Effects of inhibitors of nonselective cation channels on the acetylcholine-induced depolarization of circular smooth muscle from the guinea-pig stomach antrum

Aya HOTTA[1], Young Chul KIM[2], Eri NAKAMURA[1], Yoshihiko KITO[1], Yoshimichi YAMAMOTO[3] and Hikaru SUZUKI[1]

[1]*Department of Physiology, Nagoya City University Medical School, Mizuho-ku, Nagoya 467-8601, Japan*
[2]*Department of Physiology, Chungbuk National University,*
[3]*Department of Physiology, Nagoya City University Nursing School*

Abstract

In circular smooth muscle bundles isolated from the guinea-pig stomach antrum, the effects of quinidine, Ni^{2+}, flufenamic acid, niflumic acid, La^{3+}, SKF-96365 and 4'-diisothiocyanatostilbene-2,2'-disulphonic acid (DIDS) on acetylcholine (ACh)-induced depolarization were investigated. Recording membrane potentials from smooth muscle cells with intracellular microelectrodes revealed that ACh (1 μM) depolarized the membrane by 5–8 mV and increased the amplitude and frequency of slow potentials. These effects were inhibited by atropine. Quinidine (10 μM) increased the amplitude of ACh-induced depolarization, with no alteration to the properties of slow potentials. Ni^{2+} (50 μM) transiently (5–10 min) depolarized the membrane by about 5 mV, with an associated increase in frequency and amplitude of slow potentials. In the stabilized condition with Ni^{2+}, the amplitude of ACh-induced depolarization remained unchanged. Flufenamic acid (10 μM) inhibited the generation of slow potentials, with no change in either the amplitude of ACh-induced depolarization or of the amplitude and frequency of slow potentials generated during ACh stimulation. A high concentration of flufenamic acid (100 μM) depolarized the membrane and increased the amplitude of ACh-induced depolarization. Niflumic acid (10 μM) hyperpolarized the membrane and increased the amplitude and frequency of slow potentials and also the amplitude of ACh-induced depolarization. DIDS (100 μM) hyperpolarized the membrane and inhibited the amplitude and frequency of slow potentials, with no alteration to the amplitude of ACh-induced depolarization. SKF-96365 (3–50 μM) depolarized the membrane in a concentration-dependent manner, but did not change the level of ACh-induced depolarization. La^{3+} (50 μM) did not alter the properties of the slow potentials or the ACh-induced responses. These results provide evidence that ACh-induced depolarization is not inhibited by chemicals known to inhibit non-selective cation channels. We suggest that muscarinic receptor-mediated signal transduction may be different in smooth muscle and interstitial cells.

Key words: acetylcholine, depolarization, non-selective cation channel, gastric muscle, slow potential

Correspondence to: Hikaru Suzuki, Ph.D., Department of Physiology, Nagoya City University Medical School, Mizuho-ku, Nagoya 467-8601, Japan
Phone: +81-52-853-8131 Fax: +81-52-842-1538 e-mail: hisuzuki@med.nagoya-cu.ac.jp

Introduction

The spontaneously generated rhythmic activity of gastrointestinal smooth muscle, with associated slow waves or spike potentials (Tomita, 1981), is initiated by interstitial cells of Cajal distributed in the myenteric region (ICC-MY) (Sanders, 1996; Huizinga *et al.* 1997; Sanders *et al.* 1999; Suzuki, 2000; Hirst and Ward, 2003; Takaki, 2003). In gastric muscle, pacemaker potentials generated in ICC-MY are propagated to the longitudinal muscle to form follower potentials and to circular muscle to elicit slow waves (Dickens *et al.*, 1999; Cousins *et al.*, 2002; Hirst and Ward, 2003). However, a bundle of circular muscle free of longitudinal muscle and ICC-MY from the guinea-pig stomach antrum could periodically generate regenerative potentials with a slow time course (slow potentials) (Suzuki and Hirst, 1999). The circular muscle bundle has a rich distribution of interstitial cells of Cajal within the muscle bundles (ICC-IM) which generate unitary potentials (Dickens *et al.*, 2001). The frequency analysis of these unitary potentials indicates that a slow potential may be formed by summation of unitary potentials (Edwards *et al.*, 1999). Slow potentials differ from slow waves in that the former but not the latter can be abolished easily by low concentrations (0.5–1 mM) of caffeine (Suzuki and Hirst, 1999; Nose *et al.* 2000; Suzuki, 2000; Dickens *et al.*, 2001; Hirst and Ward, 2003).

Gastric smooth muscle is innervated by cholinergic nerves, and electrical stimulation of these nerves elicits excitatory responses in the smooth muscle, such as the generation of excitatory junction potentials (e. j. p.), an increase in contractile force and an increase in the frequency of spontaneous activity. All of these can be mimicked by stimulation of muscarinic receptors with exogenously applied acetylcholine (ACh) or related muscarinic agonists (Bolton, 1978). In single smooth muscle cells or myocytes prepared from gastrointestinal tissues using enzymatic treatment, ACh activates G-protein coupled non-selective cation channels to produce depolarization (Benham *et al.*, 1985; Inoue and Isenberg, 1990; Vogalis and Sanders, 1990; Kim *et al.*, 1995; Kuriyama *et al.*, 1998; Kim *et al.*, 2003; Lee *et al.*, 2003b; So and Kim, 2003). The activation of these channels is inhibited by a group of K-channel blockers such as quinidine, TEA and 4-aminopyridine (Chen *et al.*, 1993; Kim *et al.*, 1995) or divalent cations such as Cd^{2+}, Ni^{2+}, Mn^{2+} and Co^{2+} (Inoue, 1991; Lee *et al.*, 1993; Zholos and Bolton, 1995). Strong inhibition of muscarinic non-selective cation conductance by flufenamic acid is also noted in intestinal smooth muscle (Chen *et al.*, 1993; Yamada *et al.*, 1996; Hill *et al.*, 2004). The selectivity of these chemicals to ion channels does not seem to be high, as they have been shown to inhibit other types of ion channels, including a group of non-selective cation channels (Kuriyama *et al.*, 1998).

