP receptor antagonist and a nonselective P2 receptor antagonist and P2X2 receptor antagonist on DMPP-induced relaxation in the porcine LES. A, B. Representative recordings of 30 μM DMPP-induced relaxation in the presence of 1 μM VIP (6–28), a VIP receptor antagonist (A), or 1 mM suramin, a non-selective P2 receptor antagonist (B, n = 6). C. The cumulative data of VIP (6–28) (n = 7) and suramin (n = 6) are shown. D. Representative recordings of 30 μM DMPP-induced relaxation in the presence of 10 μM NF279 (a P2X2 receptor antagonist). E. The cumulative data of NF279 are shown (n = 5). The data are shown as the mean ± S.E.M. *$P < 0.05$ vs. control. DMPP, 1,1-dimethyl-4-phenyl-piperazinium.

present study demonstrated for the first time that the ICCs function as the interface of neurotransmission of nicotinic acetylcholine receptors between enteric neurons and smooth muscle cells by causing membrane hyperpolarization of the ICCs themselves via ANO1 activation.

In this study, the application of DMPP (a selective nicotinic acetylcholine receptor agonist) to porcine LES circular smooth muscle strips caused relaxation in a similar manner to the relaxant response to nicotine stimulation in humans (González et al., 2004). Since coordinated esophageal motility is considered to be regulated by a close network among enteric neurons, ICCs and smooth muscle cells (Ward et al., 1998), it was necessary to assess whether and how they were involved in the LES relaxation induced by stimulation of nicotinic acetylcholine receptors in the porcine LES. The DMPP-induced relaxation was reasonably abolished by tetrodotoxin, indicating the involvement of enteric nerve activity in DMPP-induced relaxation. Esophageal motility is regulated by the balance of excitatory and inhibitory enteric neurons (Hornby and Abrahams, 2000). Thus far, it has been demonstrated, based on the morphological and physiological studies, that acetylcholine and substance P are neurotransmitters for excitatory enteric neurons, while NO, hyperpolarizing factors, VIP and ATP are neurotransmitters for inhibitory enteric neurons (Biancani et al., 1984; Conklin et al., 1993; Daniel et al., 1989; De Man et al., 1991; Goyal et al., 1980; Ny et al., 1995; Rattan, 1986; Tøttrup et al., 1991; Hoyle and Burnstock, 1989). In the present study, neither 4-DAMP nor L-76035 affected the extent of DMPP-induced relaxation; however, after pretreatment with 4-DAMP, more time was required to recover to the baseline after DMPP-induced relaxation. The stimulation of nicotinic acetylcholine receptors by DMPP activates both the enteric inhibitory and excitatory neurons in the porcine LES circular smooth muscle. The DMPP-induced response is formed by the relaxant effect of inhibitory neurons and the contractile effect of excitatory neurons. It has been reported that the two muscarinic receptors, M2 and M3, exert opposing effects on neurotransmitter release from postganglionic nerve varicosities in the GI tract (Vieira et al., 2009). Generally, 4-DAMP is used as an M3 muscarinic receptor antagonist, but it is also known to act on M2 muscarinic receptor (Ki values for M2 and M3 muscarinic receptors: 7.3 and 0.37 nM, respectively). Therefore, the possible involvement of the M2 as well as the M3 muscarinic receptor in the recovery to the baseline after DMPP-induced relaxation in the present study cannot be completely denied due to a lack of more selective compounds.

We next examined the types of relaxant neurotransmitters involved in DMPP-induced relaxation. Unexpectedly, NO was not a main relaxant

