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Mibefradil-sensitive component involved in the plateau potential in submucosal interstitial cells of the murine proximal colon

Aya Hotta [a,b,*], Noriko Okada [b], Hikaru Suzuki [a]

[a] Department of Regulatory Cell Physiology, Graduate School of Medical Sciences, Nagoya City University, Nagoya 467-8601, Japan
[b] Department of Biodefence, Graduate School of Medical Sciences, Nagoya City University, Nagoya 467-8601, Japan

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Abstract

Submucosal interstitial cells of Cajal (ICC_{SM}) produce plateau potentials comprised of initial fast and subsequent plateau components. The possible involvement of voltage-dependent Ca^{2+} channels in plateau potentials was examined in ICC_{SM} of the murine proximal colon. Increases in external K^+ concentration ($[K^+]_o$) changed the rise rate of the initial component in a biphasic way, an increase in 10.6 or 15.3 mM $[K^+]_o$ and a decrease in 20.0 mM $[K^+]_o$. The rise rate of plateau potentials was significantly reduced by the application of 3 μM mibefradil or 100 μM Ni^{2+} but not by 0.3 μM nifedipine. The inhibitory effect of mibefradil on the rise rate of plateau potentials was concentration-dependent with an IC_{50} value of 1.0 μM. In conclusion, the initial phase of plateau potentials is partly due to the activation of T-type Ca^{2+} channel in ICC_{SM} from the murine proximal colon.

Keywords: Interstitial cells of Cajal; Plateau potential; T-type Ca^{2+} channel; Mibefradil; Proximal colon

Gastrointestinal smooth muscle generates spontaneous electrical activity in the form of slow waves or spike potentials [1,2]. The rhythmic activity of smooth muscle is thought to be initiated from interstitial cells of Cajal (ICC) from evidence that slow waves are absent in the intestinal smooth muscles of ICC-deficient mice [3]. ICC is identified by the expression of tyrosine kinase receptor, c-kit, and is also characterized from histological features such as the presence of many mitochondria, bundles of intermediate filaments, and gap junctions [4]. Based on their functional and regional variations, many subtypes of ICC have been recognized. Of them, ICC distributed in the myenteric layer of the gastrointestinal tissues (ICC_{MY}) produces pacemaker potentials for slow waves [1,2]. In addition to ICC_{MY}, the colon of some species also has a network of ICC in the submucosal layer (ICC_{SM}) [5,6]. Although the physiological and pathological roles of ICC_{SM} remain unclear, ICC_{SM} of the mouse proximal colon is c-kit positive and shows periodical generation of plateau potentials [7], suggesting a possible contribution to special functions.

In isolated gastrointestinal ICC, the expression of many types of ion channels has been reported; voltage-gated K^+ channel [8,9], Cl^- channel conductance [10,11], non-selective cation channel [12], and voltage-dependent Ca^{2+} channel (VDCC) [7,13]. In addition, Ca^{2+} released from internal stores through the activation of inositol 1,4,5-trisphosphate (IP_3) and ryanodine receptors seems to be required for generating spontaneous activity in gastrointestinal pacemaker cells [14–17]. However, the contribution of any of these ion channels to the formation of pacemaker potentials generated in ICC remains unclear. Pacemaker potentials recorded from ICC_{MY} distributed in intact tissues were revealed to be formed by the activation of IP_3-induced Ca^{2+} release and subsequent Ca^{2+}-activated Cl^- channel [15,18].

In this investigation, we examined to elucidate the channel profiles involved in plateau potentials observed in

* Corresponding author. Fax: +81 52 842 1538.
E-mail address: ayahotta@med.nagoya-cu.ac.jp (A. Hotta).

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ICC_{SM} of the mouse proximal colon using intracellular microelectrode recording under different ionic conditions or in the presence of chemical compounds which selectively interfere with VDCCs (mibefradil, Ni^{2+}, and nifedipine). Our data indicate that mibefradil-sensitive T-type Ca^{2+} channel is involved in the primary fast component of plateau potentials generated in ICC_{SM} of the murine proximal colon.