Attempts were made to test the effects of chemicals (such as quinidine, Ni^{2+}, flufenamic acid) known to inhibit muscarinic non-selective cation channels on ACh-induced depolarization in circular smooth muscle tissue isolated from the guinea-pig stomach antrum. As flufenamic acid has inhibitory actions on Ca^{2+}-sensitive Cl^{-}-channels, the effects of chemicals (such as niflumic acid and DIDS) with similar actions to flufenamic acid (Large and Wang, 1996) were also tested. The effects of SKF-96365 and La^{3+}, known inhibitors of some types of Ca^{2+} influx (Kuriyama *et al.*, 1998), on ACh-induced depolarization were also examined. The results have indicated that none of these chemicals inhibited the ACh-induced depolarization, although some were effective in modulating spontaneously generating slow potentials. Possible discrepancy of

these results from those obtained in experiments using dispersed single cells is discussed.

Methods

Guinea-pigs of either sex, weighing 200–500 g, were anaesthetized with fluoromethyl 2,2,2-trifluoro-1-(trifluoromethyl) ethyl ether (Sevoflurane; Maruishi Pharmaceutical, Osaka, Japan) and decapitated. All animals were treated ethically according to the Guiding Principles for the Care and Use of Animals in the Field of Physiological Sciences, as approved by The Physiological Society of Japan. The stomach was excised and opened in Krebs solution by cutting along the small curvature. The mucosa was removed by cutting with fine scissors, and smooth muscle tissue was isolated from the antrum region. A segment of circular muscle bundles (single bundle, 80–100 μm wide and 200–300 μm long) was prepared by using fine forceps to mechanically remove the longitudinal muscle layer with the attached myenteric layers,. The preparation was pinned out on a Sylgard plate (silicone elastomer, Dow Corning, Midland, MI, USA) at the bottom of the recording chamber (25 mm wide, 2.5 mm deep, 1.7 mm long), and superfused with warmed (35°C) Krebs solution at a constant flow rate (about 2 ml min^{-1}). The recording chamber was mounted onto the stage of an inverted microscope (Nikon IX-70, Tokyo, Japan).

Electrical responses of smooth muscle cells were recorded using conventional microelectrode methods. The glass capillary microelectrodes (outer diameter 1.5 mm, inner diameter 0.8 mm, Hilgenberg, Germany) filled with 3 M KCl had tip resistances ranging between 50 and 80 MΩ. Electrical responses recorded via a high-input-impedance amplifier (Microelectrode Amplifier MEZ-8300, Nihon Kohden, Tokyo, Japan), were displayed on a cathode-ray oscilloscope (SS-7602, Iwatsu, Osaka, Japan) and stored on a personal computer for later analysis. Possible contamination of the preparations by myenteric interstitial cells of Cajal (ICC-MY) in the circular muscle bundle was tested using 1 mM caffeine, and preparations whose slow potentials were not abolished were excluded from the present experiments (Nose *et al.*, 2000).

The ionic composition of the Krebs solution was as follows (in mM): Na^+ 137.4, K^+ 5.9, Mg^{2+} 1.2, Ca^{2+} 2.5, HCO_3^- 15.5, $H_2PO_4^-$ 1.2, Cl^- 134, glucose 11.5. Nifedipine (1 μM) was added to all Krebs solution. The solution was aerated with O_2 containing 5% CO_2, and had a pH of 7.2–7.3.

Drugs used were acetylcholine chloride (ACh), atropine sulphate, caffeine, flufenamic acid, $LaCl_3$, $NiCl_2$, nifedipine, niflumic acid, SKF-96865, quinidine and 4,4'-diisothiocyanatostilbene-2,2'-disulphonic acid (DIDS) (purchased from Calbiochem, San Diego, CA, USA). DIDS, flufenamic acid, niflumic acid, nifedipine, SKF-96865 and mibefradil were dissolved in dimethyl sulphoxide (DMSO) to make a stock solution. Other chemicals were dissolved in distilled water to make the stock solution. These chemicals were diluted further with Krebs solution to prepare desired concentrations (the volume ratios of the dilution were over 1:1000). The dilution procedures did not alter the pH of the Krebs solution.

Experimental values were expressed as the mean value ± standard deviation (SD). Statistical significance was tested using Student's *t*-test, and probabilities less than 5% ($P<0.05$) were considered to be significant.

Results

ACh-induced electrical responses of antrum circular muscle

The effects of ACh (1 nM–3 μM) on the amplitude and frequency of slow potentials were examined in isolated circular muscle preparations of the stomach antrum. Preliminary experiments revealed a dual concentration-dependent action of ACh on the electrical responses of the membrane. At low concentrations (< 0.1 μM), ACh increased the amplitude and frequency of slow potentials with no change in the resting membrane potential, while at high concentrations (0.3–3 μM), ACh depolarized the membrane and increased the frequency of slow potentials further in a concentration-dependent manner. The amplitude of the slow potentials was not significantly changed in the presence of 1 μM ACh, but was smaller than in the absence of ACh during exposure to 3 μM ACh, however with an associated depolarization of the membrane at both concentrations. These excitatory actions of ACh were abolished by 1 μM atropine, with no significant alteration to the resting membrane potential (control, –65.7 ± 2.1 mV; in atropine, –65.0 ± 3.1 mV; n=5; $P>0.05$). These results confirmed previous observations (Nakamura and Suzuki, 2004). Experiments were carried out to investigate the effects of either quinidine, Ni^{2+} or flufenamic acid, known inhibitors of muscarinic non-selective cation channels (Kuriyama *et al.*, 1998), on the depolarization produced by 1 μM ACh. All experiments were carried out in the presence of 1 μM nifedipine.