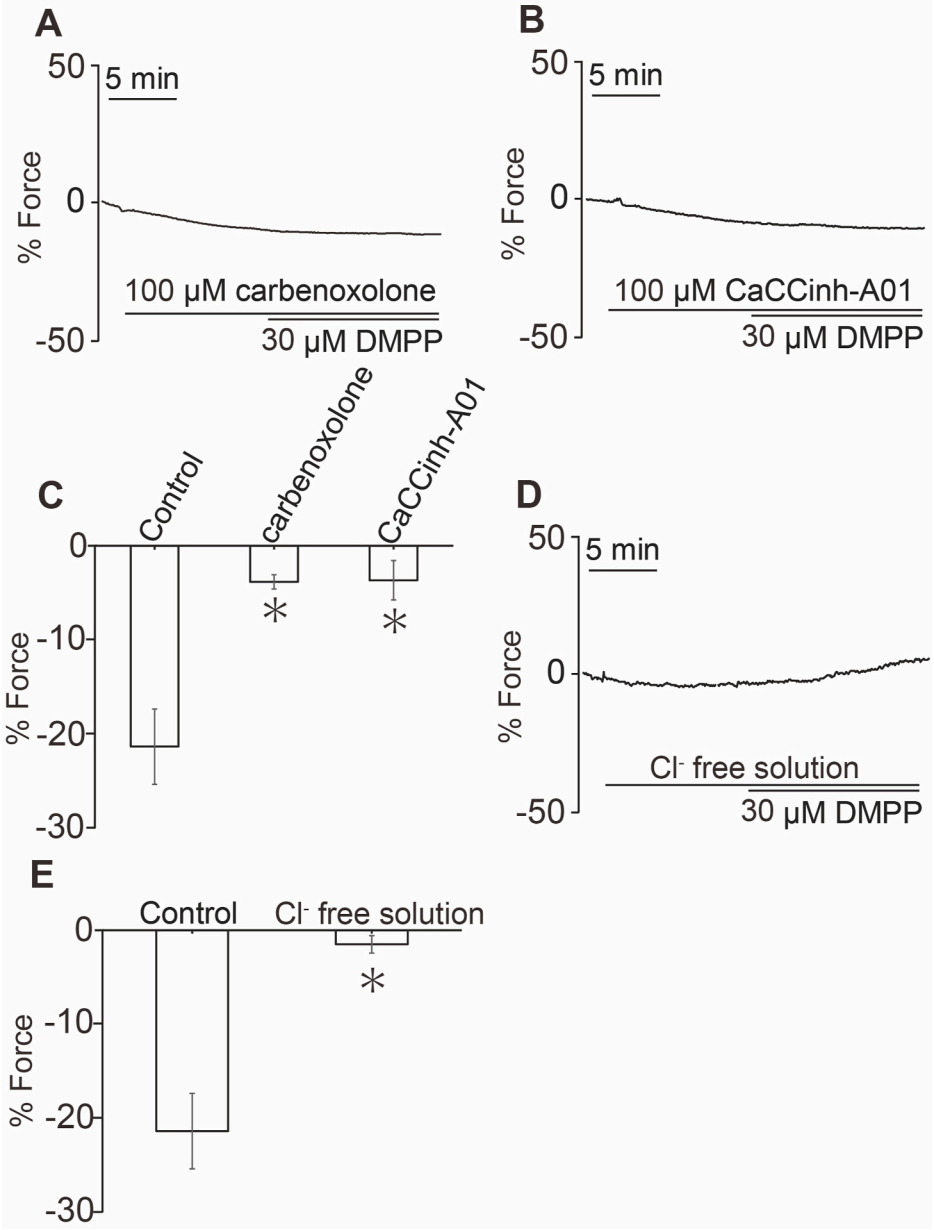

Fig. 4. Involvement of interstitial cells of Cajal activity in DMPP-induced relaxation in the porcine LES. A-C. Representative recordings showing the effects of 100 μM carbenoxolone (A) or 100 μM CaCCinh-A01 (B) on 30 μM DMPP-induced relaxation, and the cumulative data for carbenoxolone (n = 8) and CaCCinh-A01 (n = 5) (C). D, E. Representative recordings of the 30 μM DMPP-induced relaxation in the Cl^--free-ES (D) and the cumulative data for Cl^--free-ES (n = 6) (E). The data are shown as the mean ± S.E.M. *$P < 0.05$ vs. control. DMPP, 1,1-dimethyl-4-phenyl-piperazinium.

neurotransmitter in DMPP-induced relaxation in the porcine LES. NO is a major contributor to smooth muscle relaxation throughout the body, and it was shown to be involved in the LES relaxation in previous studies (Farré et al., 2006). We therefore assessed whether or not NO-producing inhibitory neurons existed in our experimental conditions by examining the effects of L-NAME on the contraction induced by 118-KES, which causes membrane depolarization of the NO producing inhibitory neurons if present. As a result, pretreatment with L-NAME significantly abolished the 118-KES-induced initial transient relaxation and augmented the 118-KES-induced contraction, indicating that NO was released by enteric inhibitory neurons upon appropriate stimulation. Taken together, these findings suggest that NO was unlikely to be involved in DMPP-induced relaxation.

