Arecoline Hydrobromide Enhances Jejunum Smooth Muscle Contractility via Voltage-Dependent Potassium Channels in W/W^v Mice

Qicheng CHEN[1], Zhi JIANG[1], Junhong ZHANG[1], Lixing CAO[1], Zhiqiang CHEN[1]

[1]The Research Team of Traditional Chinese Medicine Applications of Perioperative, The Second Affiliated Hospital of Guangzhou University of Chinese Medicine, Guangzhou, Guangdong, China

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Summary

Gastrointestinal motility was disturbed in W/W^v, which were lacking of interstitial cells of Cajal (ICC). In this study, we have investigated the role of arecoline hydrobromide (AH) on smooth muscle motility in the jejunum of W/W^v and wild-type (WT) mice. The jejunum tension was recorded by an isometric force transducer. Intracellular recording was used to identify whether AH affects slow wave and resting membrane potential (RMP) *in vitro*. The whole-cell patch clamp technique was used to explore the effects of AH on voltage-dependent potassium channels for jejunum smooth muscle cells. AH enhanced W/W^v and WT jejunum contractility in a dose-dependent manner. Atropine and nicardipine completely blocked the excitatory effect of AH in both W/Wv and WT. TEA did not reduce the effect of AH in WT, but was sufficient to block the excitatory effect of AH in W/W^v. AH significantly depolarized the RMP of jejunum cells in W/W^v and WT. After pretreatment with TEA, the RMP of jejunum cells indicated depolarization in W/W^v and WT, but subsequently perfused AH had no additional effect on RMP. AH inhibited the voltage-dependent K^+ currents of acutely isolated mouse jejunum smooth muscle cells. Our study demonstrate that AH enhances the contraction activity of jejunum smooth muscle, an effect which is mediated by voltage-dependent potassium channels that acts to enhance the excitability of jejunum smooth muscle cells in mice.

Key words

Arecoline hydrobromide • Gastrointestinal motility • W/W^v • Voltage-dependent potassium channels

Corresponding author

Z. Chen, Guangdong Provincial Hospital of T.C.M, No. 111 Dade Road, Guangzhou, 510120, China. E-mail: wssq@gzucm.edu.cn

Introduction

Under physiological conditions, rhythmic depolarization waves (also known as slow waves) are observed in gastrointestinal smooth muscle tissue. It was previously believed that gastrointestinal contractions were generated by action potentials superimposed on slow waves (Sanders *et al.* 2016). However, the work of both Sanders and Huizinga demonstrated that, slow wave activity is abolished, while scattered action potentials persist in W/W^v which lack of c-kit-positive interstitial cells of Cajal (ICC) (Hulzinga *et al.* 1995, Ward *et al.* 1994). Other studies have shown that W/W^v exhibit irregular action potentials and small intestinal tissue contractions in addition to decreases in both gastrointestinal contraction coordination and intestinal propulsion (Der-Silaphet *et al.* 1998). Smooth muscle cells (SMCs) receive electrical signals from the intestinal nervous system and interstitial cells. Based on these inputs, the SMCs are stimulated to either contract or relax, thereby regulating gastrointestinal motility (Sung *et al.* 2015). The excitability of these SMCs is closely related to the ion channels present on their cell membranes. For example, potassium channel activity contributes to the maintenance of membrane hyperpolarization in SMCs. This reduces cell excitability, ultimately promoting smooth muscle relaxation (Haick

Fax +420 241 062 164, e-mail: physres@fgu.cas.cz, www.biomed.cas.cz/physiolres

and Byron 2016). When voltage-gated potassium channels are inhibited, the resultant membrane depolarization can induce the activation of L-type calcium channels, resulting in action potential-induced contractions (Chang 2005, Sanders *et al.* 2012, McClain *et al.* 2015). Therefore, it is of great physiological and pathophysiological significance to study the relationship between voltage-gated potassium channels and smooth muscle motility in gastrointestinal SMCs.

Our previous studies have shown that oral consumption of areca nut water extract by healthy volunteers can increase gastrointestinal motility by enhancing electrogastrogram parameters (Sun *et al.* 2016). Arecoline is the main active ingredient found in the areca nut. The arecoline content in Xiangbin exceeds 2.7 % (Jiang *et al.* 2019). We also found that arecoline enhances gastrointestinal muscle strip contraction in rats through the activation of muscarinic receptors (Zhang *et al.* 2016). However, it remains unclear whether arecoline regulates gastrointestinal motility by modulating SMC excitability through voltage-gated potassium channels.