Effects of quinidine, Ni^{2+} or flufenamic acid on the ACh-induced depolarization

At concentrations of quinidine ranging between 0.1 and 10 μM, there was no marked effect on the electrical responses (the resting membrane potential, amplitude and frequency of slow potentials) of the antrum muscle. Figure 1 shows the effects of 10 μM quinidine on the ACh-induced responses recorded from circular smooth muscle cells of the stomach antrum. Application of 10 μM quinidine did not alter either the resting membrane potential (control, –67.5 ± 2.2 mV; in quinidine, –67.1 ± 2.0 mV; n=12, $P>0.05$) or the frequency and amplitude of slow potentials (Fig. 1, D and E, respectively). ACh depolarized the membrane in the absence and presence of quinidine (Fig. 1, A and B, respectively), and the amplitude was significantly larger in the presence of quinidine than in its absence (Fig. 1C). The increase in frequency and amplitude of slow potentials caused by ACh was also not changed by quinidine (Fig. 1, D and E). Thus, quinidine increased the amplitude of ACh-induced depolarization with no alteration to the amplitude and frequency of slow potentials.

The effects of 50 μM Ni^{2+} on both slow potentials and ACh-induced depolarization are summarized in Fig. 2. Application of Ni^{2+} produced transient depolarization with an associated increase in both the amplitude and frequency of slow potentials and also of unitary potentials (Fig. 2B). In the continued presence of Ni^{2+}, the membrane was repolarized to the resting level within 5–10 min. In the stabilized condition to Ni^{2+}, slow potentials with increased amplitude were generated in frequencies higher than those generated in the absence of Ni^{2+} (Fig. 2, E and F). ACh (1 μM) depolarized the membrane and increased both the amplitude and frequency of slow potentials to a similar extent, either in the absence or presence of Ni^{2+} (Fig. 2, A and C, respectively). The quantified data confirmed that Ni^{2+} did not significantly alter the amplitude of

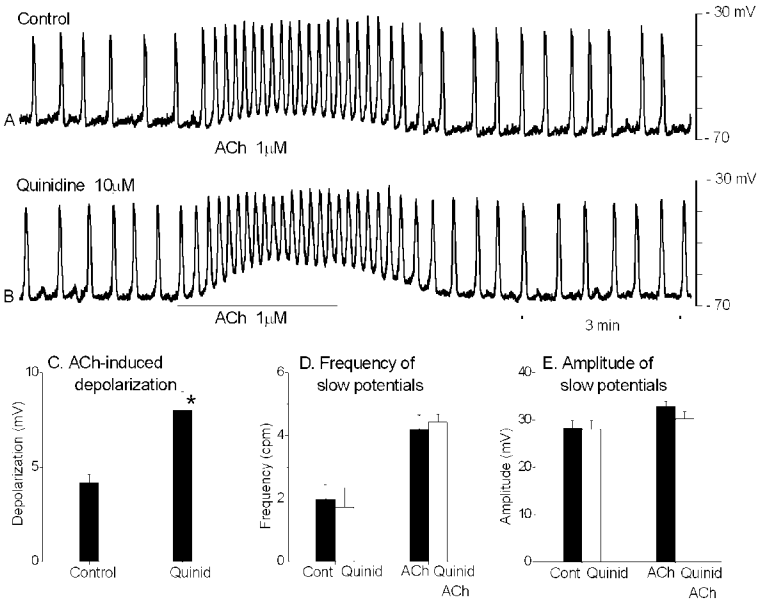

Fig. 1. Effects of quinidine on the ACh-induced responses. ACh (1 μM) was applied for 3 min in the absence (A) and presence of 10 μM quinidine (B). The effects of quinidine on the amplitude of ACh-induced depolarization (C) and the frequency (D) and amplitude (E) of slow potentials in the absence and presence of ACh are summarized as the mean + S.D. obtained from 5–12 tissues. In D and E, filled and open columns represent the values obtained in the absence and presence of 10 μM quinidine, respectively. *, significant from individual control values (P<0.05).

ACh-induced depolarization (Fig. 2D).

Experiments were carried out to test the effects of two concentrations of flufenamic acid (10 and 100 μM) on the ACh-induced responses. As reported previously (Hotta *et al.*, 2005), flufenamic acid (>10 μM) inhibited the generation of slow potentials with no alteration to the resting membrane potential (Fig. 3, D). However, application of 100 μM flufenamic acid depolarized the membrane by 3–5 mV and inhibited the generation of both slow potentials and unitary potentials (Fig. 3, C and D). Application of 1 μM ACh elicited generation of slow potentials with increased amplitude and frequency, either in the absence (Fig. 3A) or presence of 10 μM flufenamic acid (Fig. 3, B). The amplitude of the slow potentials and the membrane depolarization produced by ACh were not significantly changed by 10 μM flufenamic acid (Fig. 3, D and E). In the presence of 100 μM flufenamic acid, while the amplitude of ACh-induced depolarization was larger, the amplitude of slow potentials was smaller than in the absence of flufenamic acid, and as a consequence the peak amplitude of slow potentials generated in the presence of ACh was not significantly changed (Fig. 3D). The frequency of slow potentials generated during stimulation with ACh was not changed by either concentration of flufenamic acid (Fig. 3E). Thus, flufenamic acid mainly inhibited the amplitude of slow potentials, with no marked effect on their frequency. The amplitude of the ACh-induced depolarization was increased by a high concentration of flufenamic acid.