To evaluate the involvement of hyperpolarizing factors in DMPP-induced relaxation, DMPP was applied during the precontraction induced by 40 mM K^+. Although several kinds of hyperpolarizing factors have been suggested (Edwards et al., 1998; Matoba et al., 2000; Popp et al., 1996), others remain to be identified, and the potency of hyperpolarizing factors is considered to be lost in the precontraction induced by 40 mM K^+ (Ihara et al., 2000). Interestingly, the present study showed that NO was not a major contributor to DMPP-induced relaxation in the porcine LES (Fig. 2A and C), although nitrergic neurons existed under our experimental conditions. DMPP-induced relaxation was significantly attenuated during the precontraction induced by 40 mM K^+, indicating that it was caused by the induction of membrane hyperpolarization of LES smooth muscle cells. The K^+ channels play a main role in changes to the membrane polarization of smooth muscles, and it was suggested that apamin-sensitive, small conductance Ca^{2+}-activated K^+ channels were involved in the nicotine-induced relaxation in the porcine LES (Farré et al., 2006). Thus, we examined the effects of four types of K^+ channel blockers on the LES relaxation induced by stimulation of nicotinic acetylcholine receptor. However, none of them contributed to this relaxation, indicating that the K^+ channels of smooth muscle are unlikely to be involved in DMPP-induced membrane hyperpolarization; therefore, some other mechanisms of membrane hyperpolarization are likely to be involved. This turned out to be attributed to changes in the ANO1 activity on ICCs. The reason for the discrepancies regarding the involvement of NO and K^+ channels