Materials and methods

Tissue preparation. All experiments were performed according to the Guiding Principles for the Care and Use of Animals in the Field of Physiological Sciences, approved by the Physiological Society of Japan. Male mice (BALB/c) weighing 20–25 g, were anaesthetized with fluoromethyl 2,2,2-trifluoro-1-(trifluoromethyl)ethyl ether (sevoflurane; Maruishi Pharmaceutical, Osaka, Japan), and then decapitated. The proximal colon was isolated and opened along the mesenteric border, and thereafter the mucosal layer was removed in Krebs' solution. A small piece of tissue (2×3 mm) was immobilized on a silicon rubber plate fixed at the bottom of a recording chamber (1 ml in volume) with the submucosal layer uppermost. The tissue was superfused with aerated and warmed (35 °C) Krebs' solution at a flow rate of 3 ml/min.

Electrophysiology. Electrophysiological recordings were performed using a conventional microelectrode technique with a microelectrode amplifier (CEZ-500; Nihon Kohden, Tokyo, Japan) and pCLAMP 8 software (Axon Instruments, Foster City, USA), as described previously [19,20]. Glass capillary microelectrodes (borosilicate glass tube, 1.2 mm in outer diameter) filled with 3 M KCl had a tip resistance ranging between 50 and 80 MΩ. The rise rate and half width of plateau potentials were defined as the maximum changes in voltage of the upstroke phase of the initial component during one second and the duration of plateau potentials measured at the half of the peak amplitude, respectively.

Solutions. Krebs' solution had an ionic composition of 137.4 mM Na^+, 5.9 mM K^+, 1.2 mM Mg^{2+}, 2.5 mM Ca^{2+}, 15.5 mM HCO_3^-, 1.2 mM $H_2PO_4^-$, 134 mM Cl^-, and 11.5 mM glucose. Solutions with high external K^+ concentration ($[K^+]_o$) were prepared by replacing NaCl with the equivalent KCl. These solutions were aerated with O_2 containing 5% CO_2, and the pH of these solutions was maintained at 7.2.

Chemicals. Pharmacological reagents were obtained from Sigma–Aldrich (St. Louis, USA). Mibefradil or nifedipine was dissolved in dimethyl sulfoxide at a concentration of 10 mM to prepare as a stock solution. It was confirmed that up to 0.1% of dimethyl sulfoxide did not affect the electrophysiological recordings.

Statistics. Pooled data are shown as means ± SE. Statistical significance between the two groups and among groups was determined by Student's t test and Scheffé's test after one-way analysis of variance, respectively. Significant difference is expressed in figures ($^*p < 0.05$ or