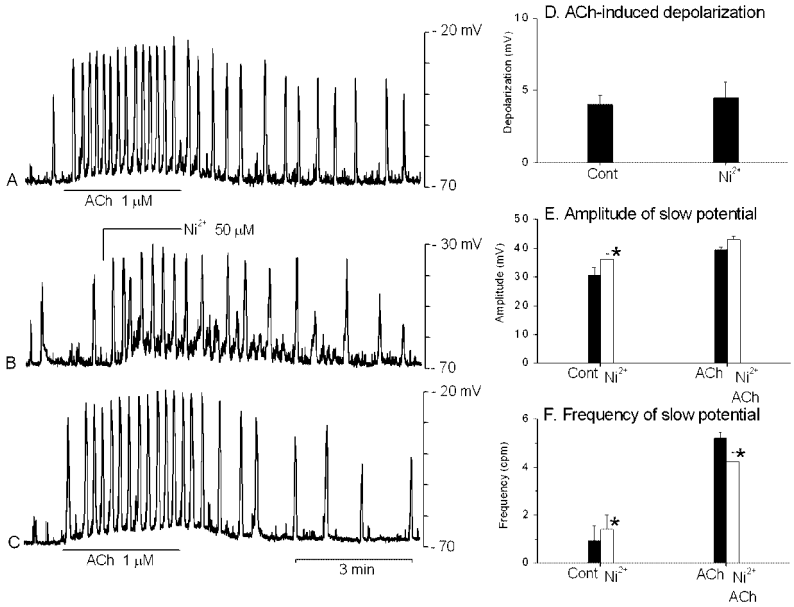

Fig. 2. Effects of Ni^{2+} on the ACh-induced responses. Electrical responses were recorded from circular smooth muscle tissue isolated from the guinea-pig gastric antrum, in the absence (A) and presence of 50 μM Ni^{2+} (C). B. Responses produced by 50 μM Ni^{2+}. A–C were recorded from the same cell. The effects of Ni^{2+} on the ACh-induced depolarization (D) and on the amplitude (E) and frequency (F) of slow potentials are summarized. In E and F, values obtained in the absence (filled column) and presence of Ni^{2+} (open column) are shown by the mean ± S.D. (n=3–5). Nifedipine (1 μM) was present throughout. *, significant from values obtained in the absence of Ni^{2+} (P<0.05).

Effects of niflumic acid or DIDS on the ACh-induced depolarization

Flufenamic acid has multiple actions on different types of ion channel, with an inhibitory action on Ca^{2+}-sensitive Cl^--channels in gastric muscle (Hotta *et al.*, 2005), as well as an inhibitory action on non-selective cation channels (Chen *et al.*, 1993; Hill *et al.*, 2004). Attempts were therefore made to examine the effects of niflumic acid and DIDS on the ACh-induced responses, since these chemicals had also been known to inhibit Ca^{2+}-sensitive Cl^--channels in smooth muscle of the mouse (Large and Wang, 1996).

Niflumic acid (10 μM) hyperpolarized the membrane (control, –66.0 ± 1.9 mV; in niflumic acid, –68.9 ± 1.6 mV; n=12, P<0.05) and increased the amplitude and frequency of slow potentials (Fig. 4, B, E and F). The amplitude of depolarization produced by 1 μM ACh was larger in the presence of niflumic acid than in its absence (Fig. 4, A and C). The quantified data indicated that the amplitude of ACh-induced depolarization was significantly increased by niflumic acid (Fig. 4D), with an associated increase in the frequency of slow potentials (Fig. 4F), but with no alteration to the amplitude of spontaneous and ACh-evoked slow potentials (Fig. 4E).

In contrast to niflumic acid, DIDS (100 μM) produced inhibitory effects on slow potentials, with an associated hyperpolarization of the membrane (control, –66.5 ± 2.3 mV, in DIDS, –68.9 ± 2.1 mV, n=5, P<0.05). In the presence of DIDS, the amplitude of slow potentials was reduced