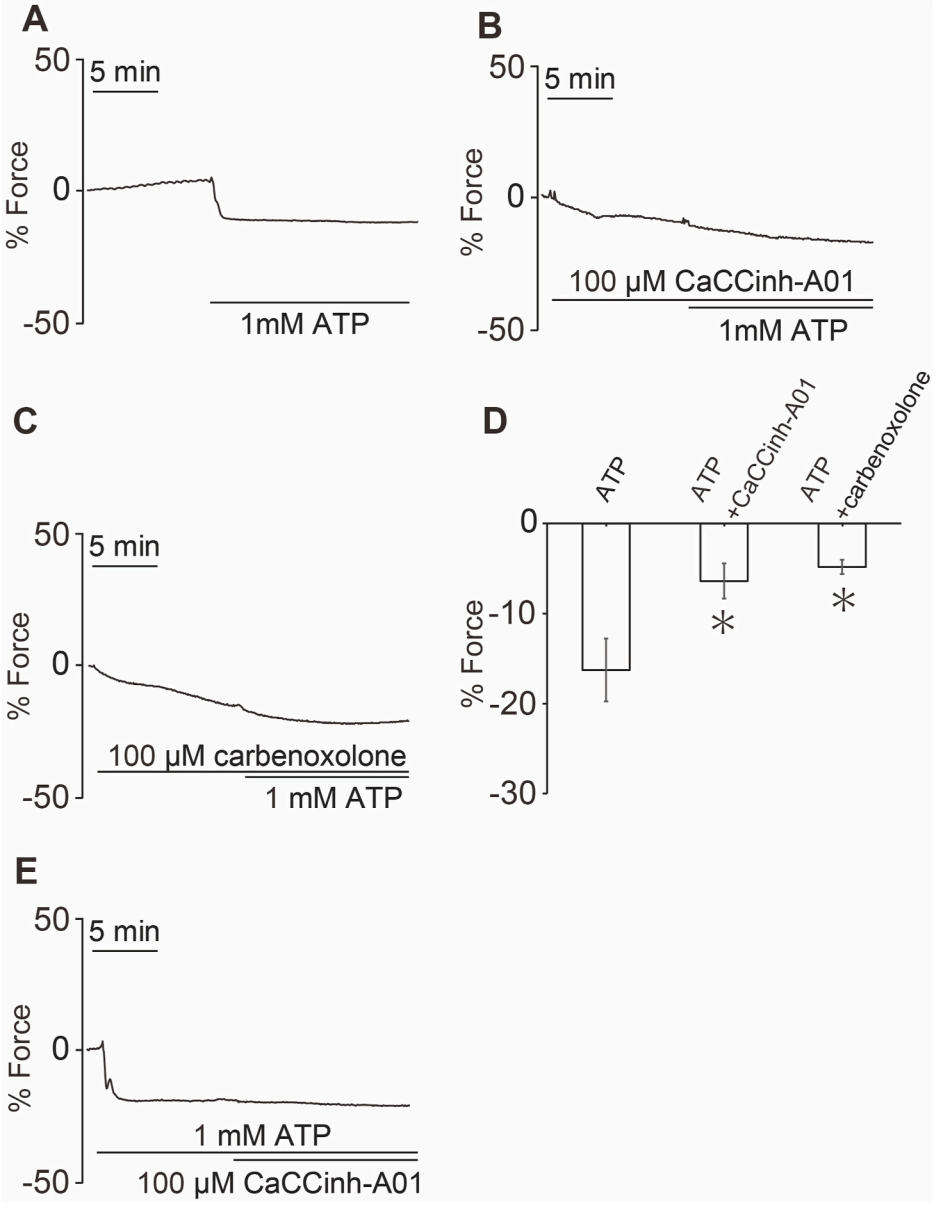

Fig. 5. Relationship between ATP/P2 receptor signaling and interstitial cells of Cajal activity in DMPP-induced relaxation in the porcine LES. A-C, Representative recordings of 100 μM ATP-induced relaxation without any pretreatment (A) and in the presence of 100 μM CaCCinh-A01 (B) and 100 μM carbenoxolone (C). D, The cumulative data for ATP (n = 6), ATP with CaCCinh-A01 (n = 6) and ATP with carbenoxolone (n = 5). E, A representative recording showing the effect of 100 μM CaCCinh-A01 on the sustained phase of ATP-induced relaxation. The data are shown as the mean ± S.E.M. *P < 0.05 vs. control. ATP, adenosine triphosphate.

between the present and previous studies could not be determined; however, one possibility is that the responsiveness of DMPP to the nicotinic acetylcholine receptors differs from that of nicotine. Both VIP and ATP are neurotransmitters that may be used to assess inhibitory enteric neurons. VIP was not involved in DMPP-induced relaxation. In contrast, the P2 receptor antagonists suramin and NF279 inhibited this relaxation, suggesting that ATP might be a primary neurotransmitter for inhibitory enteric neurons released by the stimulation of nicotinic acetylcholine receptors, and an ATP/P2 signaling pathway and membrane hyperpolarization might be involved in DMPP-induced relaxation.

The classical concept of enteric neurotransmission is that neurotransmitters released from nerve varicosities have direct effects on smooth muscle cells (Sarna, 2008). However, the neurojunctional environment is fine-tuned by ICCs, which not only make synaptic-like contacts with enteric neurons but also connect electrically to smooth muscle cells. It has been shown that the ICCs serve as electrical pacemakers and generate spontaneous electrical slow waves in the gastrointestinal tract. The spreading of electrical slow waves from ICCs to smooth muscle cells causes contraction via membrane depolarization (Takaki, 2003). Although the ICCs usually contribute to contraction in other parts of the gastrointestinal tract (other than the esophagus), we examined whether or not ICCs play any roles in DMPP-induced relaxation. Electron microscopy revealed that gap junctions exist between the ICCs and the smooth muscle cells of the gastrointestinal tract (Horiguchi et al., 2001), and coupling between the ICCs and smooth muscle cells has been shown to play an important role in the regulation of gastrointestinal motility (Negreanu et al., 2008; Sanders and Ward, 2006; Sung et al., 2015). As a result, the blocking of gap junctions between the ICCs and smooth muscles with carbenoxolone abolished DMPP-induced relaxation. Importantly we confirmed that carbenoxolone did not affect the contractions induced by 118-KES or carbachol, which act directly on smooth muscle, indicating that carbenoxolone predominantly affected the gap junctions between the ICCs and smooth muscle cells. These findings indicate that the coupling between ICCs and smooth muscle cells is indispensable for the LES relaxation induced by stimulation of the nicotinic acetylcholine receptors.