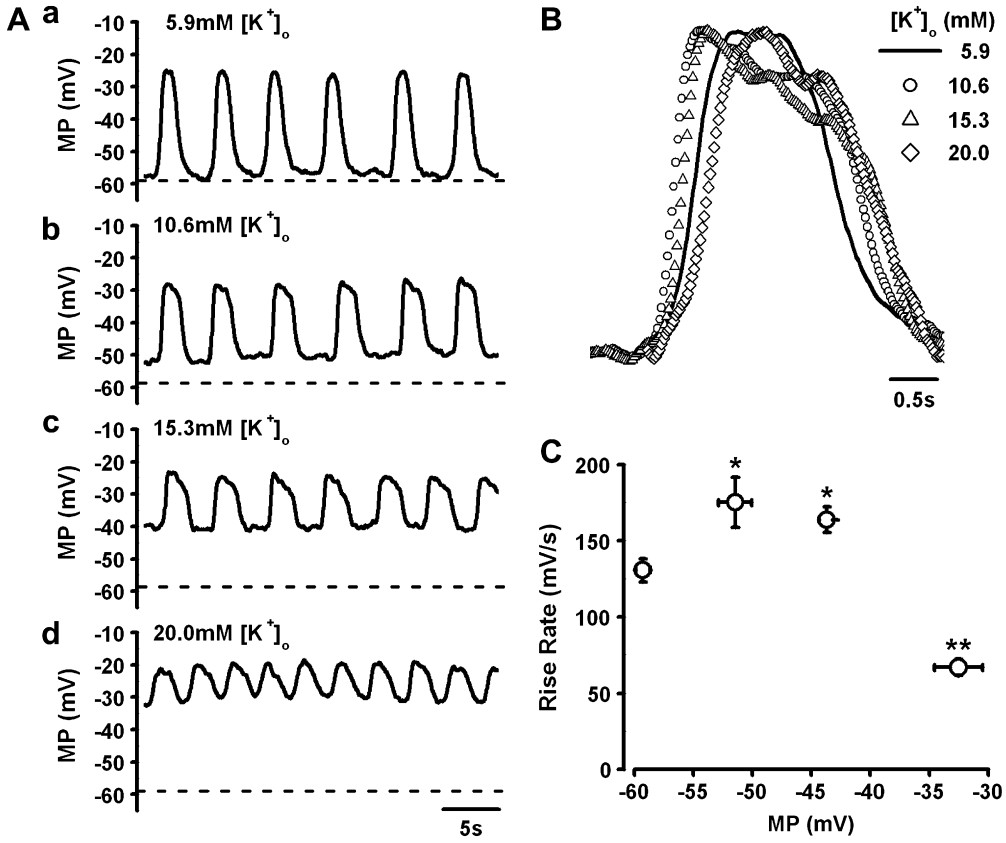

Fig. 1. Membrane potential and rise rate of plateau potentials in ICC_{SM}. Plateau potentials were recorded from ICC_{SM} of murine proximal colon in different $[K^+]_o$ solutions using a conventional microelectrode technique. (A) Representative traces of changes in membrane potential (MP) in $[K^+]_o$ solutions at concentrations of 5.9 (a), 10.6 (b), 15.3 (c), and 20 (d) mM are shown. Note that ICC_{SM} possessed spontaneous and oscillatory activity of membrane potential under resting conditions with about 15 cycles/min. Dotted lines indicate the control level of membrane potential in 5.9 mM $[K^+]_o$ solution. (B) Representative traces of plateau potentials generated in 5.9 (contentious line), 10.6 (circle), 15.3 (triangle), and 20 (diamond) mM $[K^+]_o$ solutions are superimposed after normalization by the amplitude of membrane potential on the same time scale. (C) Relationship between membrane potential and the rise rate of plateau potentials is plotted. Experimental data were obtained from 10 tissues. Statistical significance of the difference is expressed as $^*p < 0.05$ or $^{**}p < 0.01$ vs. rise rate at 5.9 mM $[K^+]_o$.

$^{**}p < 0.01$). Data of the relationship between the mibefradil concentration and the rise rate of plateau potentials were fitted using the following equation, rise rate $= C_0 - (C_0 - C)/\{1 + (K_d/[\text{Mibefradil}])^n\}$, where C_0 is the component in the absence of mibefradil, C is the resistance component, K_d is the apparent dissociation constant, [Mibefradil] is the concentration of mibefradil, and n is the Hill coefficient.

Results

Membrane potential and rise rate of plateau potentials in ICC_{SM}

We examined whether the plateau potentials generated from ICC_{SM} of the mouse proximal colon were involved in the activity of VDCCs using a conventional microelectrode technique. The colonic ICC_{SM} possessed spontaneous and oscillatory activity of membrane potential under resting conditions (5.9 mM $[K^+]_o$) with about 15 cycles/min in all tissues examined (over 100 preparations; Fig. 1Aa). Membrane potential-dependent changes in the parameters of plateau potentials were analyzed in different $[K^+]_o$ solutions. At rest (5.9 mM $[K^+]_o$), the mean resting membrane potential was -59.3 ± 0.6 mV and plateau potentials of 28.7 ± 2.3 mV in amplitude were generated periodically at a frequency of $14.3 \pm 0.2\ \text{min}^{-1}$ ($n = 10$). When different $[K^+]_o$ solutions at concentration of 10.6, 15.3, or 20.0 mM were applied, the membrane potential was depolarized to -51.4 ± 1.4, -43.7 ± 0.7, or -32.5 ± 2.0 mV ($n = 10$, $p < 0.01$), respectively, and thereby the amplitude of plateau potentials was decreased in a concentration-dependent manner (Fig. 1A and C). Interestingly, the superimposed comparison of the initial component of plateau potentials detected in different $[K^+]_o$ solutions (Fig. 1B) showed that the rise rate changed in a biphasic way, increasing in 10.6 (175 ± 16 mV/s, $n = 10$, $p < 0.05$ vs. 5.9 mM $[K^+]_o$ of 131 ± 8 mV/s, $n = 10$) and 15.3 mM $[K^+]_o$ solutions (164 ± 9 mV/s, $n = 10$, $p < 0.05$) and decreasing in 20.0 mM $[K^+]_o$ solution (67 ± 5 mV/s, $n = 10$, $p < 0.01$; Fig. 1C).