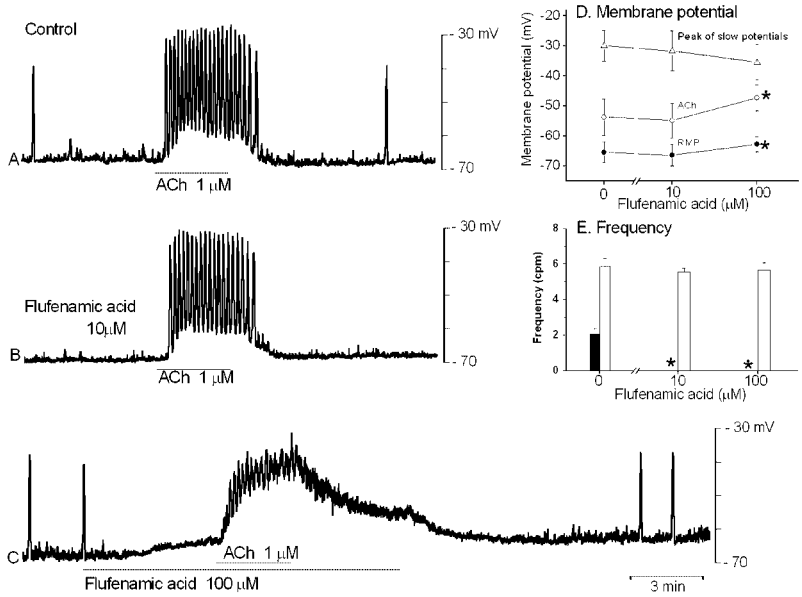

Fig. 3. Effects of flufenamic acid on the ACh-induced responses. Electrical responses produced by 1 μM ACh were recorded in the absence (*A*) and presence of flufenamic acid (B, 10 μM, C, 100 μM). D. Membrane potentials were measured in the absence (RMP, filled circles) and presence of 1 μM ACh (ACh, open circles), and also the peak of slow potentials generated during stimulation with ACh (open triangles), in the presence of different concentrations of flufenamic acid (0, 10 and 100 μM). E. Frequency of slow potentials was measured in the presence of different concentrations of flufenamic acid (0, 10 and 100 μM), in the absence (filled column) and presence of 1 μM ACh (open column). Mean ± S.D. (n=4–6 for each value). *, significant from individual control values (P<0.05).

(Fig. 5E), with no significant change in their frequency (Fig. 5D). The amplitude of slow potentials evoked in the presence of ACh was also smaller in the presence of DIDS than in its absence (Fig. 5E), but the amplitude of ACh-induced depolarization and the frequency of slow potentials were not significantly changed by DIDS (Fig. 5, C and D, respectively). These results suggest that Ca^{2+}-sensitive Cl^--channels are not activated during the ACh-induced depolarization.

Effects of SKF-96365 or La^{3+} on ACh-induced depolarization

Activation of muscarinic receptors with ACh enhances the capacitative entry of Ca^{2+} indirectly as a result of depletion of internal Ca^{2+} stores through elevated production of inositol trisphosphate (IP_3), and this Ca^{2+} entry is inhibited by high concentrations (in the order of mM) of divalent ions such as Ni^{2+}, Cd^{2+} and Zn^{2+}, and also by SKF-96365 or La^{3+} (Kuriyama *et al.*, 1998). Attempts were made to investigate the effects of SKF-96365 or La^{3+} on the ACh-induced depolarization in circular muscle of the guinea-pig stomach.

Application of SKF-96365 (3–50 μM) depolarized the membrane in a concentration-dependent manner, with an associated increase in the frequency of slow potentials (Fig. 6, B, D and F). The amplitude of the slow potentials was not significantly changed by 3–30 μM SKF-

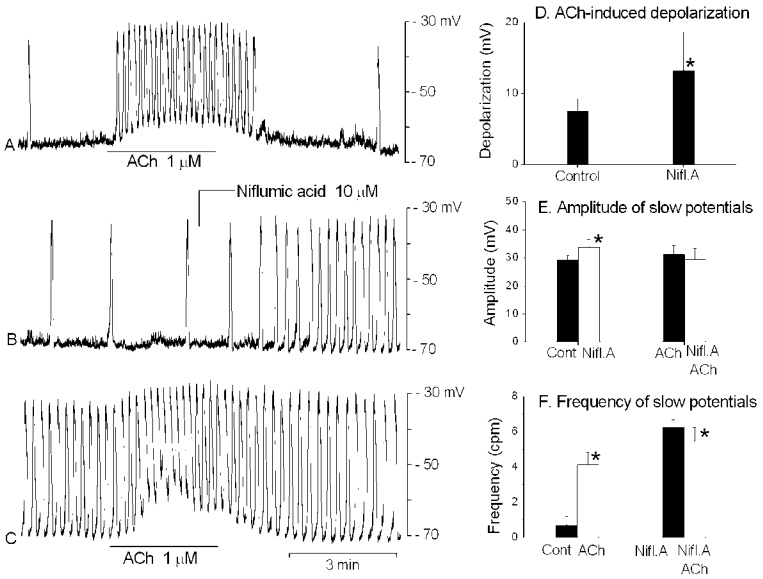

Fig. 4. Effects of niflumic acid on the ACh-induced responses. Electrical responses produced by 1 μM ACh were recorded in the absence (A) and presence of 10 μM niflumic acid (C). B, Electrical responses produced by 10 μM niflumic acid. A–C were recorded from the same cell. The effects of niflumic acid on the amplitude of ACh-induced depolarization (D) and the amplitude of slow potentials (E) are summarized. In F, the frequency of slow potentials generated in the absence (filled column) and presence of niflumic acid (open column) is shown by the mean ± S.D. (n=5–10). *, significant from control (P<0.05).

96365 and was reduced by 50 μM SKF-96365 (Fig. 6E), the latter being possibly related to a depolarization of the membrane. The amplitude of ACh-induced depolarization was reduced by SKF-96365 in a concentration-dependent manner, and as a consequence the level of the ACh-induced depolarization remained constant up to a concentration of 30 μM SKF-96365 (Fig. 6D). In the presence of 50 μM SKF-96365, the level of the depolarization was significantly shifted to a positive level and the ACh-induced depolarization was abolished (Fig. 6D). The amplitude of the slow potentials was increased by ACh in the absence and presence of SKF-96365, and the concentration-dependent changes were parallel to those produced by SKF-96365 alone (Fig. 6E). The frequency of slow potentials was increased to about 6 cpm by ACh, either in the absence or presence of any concentration of SKF-96365 (Fig. 6F). Thus, the results indicated that SKF-96365 did not show marked effects on the ACh-induced responses.