It was found that Anoctamin-1 (ANO1/TMEM16A), which is a Ca^{2+}-activated Cl^- channel, is highly expressed in ICCs (Gomez-Pinilla et al., 2009). Several studies have investigated the function of ANO1 in ICCs. Slow waves failed to develop in *Ano1*-null mice (Hwang et al., 2009),

and slow waves and phasic contractions were diminished or almost abolished by blocking ANO1 in the stomach and intestine (Hwang et al., 2016; Sanders et al., 2012; Singh et al., 2014). ANO1 has been shown to be responsible for controlling membrane potential, which is necessary to maintain the pacemaker activity of ICCs in gastrointestinal smooth muscles. It has also been shown that ICCs are required for the achievement of neurotransmission, with the help of ANO1, since the down-regulation of ANO1 in the ICCs caused a decrease in gastrointestinal inhibitory neurotransmission (Lu et al., 2019). In the present study, inhibition of the ICC function by carbenoxolone, an ANO1 inhibitor or Cl^--free-ES reduced the basal tone of the LES. Since smooth muscle cells do not express ANO1 (Gomez-Pinilla et al., 2009; Hwang et al., 2009; Miettinen et al., 2009), ICCs are considered to be involved in maintaining the basal tone of the LES. Furthermore, complete blocking of DMPP-induced relaxation by either an ANO1 inhibitor or Cl^--free-ES, indicated that ICCs are essential for the inhibitory neurotransmission activated by the stimulation of the nicotinic acetylcholine receptors. Discrepancies in the ICC function seem to exist between at rest and during stimulation of nicotinic acetylcholine receptors. A previous study found that the LES in W/Wv mice was hypotensive (Sivarao et al., 2001), suggesting that ICCs help maintain the basal tone at rest. In contrast, the relaxation induced by neurotransmitters was reportedly suppressed in W/Wv mice (Burns et al., 1996; Ward et al., 1998). It is thus considered that ICCs maintain the balance of LES contractility to induce both contraction and relaxation, depending on the condition. Since treatment with carbenoxolone and CaCCinh-A01 simply blocks the function of ICCs, both contractile and relaxant responses will be observed, depending on the condition.

It is important to assess how ICCs are associated with ATP as an inhibitory neurotransmitter and membrane hyperpolarization induced by stimulation of the nicotinic acetylcholine receptors. The application of ATP induced significant relaxation (Fig. 5A). This was consistent with the notion that ATP might be an inhibitory neurotransmitter induced by the stimulation of nicotinic acetylcholine receptors. Concerning the possible hydrolysis of ATP, the findings obtained with an inhibitor of NTPDases further confirmed the relaxant effect of ATP. Interestingly, the ATP-induced relaxation was blocked by an ANO1 inhibitor or a gap junction inhibitor (Fig. 5B and C). Taken together with the findings that P2 receptor antagonists significantly blocked DMPP-induced relaxation, the P2 receptors on ICCs are suggested to be the targets of ATP released from inhibitory enteric neurons.

However, ATP-induced relaxation was not suppressed by posttreatment with CaCCinh-A01. Application of CaCCinh-A01 blocks ICC hyperpolarization by acting on the ANO1 channel in ICCs and induces smooth muscle relaxation. Our present findings suggest that once hyperpolarization is transmitted from ICCs to smooth muscle, hyperpolarization of the smooth muscle cannot be cancelled by blocking hyperpolarization of ICCs. This may be due to the difference in the amount of smooth muscle and ICCs. Taken together, these findings suggest that the ANO1 activation induced by the ATP might cause membrane hyperpolarization in the ICCs, which would then be electrically transmitted to the LES smooth muscle via the gap junctions (Fig. 6).

5. Conclusions

The porcine LES possesses a basal tone where transient relaxation is caused by the stimulation of nicotinic acetylcholine receptors. ATP released from the inhibitory enteric neurons stimulates the ICCs, causing membrane hyperpolarization of the ICCs via ANO1 activation. The membrane hyperpolarization of the ICCs, in turn, is electrically transmitted to the LES smooth muscle via the gap junctions to induce relaxation of the LES. ICCs are indispensable for this relaxation, acting as an interface for the neurotransmission of nicotinic acetylcholine receptors.