Involvement of VDCCs in plateau potentials of ICC_{SM}

To explore the involvement of VDCCs in the primary component of plateau potentials, the effects of chemical modulators for VDCCs were examined on the plateau

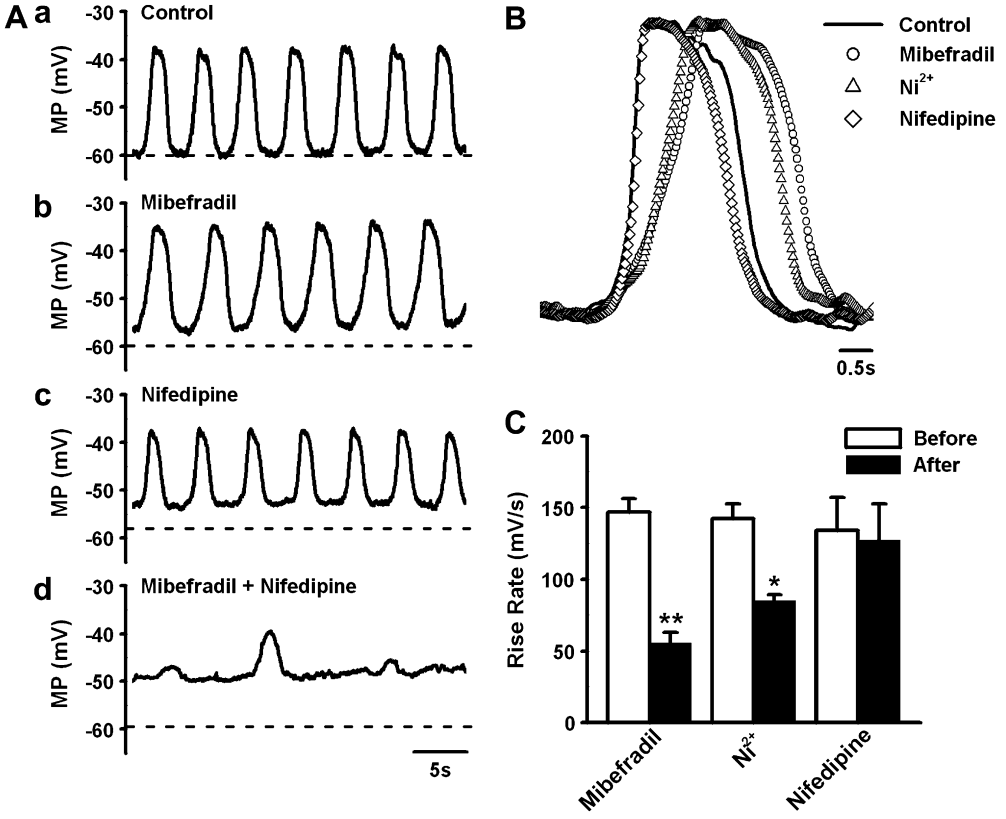

Fig. 2. Modulation by inhibitors for VDCCs on plateau potentials of ICC_{SM}. Effects of various inhibitors for VDCCs on plateau potentials were observed from ICC_{SM}. (A) Representative traces of changes in membrane potential (MP) before (a) and after the application of 3 μM mibefradil (b), 0.3 μM nifedipine (c), or the mixture (d) are plotted. Dotted lines indicate the resting level of membrane potential in the absence of chemical compounds. Note that, in the coapplication of mibefradil and nifedipine, plateau potentials were mostly abolished or, in some occasions, plateau potentials with small and irregular amplitudes were generated. (B) Representative traces of plateau potentials in the absence (continuous line) and presence of 3 μM mibefradil (circle), 100 μM Ni^{2+} (triangle), and 0.3 μM nifedipine (diamond) are superimposed after normalization by the amplitude of membrane potential on the same time scale. (C) Effects on the rise rate of plateau potentials in the presence of 3 μM mibefradil, 100 μM Ni^{2+}, or 0.3 μM nifedipine are summarized. Open and closed columns indicate before and after application of chemical compounds, respectively. Experimental data were obtained from 3 to 12 tissues. Statistical significance of the difference is expressed as $^{*}p < 0.05$ or $^{**}p < 0.01$ vs. before application.

potentials in ICC_{SM}. By the application of 3 μM mibefradil, a selective inhibitor of T-type Ca^{2+} channel [21,22], the resting membrane potential was significantly depolarized from -58.9 ± 1.1 to -54.8 ± 1.6 mV ($n = 12$, $p < 0.05$; Fig. 2A) and the amplitude of plateau potentials was slightly decreased from 28.7 ± 2.3 to 22.0 ± 1.8 mV ($n = 12$, $p < 0.05$). Quantified data indicated that the amplitude of plateau potentials and membrane potential were reciprocally related, the amplitude decreased parallel to the increase in amplitude of depolarization. The frequency of plateau potentials was reduced in the presence of mibefradil from 14.3 ± 0.2 to 13.2 ± 0.2 min^{-1} ($n = 12$, $p < 0.01$). The most striking finding was that mibefradil markedly reduced the rise rate of plateau potentials from 147 ± 10 to 55 ± 8 mV/s ($n = 12$, $p < 0.01$) and lengthened the half width from 1.51 ± 0.05 to 1.66 ± 0.04 s ($n = 12$, $p < 0.05$; Fig. 2B and C). A similar effect on the rise time of plateau potentials was observed by the application of 100 μM Ni^{2+} (142 ± 10 to 85 ± 4 mV/s, $n = 3$, $p < 0.05$), which blocks the T-type Ca^{2+} channel [23]. On the other hand, the addition of 0.3 μM nifedipine, an L-type Ca^{2+} channel antagonist, did not affect on the rise rate of plateau potentials ($n = 6$, $p > 0.05$), although nifedipine caused membrane depolarization at the same level as mibefradil (by 6.4 ± 0.9 mV depolarization, $n = 6$, $p > 0.05$ vs. mibefradil of 4.2 ± 1.5 mV, $n = 12$). In contrast to mibefradil, the half width of plateau potentials was shortened during the application of nifedipine (1.52 ± 0.02 to 1.30 ± 0.04 s, $n = 6$, $p < 0.05$), as described previously [7]. As plateau potentials were generated even in the presence of mibefradil at a high concentration of 3 μM with a decrease in the rise rate, the effects of a mixture of mibefradil and nifedipine on plateau potentials were examined. In the copresence of 3 μM mibefradil and 0.3 μM nifedipine, plateau potentials were abolished or, on some occasions, plateau potentials with small and irregular amplitudes were generated ($n = 5$; Fig. 2Ad).

Effects of mibefradil on plateau potentials of ICC_{SM} during high $[K^+]_o$ solutions

To obtain further evidence that the initial phase of plateau potentials was due to the activation of T-type Ca^{2+} channel, the effects of mibefradil and Ni^{2+} on the relationship between the membrane potential and the rise rate of plateau potentials were examined in solutions with different