La^{3+} (50 μM) produced negligibly small effects on electrical responses of the circular muscle tissue isolated from the guinea-pig stomach antrum. The amplitude of depolarization and the increase in amplitude and frequency of slow potentials produced by 1 μM ACh were not significantly different between either the absence or presence of 50 μM La^{3+} (Fig. 7, A–C), as confirmed in the quantified data (Fig. 7D, ACh-induced depolarization; Fig. 7E, frequency of slow potentials; Fig. 7F, amplitude of slow potentials).

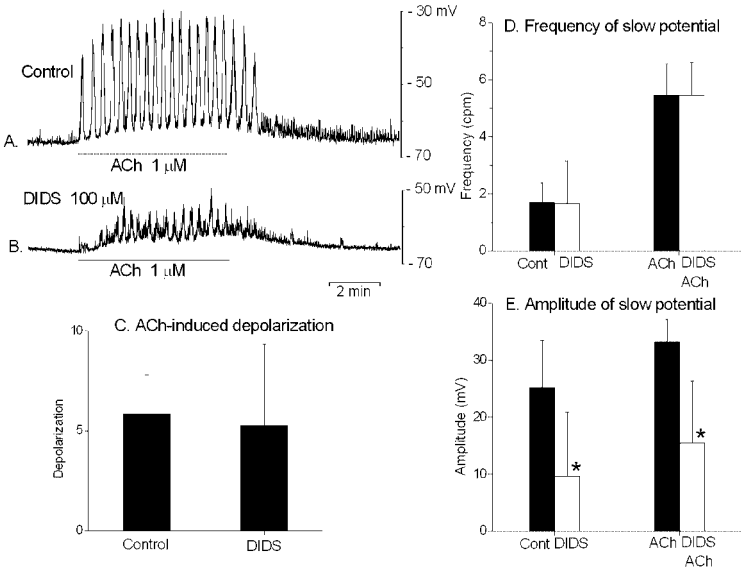

Fig. 5. Effects of DIDS on the ACh-induced responses. The effects of 1 μM ACh on electrical responses of circular smooth muscle tissue isolated from the guinea-pig gastric antrum were observed in the absence (A) and presence of 100 μM DIDS (B). Records A and B were obtained from the same cell. Nifedipine (1 μM) was present throughout. The effects of DIDS on the amplitude of ACh-induced depolarization (C) and on the frequency (D) and amplitude (E) of slow potentials recorded from 3 tissues are summarized. In D and E, slow potentials generated spontaneously or in the presence of ACh, in the absence (Control, filled column) and presence of DIDS (open column), are shown as the mean ± S.D. (n=5). *, significant from control (P<0.05).

Discussion

The present experiments were carried out to investigate the properties of depolarization produced by ACh in circular muscle isolated from the guinea-pig stomach antrum. The chemicals tested were quinidine, Ni^{2+}, flufenamic acid, niflumic acid, DIDS, La^{3+} and SKF-96365, and the results indicated that none of them could inhibit ACh-induced depolarization. These chemicals are known to inhibit muscarinic receptor coupled non-selective cation channels (quinidine, Ni^{2+}, flufenamic acid), Ca^{2+}-sensitive Cl^--channels (flufenamic acid, niflumic acid, DIDS) or capacitative Ca^{2+} channels (Ni^{2+}, La^{3+}, SKF-96365) (Kuriyama *et al.*, 1998), suggesting that any one of these channels is important for ACh-induced depolarization. Thus, these results did not support the conclusion obtained in patch clamp experiments using dispersed single smooth muscle cells (Inoue and Isenberg, 1990; Chen *et al.*, 1993; Kim *et al.*, 1995; Zholos and Bolton, 1995; So and Kim, 2003). Although the discrepancy between the results reported previously and those presented here remains unclear, the following two possibilities are considered. One possibility is related to the difference in the experimental milieu of the smooth muscle cells; in patch clamp experiments, single smooth muscle cells are studied and therefore the receptors stimulated by ACh or muscarinic agonists are distributed in the smooth muscle membrane, while in the present experiments, the bundle of circular smooth muscle tissue