Because of the absence of slow wave behaviors in W mutant mice small intestine, only stable resting membrane potential (RMP) of SMC (Hulzinga *et al.* 1995, Ward *et al.* 1994). Therefore, this may be a good model system for studying the basic excitability of small intestinal SMCs. In this study, W/W^v were used to assess the effects of jejunum contraction and resting membrane potential on arecoline hydrobromide (AH) promoting gastrointestinal motility.

Methods

Animals

W/W^v and WT mice aged 8-12 weeks (20-25 g) were provided by Jackson Laboratory (USA) and fed in Guangdong Experimental Animal Center. The mice were housed at a constant temperature (20-25 ℃) under a 12 h light/dark cycle with free access to water and food.

Ethics

The protocol was approved by the Committee on the Ethics of Animal Experiments (No. 2014017-3). Animals were treated in accordance with Guide for the Care and Use of Laboratory Animals (8^{th} edition, National Academies Press). All surgeries were performed with isoflurane, and all efforts were made to minimize animal suffering.

Tissue preparation and tension measurement of muscle strips

The mice were initially anesthetized with isoflurane before being killed by cervical dislocation. The jejunum, found 5-10 cm above the ileocecal region, was removed quickly and placed in Krebs solution (121.9 mM NaCl, 15.5 mM $NaHCO_3$, 5.9 mM KCl, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4, 11.5 mM glucose, and 2.5 mM $CaCl_2$) which aerated (95 % O_2 and 5 % CO_2). The jejunum was cut along the mesentery, washed with ice-cold Krebs solution with the mucosa facing upward, after removed the mucosa and submucosa. All thickness muscle strips (2 mm×8 mm) were taken along the longitudinal axis of intestine. The strips were hung in the 10 ml organ baths perfused with 37 ℃ Krebs solution. Mechanical activity of jejunum smooth muscle was recorded by an isometric force transducer (multi-chamber Organ Bath Systems, Panlab, Spain).

Intracellular microelectrode recording

Full-thickness muscle strips (8 mm×4 mm) were cut from the intestine and pinned onto the base of a silica layer and continuously perfused with oxygenated Krebs solution at 37 ℃. Before recording, strips incubated for 2 h. The glass microelectrodes with resistances of 50-80 MΩ were filled with 1 M KCl for piercing cells. Recording and amplifying electrical response through a high input impedance amplifier (AXON210B, Molecular Devices, USA). The data were recorded by personal computer using Clampfit 10.4.

Cell preparation

As described above, the muscularis was cut into segments after the mucosal and submucosal layers had been removed. These segments were stored in Kraft-Bruhe (KB, 0.5 mM EGTA, 10 mM HEPES, 3 mM $MgCl_2$, 50 mM KCl, 10 mM glucose, 20 mM KH_2PO_4, 20 mM taurine, and 50 mM glutamic acid) solution for 15 min at 4 °C. They were then incubated at 37 °C in 1 ml of digestion medium (containing 2 mg collagenase II, 1.5 mg trypsin inhibitor, 500 μg papain, 1.5 mg dithiothreitol, and 3 mg bovine serum albumin) for 10-15 min. Then discarded the supernatant, and washed the muscle segments with the modified KB solution. Tissue pieces were blowed to create a cell suspension. The freshly dispersed cells were stored in the KB solution at 4 °C for patch-clamp.

Voltage patch-clamp experiment

Transferred the cell suspension to a perfusion chamber on the stage of an inverted microscope. Cells were adherent for 20 min before recording. The transient outward K^+ current (IKv) was recorded and amplified using an AXON 700B amplifier (Molecular Devices, USA). For recording the IKv, the pipette with resistances of 3-5 MΩ was filled with a potassium ion electrode solutions. IKv was a step voltage command pulse from -40 mV to +100 mV for 400 ms, increasing by 20 mV at 10 s intervals.

Drugs

AH was purchased from Adamas (Adamas Reagent, Ltd., Shanghai, China). Atropine, tetraethylammonium (TEA), nicardipine and other chemicals were all acquired from Sigma (Sigma-Aldrich, St. Louis, MO, USA) unless indicated otherwise. Nicardipine was dissolved in dimethyl sulfoxide (DMSO). Other drugs were dissolved in distilled water.

Statistical analysis

The data were shown as mean ± S.E.M.; n refers to the number of animals. A Student's *t*-test was used to determine whether data sets were different, with a $P<0.05$ expressed as significant differences. Data statistics were calculated with GraphPad Prism 5 (GraphPad Software, La Jolla, USA).