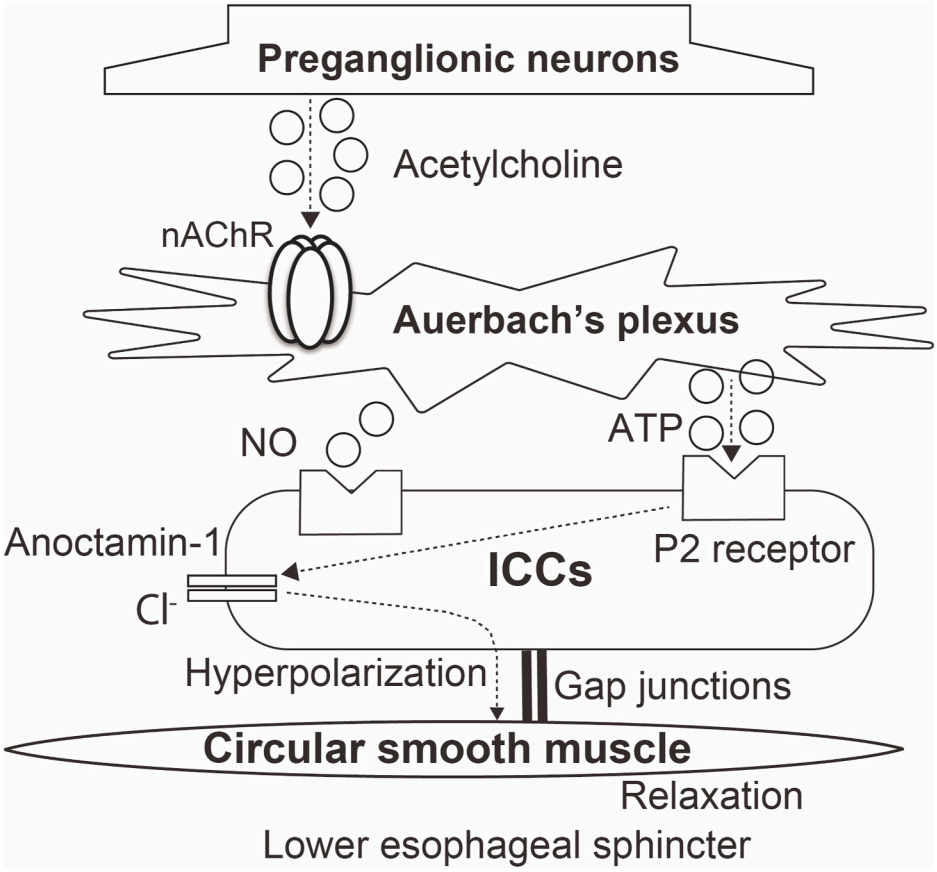

Fig. 6. Proposed mechanisms underlying DMPP-induced relaxation in the circular smooth muscle of the porcine LES. Acetylcholine released from the preganglionic nerves binds to the nicotinic acetylcholine receptors on inhibitory neurons in Auerbach's plexus. Activation of the inhibitory neurons results in the release of ATP, which acts on the P2 receptor on ICCs. Stimulation of the nicotinic acetylcholine receptors by acetylcholine is converted into electrical signals in ICCs by ATP via the activation of anoctamin-1, which is transmitted to the smooth muscle via the gap junctions between ICCs and smooth muscle. nAChR, nicotinic acetylcholine receptor; NO, nitric oxide; ATP, adenosine triphosphate; ICCs, interstitial cells of Cajal; P2 receptor, purinergic P2 receptor.

CRediT authorship contribution statement

Yoshihiro Otsuka: Methodology, Validation, Formal analysis, Investigation, Data curation, Writing – original draft, Visualization. **Xiaopeng Bai:** Methodology, Validation, Formal analysis, Investigation. **Yoshimasa Tanaka:** Conceptualization, Methodology, Writing – review & editing. **Eikichi Ihara:** Conceptualization, Writing – review & editing, Supervision, Project administration, Funding acquisition. **Takatoshi Chinen:** Supervision, Funding acquisition. **Haruei Ogino:** Supervision, Funding acquisition. **Yoshihiro Ogawa:** Conceptualization, Supervision, Project administration, Funding acquisition.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Eikichi Ihara participated in the funded research of Takeda Pharmaceutical Co., Ltd. and belongs to an endowed course supported by companies, including Ono Pharmaceutical Co., Ltd., Miyarisan Pharmaceutical Co. Ltd., Sanwa Kagaku Kenkyusho Co., Ltd., Otsuka Pharmaceutical Factory, Inc., Fujifilm Medical Co., Ltd., Terumo Corporation, FANCL Corporation, Ohga Pharmacy, and Abbott Japan, LLC. Yoshihiro Ogawa is conducting a joint study with FANCL Corporation and Fujifilm Medical Co., Ltd. The authors declare no other conflicts of interest in association with this study.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ejphar.2021.174491.

Disclosures

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