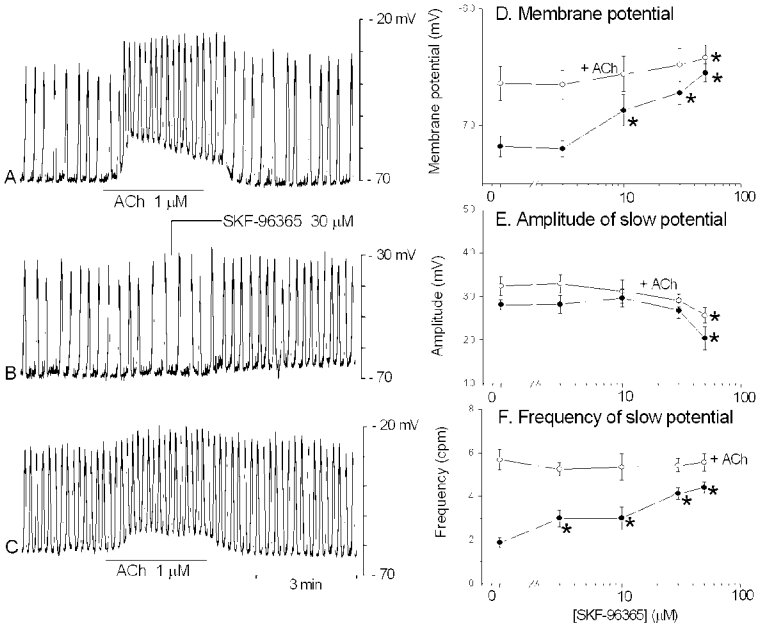

Fig. 6. Effects of SKF-96365 on the ACh-induced responses. ACh (1 μM) was applied for 3 min in the absence (A) and presence of 10 μM SKF-96365 (C; B shows the initial responses produced by SKF-96365). D. The membrane potentials measured in the absence and presence of 1 μM ACh (filled and open circles, respectively) are shown as a function of the concentration of SKF-96365 (5–50 μM). Mean ± S.D. of values obtained from 3–5 preparations. The amplitude (E) and frequency (F) of slow potentials generate in the absence and presence of 1 μM ACh (filled and open circles, respectively) are shown as a function of the concentration of SKF-96365. *, significant from the value measured in the absence of SKF-96365 (P<0.05).

contained both smooth muscle cells and ICC-IM (Komuro *et al.*, 1996). Although recordings were made from individual smooth muscle cells, it remains unclear which cell is stimulated by ACh, since both cell types are electrically coupled (Hirst and Ward, 2003). It has recently been proposed that in gastrointestinal smooth muscle, cholinergic nerves terminate mainly on ICC-IM, and their stimulation produces excitatory cholinergic junction potentials in ICC-IM first, before the potential is conducted to smooth muscle cells in an electrotonic manner (Ward *et al.*, 2000; Hirst and Ward, 2003). If this is the case, it is reasonable to consider the heterogeneous distribution of muscarinic receptors on both ICC-IM and smooth muscle cells: muscarinic receptor-coupled non-selective cation channels activated by ACh are distributed only on smooth muscle cells, while those that are activated by ACh but which cannot be inhibited by a group of known inhibitors of non-selective cation channels are distributed in ICC-IM.

The second possibility is that the population of muscarinic receptors has a heterogeneous threshold for excitation. In experiments using single smooth muscle cells, the concentrations of ACh or muscarinic agonists tested were extremely high (equal to 100–300 μM; Inoue, 1991; Chen *et al.*, 1993; Kim *et al.*, 2003), in comparison with those tested in the present experiments (equal to 1 μM). In circular smooth muscle bundles from the guinea-pig stomach antrum, ACh

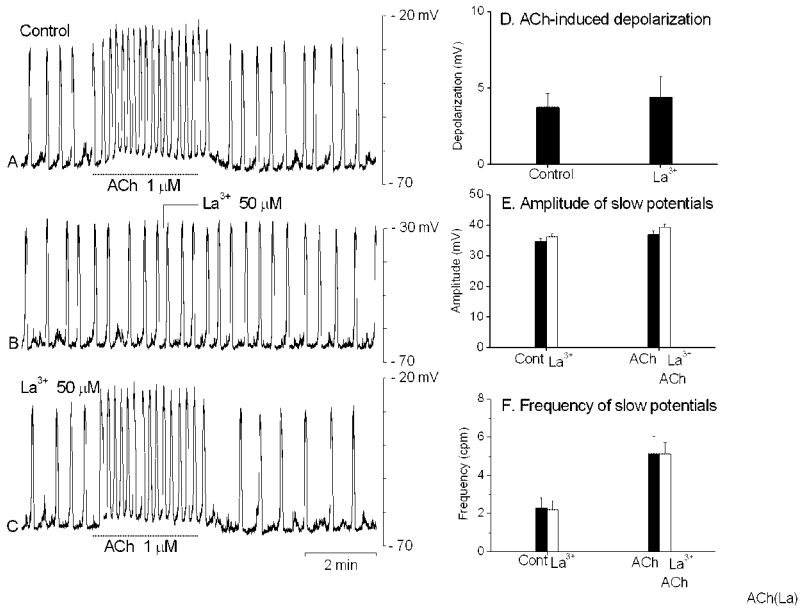

Fig. 7. Effects of La^{3+} on the ACh-induced responses. Electrical responses produced by 1 μM ACh were recorded from circular smooth muscle tissue isolated from the guinea-pig gastric antrum, in the absence (A) and presence of 50 μM La^{3+} (C). Application of 50 μM La^{3+} did not produce marked effects on electrical responses (B). The effects of La^{3+} on the amplitude of ACh-induced depolarization (D) and on the amplitude (E) and frequency (F) of spontaneous and ACh-evoked slow potentials are summarized. In E and F, values obtained in the absence (filled column) and presence of La^{3+} (open column) are shown as the mean ± S.D. (n=6–7).

produces excitatory responses in two ways. At low concentrations (1–100 nM), ACh increased the amplitude and frequency of slow potentials with no change in the resting membrane potential, while at high concentrations (> 300 nM) ACh depolarized the membrane and further increased the frequency of slow potentials, but with a decreased amplitude due to concomitant depolarization of the membrane (Kim *et al.*, 2003; Nakamura and Suzuki, 2004). The concentration of ACh tested in the present experiments was just above the threshold for depolarization, since these concentrations of ACh are considered comparable to those effective in physiological conditions (Komori and Suzuki, 1988). The amplitude of depolarization produced by 1 μM ACh (equal to 5–8 mV) was comparable to that produced by repeated excitation of cholinergic nerves at 5–10 Hz frequency (Komori and Suzuki, 1986; Lee *et al.*, 2003a). The frequency of slow potentials is a function of the concentration of intracellular Ca^{2+} (Fukuta *et al.*, 2002), and the increase in frequency of slow potentials by ACh in the absence of membrane depolarization is probably due to the activation of protein kinase C (PKC) in ICC-IM (Suzuki *et al.*, 2002; Kito *et al.*, 2002; Nakamura and Suzuki, 2004). It is therefore reasonable to speculate that there is an involvement of different types of ion channel activated by different concentrations of muscarinic stimulant; ion channels activated by high concentrations of ACh which are distributed in both ICC-IM and smooth muscle cells, while those activated by low

concentrations of ACh are distributed only in ICC-IM. Either of these two alternatives may provide an explanation for the discrepancy which has appeared between experiments using either single smooth muscle cells or intact circular muscle bundles.