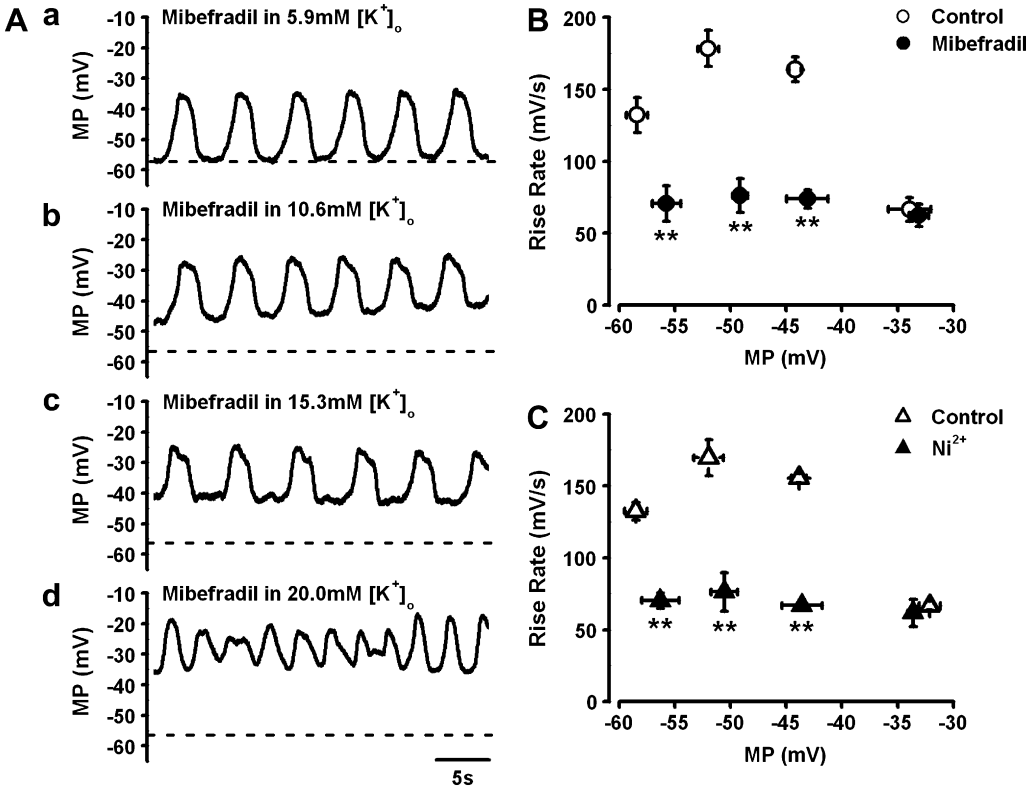

Fig. 3. Effects of mibefradil on plateau potentials of ICC_{SM} in different $[K^+]_o$ solutions. Effects of mibefradil and Ni^{2+} on plateau potentials were examined during different $[K^+]_o$ solutions in ICC_{SM}. (A) Representative traces of changes in membrane potential (MP) in $[K^+]_o$ solutions at concentrations of 5.9 (a), 10.6 (b), 15.3 (c), and 20 (d) mM in the presence of 3 μM mibefradil are shown. Dotted lines indicate the level of membrane potential in 5.9 mM $[K^+]_o$ solution after the pretreatment of 3 μM mibefradil. (B) Relationship between the membrane potential and the rise rate of plateau potentials in various $[K^+]_o$ solutions in the absence (open) and presence (closed) of 3 μM mibefradil are plotted. (C) The rise rate of plateau potentials generated in different $[K^+]_o$ solutions before (open) and after (closed) application of 100 μM Ni^{2+} are summarized on the membrane potential. Experimental data were obtained from 6 tissues. Statistical significance of the difference is expressed as ** $p < 0.01$ vs. rise rate in the absence.

$[K^+]_o$. Pretreatment with 3 μM mibefradil markedly reduced the rise rate of plateau potentials generated in 10.6 and 15.3 mM $[K^+]_o$ solutions to approximately 45% ($n = 6$) as well as in normal $[K^+]_o$ solution (to 53%, $n = 6$; Fig. 3A and B). The increase in the rise rate observed in 10.6 and 15.3 mM $[K^+]_o$ solutions, as shown in Fig. 1C, were not detectable in the presence of mibefradil. At higher $[K^+]_o$ of 20 mM, the rise time of plateau potential was not significant between before and after the application of mibefradil ($n = 6$, $p > 0.05$). Similar responses in the relationship between the membrane potential and the rise rate of plateau potential were observed with the addition of 100 μM Ni^{2+} ($n = 6$; Fig. 3C).