Results

Effect of AH on jejunum smooth muscle contractions in W/W^v and WT

We observed the effect of different concentrations of AH on jejunum smooth muscle tonic contractions in W/W^v and WT. When muscle strip tension was balanced, rhythmic contractions appeared in WT. However, in W/W^v, contraction frequency was decreased and intermittent interruptions occurred randomly under normal conditions. After treatment with AH (10^{-8}, 10^{-7}, 10^{-6}, and 10^{-5} M), jejunum contractions were enhanced in a dose-dependent manner in both WT and W/W^v. In WT, the change of contraction amplitudes were found to be significant at the concentrations of 10^{-7} and 10^{-6} M (P=0.0166 and 0.0188 respectively, n=6, Fig. 1A, C). The contraction frequency was changed from 37.4±2.5 cpm in the control to 38.1±2.7 cpm, 41.2±1.5 cpm, 36.9±1.9 cpm and 37.0±4.5 cpm following AH (10^{-8}, 10^{-7}, 10^{-6}, and 10^{-5} M) treatments in (Fig. 1A, D). For contraction frequency in WT, there was no significant difference found at any of these concentrations as compared to the control (P>0.05, n=6, Fig. 1A, D).

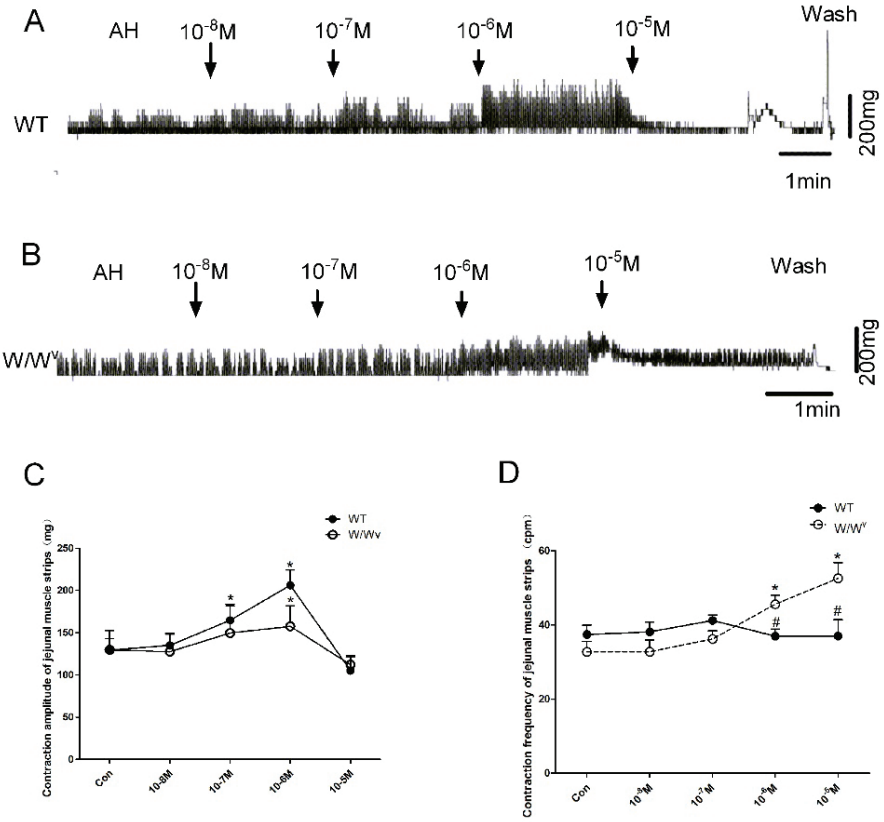

Fig. 1. Arecoline hydrobromide enhances contractions of jejunal smooth muscle strips in wild-type and W/W^v. Representative trace of changes to jejunal smooth muscle strips induced by different concentrations (10^{-8}, 10^{-7}, 10^{-6} and 10^{-5} M) of arecoline hydrobromide (AH) for wild type (WT, **A**) and W/W^v (**B**). (**C**) Line graph showing the effects of different concentrations of AH on induced contraction amplitude (C) and frequency (**D**) in jejunal smooth muscle from WT and W/W^v. Data are expressed as mean ± SE, n=6, * P<0.05, vs. the control. # P<0.05, comparing with the same concentrations between WT and W/W^v.