In addition to the failure of the inhibition of ACh-induced depolarization, some chemicals (quinidine, niflumic acid) increased the amplitude of ACh-induced depolarization. Hyperpolarization of the membrane may be a reasonable interpretation for this increase in the presence of niflumic acid, and this is supported by the increase in the amplitude of slow potentials (Kito and Suzuki, 2003). However, no associated change in the resting membrane potential was observed in the case of quinidine, and this was accompanied by an unaltered amplitude of the slow potentials. It remains unclear why the amplitude of ACh-induced depolarization was increased by quinidine. Although La^{3+} has been reported to have potentiating actions on the ACh-induced cation current in smooth muscle cells of the guinea-pig ileum (Inoue *et al.*, 1998), this has not been confirmed in the present experiments, possibly because of the difference in concentration of La^{3+} examined; the potentiating actions appear in milimolar concentrations of La^{3+} (Inoue *et al.*, 1998), while the inhibitory actions on capacitative Ca^{2+} entry appear in micromolar concentrations of La^{3+} (Kuriyama *et al.*, 1998). The amplitude of ACh-induced depolarization was also increased when muscles were exposed to a high concentration (100 μM) of flufenamic acid. This concentration of flufenamic acid inhibits spontaneous slow potentials and also pacemaker potentials generated in ICC-MY (Hotta *et al.*, 2005). Multiple actions of flufenamic acid have been recognized on different types of ion channel in smooth muscles, mostly at higher concentrations (Yamada *et al.*, 1996). Thus, it is not likely that the increase in amplitude of ACh-induced depolarization in the presence of high concentration of flufenamic acid is causally related to the inhibition of Ca^{2+}-sensitive Cl^--channels. As flufenamic acid did not alter the frequency of the slow potentials generated during stimulation with ACh, even though it altered their amplitude, the elevation of $[Ca^{2+}]_i$ may not be significantly different between the absence and presence of flufenamic acid, and this could be extrapolated to indicate that the effects of flufenamic acid on Ca^{2+}-sensitive Cl^--channels are not changed by $[Ca^{2+}]_i$, unlike in the case of niflumic acid (Piper *et al.*, 2002).

Although none of the chemicals tested in the present experiments inhibited ACh-induced depolarization, the frequency and amplitude of slow potentials were modulated by some of them. The frequency of slow potentials was increased by SKF-96365 and niflumic acid. Ni^{2+} also increased the frequency of slow potentials but transiently, possibly due to the associated depolarization of the membrane. In the stabilized condition to Ni^{2+}, the frequency and amplitude of slow potentials remained at a higher level than in the absence of Ni^{2+}. Although both Ni^{2+} and SKF-96365 are known inhibitors of capacitative Ca^{2+}-channels (Kuriyama *et al.*, 1998), it remains unclear why Ni^{2+} altered the properties of spontaneously generated slow potentials. The increase in amplitude and frequency of slow potentials by niflumic acid may be not related to its inhibitory actions on Ca^{2+}-sensitive Cl^--channels, since other inhibitors of these channels (flufemanic acid, DIDS) reduced the amplitude or frequency of slow potentials. Niflumic acid has dual actions on some type of smooth muscle, and circular smooth muscle tissue may probably be the representative case for $[Ca^{2+}]_i$-dependent dual actions on Ca^{2+}-sensitive Cl^--channels with agonistic actions at high $[Ca^{2+}]_i$ and antagonistic actions at low $[Ca^{2+}]_i$ (Piper *et al.*,

2002). The increase in amplitude of slow potentials by niflumic acid may be causally related to the hyperpolarization of the membrane. However, this is not the case for DIDS, and the amplitude of ACh-induced depolarization is similar to that in its absence during hyperpolarization with DIDS. High concentrations of DIDS often inhibit some types of ion channels, possibly due to a reduction in $[Ca^{2+}]_i$ (Kuriyama *et al.*, 1998), although this possibility is unlikely in the case of the circular smooth muscle bundles of the guinea-pig stomach, since there is no associated alteration of the frequency during the inhibition of the amplitude of the slow potentials. The changes in amplitude and frequency of slow potentials produced by different concentrations of SKF-96865 may be related to the depolarization of the membrane.

In summary, these experiments on the circular smooth muscle bundles isolated from the guinea-pig stomach antrum have shown that ACh (1 μM) depolarized the membrane and increased the amplitude and frequency of slow potentials. The ACh-induced depolarization was not inhibited by quinidine, Ni^{2+}, flufenamic acid, niflumic acid, DIDS, La^{3+} or SKF-96365, suggesting that it was not produced by activation of non-selective cation channels, Ca^{2+}-sensitive Cl^--channels or capacitative Ca^{2+}-channels distributed in smooth muscle cells. The possible involvement of a heterogeneous distribution of muscarinic receptor-coupled ion channels between smooth muscle cells and ICC-IM was considered. Alternatively, the discrepancy could be interpreted by assuming that muscarinic receptors with different thresholds to ACh are coupled to the activation of non-selective cation channels which are sensitive to quinidine, Ni^{2+} or flufenamic acid.

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