Dose dependence of the inhibitory effect by mibefradil on the rise rate of plateau potentials in ICC_{SM}

In the next set of experiments, the concentration dependency of the inhibitory effects by mibefradil was analyzed using parameters of the plateau potentials in ICC_{SM}. Changing the concentration of mibefradil in the range from 0.1 to 10 μM showed that the rise rate was significantly decreased by mibefradil at a concentration of 1 μM and more ($n = 12$, $p < 0.05$ vs. control of 139 ± 8 mV/s, $n = 12$), and the inhibitory effect was concentration-dependent (Fig. 4). The IC_{50} value of mibefradil in the rise rate of plateau potentials was 1.0 μM, and the Hill coefficient was 2.4.

Discussion

In the gut, spontaneous electrical rhythmicity generates a unique smooth muscle tonus with oscillatory frequency, often referred to as slow waves [1,2]. Recent studies have revealed that spontaneous activity paces the gastrointestinal motility, which originates from Ca^{2+} oscillations generated in ICC [12,17,24,25]. ICC can generate pacemaker activity, which is the basis for slow wave activity in gastrointestinal smooth muscles. Our data suggested that the initial component of spontaneous activity was through the activation of mibefradil-sensitive VDCCs, which was essential for the generation of plateau potentials in ICC_{SM} of the murine proximal colon.

In the present investigation, experiments for the voltage dependency of plateau potentials in ICC_{SM} by high $[K^+]_o$-induced depolarization revealed that the rise rate of the

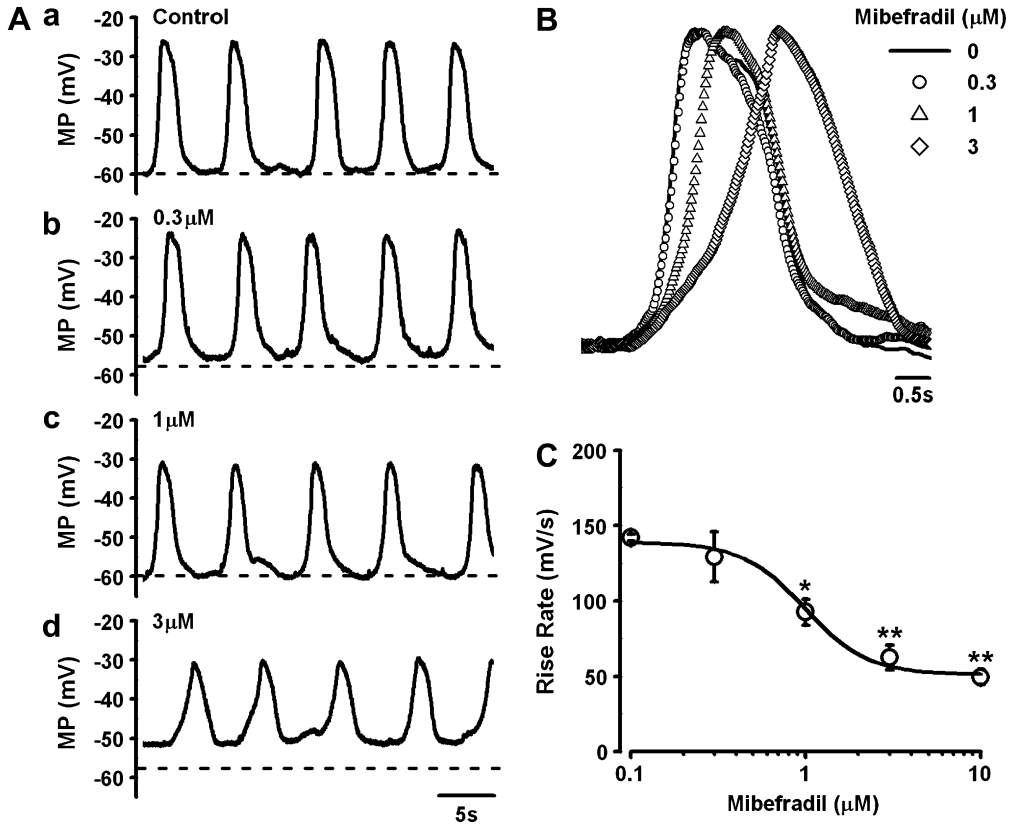

Fig. 4. Dose-dependent inhibition by mibefradil on rise rate of plateau potentials in ICC_{SM}. The concentration dependence of mibefradil on the rise rate of plateau potential was analyzed in ICC_{SM}. (A) Typical traces of changes in membrane potential (MP) in the absence (a) and presence of mibefradil at concentrations of 0.3 (b), 1 (c), and 3 (d) μM are represented. (B) Plateau potentials in the absence (continuous line) and presence of mibefradil at concentrations of 0.3 (circle), 1 (triangle), and 3 (diamond) μM are superimposed after normalization by membrane potential amplitude on the same time scale. (C) Sensitivity to mibefradil in the rise rate of plateau potentials in ICC_{SM} is summarized. The rise rate was significantly decreased by mibefradil at a concentration of 1 μM and more ($p < 0.05$). The IC_{50} value for mibefradil in the rise rate of plateau potentials was 1.0 μM, and the Hill coefficient was 2.4. Experimental data were obtained from 12 tissues. Statistical significance of the difference is expressed as * $p < 0.05$ or ** $p < 0.01$ vs. control.

initial component changed in a biphasic way, with an increase in 10.6 and 15.3 mM $[K^+]_o$ solutions and a decrease in higher $[K^+]_o$. In addition, the primary component of plateau potentials of ICC_{SM}, was inhibited by T-type Ca^{2+} channel blockers, mibefradil and Ni^{2+}, and the remainder were sensitive to an antagonist for an L-type Ca^{2+} channel, nifedipine. The properties of these channels differ in voltage sensitivity and drug selectivity; T-type Ca^{2+} channel is activated at membrane potentials around −60 mV and peaked at about −40 mV, while the activity of the L-type Ca^{2+} channel peaks at about −10 mV [26]. If these are applicable to channels activated during generation of the initial component, the increase in the rise rate during depolarization to −51 and −44 mV in 10.6 and 15.3 mM $[K^+]_o$ solution, respectively, may be mainly due to the enhanced activity of the T-type Ca^{2+} channel. The relationship between the membrane potential and the rise rate of plateau potentials was bell-shaped with peak amplitude at low voltage, probably by the activity of the VDCC current of the T-type rather than the L-type. This may be supported by the inhibition of the acceleration of the rise rate of plateau potentials in high $[K^+]_o$ solutions with mibefradil or Ni^{2+}. In 20.0 mM $[K^+]_o$ solution where membrane potentials amounted to −33 mV, the deficient effect on plateau potentials by mibefradil was because the gating via the T-type Ca^{2+} channel was mostly closed around the membrane potential. In addition to a previous report describing that the second sustained phase of plateau potentials was influenced by nifedipine [7], the absence of plateau potentials in both the presence of mibefradil and nifedipine strongly in this study strongly suggested that the L-type Ca^{2+} channel also contributes to generating and maintaining plateau potentials in ICC_{SM} of the murine proximal colon.

The physiological and pathological significances of plateau potentials generated in ICC_{SM} are unknown, but it is speculated that they may have some special role in the contraction of smooth muscle in proximal colon, which has a unique activity, termed antiperistalsis, in some species including mice, rats, and guinea-pigs. In the isolated proximal colon of guinea-pig, rhythmic contractions are generated with frequencies comparable to those of plateau potentials recorded from ICC_{SM} (10–12 min^{-1}) [27]. In mouse colonic ICC_{SM}, the rhythm of plateau potentials (about 15 min^{-1}) differs from that of smooth muscle (about 4.5 min^{-1}) [7], although the activity of ICC_{SM} is estimated to contribute partly to the contraction of circular smooth muscles [6,28]. Antiperistalsis is a specific physiological function in the proximal colon, different from other parts of the gastrointestinal tract. Thus, it seems likely that the plateau potentials generated in ICC_{SM} contributed to antiperistaltic activity.

In summary, we found that the initial fast component of plateau potentials generated in ICC_{SM} distributed in the murine proximal colon is involved in the mibefradil-sensitive T-type Ca^{2+} channel. This finding provides key evidence for elucidating the physiological and pathological functions of ICC_{SM}, which may contribute the generation of slow wave in gastrointestinal smooth muscles.

Acknowledgments

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