In W/W^v, contraction amplitude was changed from 129.6±22.9 mg in the control to 127.4±22.0 mg, 149.5±32.3 mg, 157.5±24.4 mg, and 112.1±9.2 mg after treatment with 10^{-8}, 10^{-7}, 10^{-6}, and 10^{-5} M AH, respectively (Fig. 1B, C). The effect was significant at the 10^{-6} M concentration (P=0.0121, n=6). The contraction frequency was changed from 32.7±2.8 cpm in the control to 32.8±3.1 cpm, 36.2±2.2 cpm, 45.6±2.5 cpm and 52.6±4.3 cpm after treatment with AH (10^{-8}, 10^{-7}, 10^{-6}, and 10^{-5} M) (Fig. 1A, D). When compared to the control, a significant difference was found for the 10^{-6} and 10^{-5} M concentrations (P=0.0291, 0.0278, respectively; n=6).

Furthermore, when we compared the effects of AH on jejunal contraction parameters between WT and W/W^v, it was shown that contraction frequency in W/W^v increased significantly after the addition of 10^{-6} and 10^{-5} M AH (P=0.0200 and 0.0299, respectively; n=6).

The inhibitory effects of atropine, nicardipine and TEA on the enhancement of jejunal muscle strip contraction by AH

Atropine (10^{-4} M) completely blocked the excitatory effect of AH (10^{-6} M) on jejunal tonic contraction in W/W^v and WT (Fig. 2A, B). Similarly, nicardipine (10^{-6} M), an L-Ca^{2+} channel blocker, blocked the excitatory effect of AH in W/W^v and WT (Fig. 2C, D). The K$^+$ channel blocker TEA could not inhibit the enhancement of AH (10^{-6} M) in WT, but was able to block the excitatory effect of AH (10^{-6} M) in W/W^v. Pre-treatment with TEA (10^{-4} M) increased jejunal muscle strip contraction in WT. Subsequent treatment of WT with AH (10^{-6} M) significantly enhanced the contractions of muscle strips (Fig. 2E). After treating with TEA and AH, contraction amplitude was 241.5±16.0 mg, and contraction frequency was 37.8±1.1 cpm. As compared with TEA pre-treatment alone, contraction amplitude increased after AH treatment (P=0.0002, n=6). The different changes were observed in W/W^v after TEA (10^{-4} M) pre-treatment. Basal contraction amplitude and frequency were increased to 128.5±6.2 mg and 34.4±1.3 cpm, respectively. However, the following treatment with AH (10^{-6} M) did not enhance contraction amplitude (129.9±4.7 mg) or frequency (34.9±1.2 cpm) (Fig. 2F). Compared with TEA pre-treatment alone, subsequent AH treatment did not lead to significant changes in jejunal contraction amplitude or frequency in W/W^v.

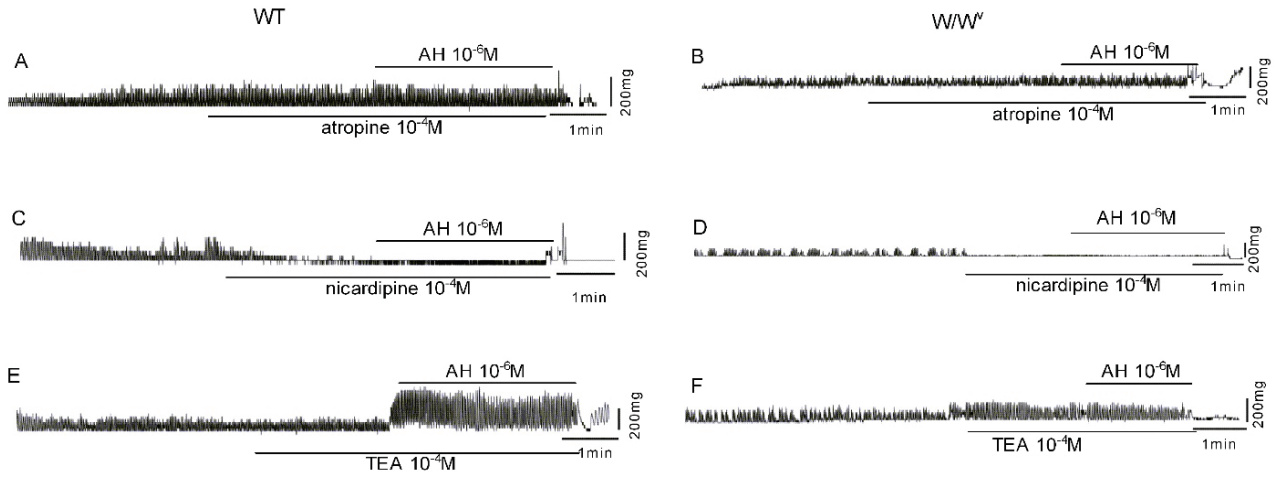

Fig. 2. Atropine, nicardipine and tetraethylammonium block arecoline hydrobromide-induced enhancement of contractions in jejunal smooth muscle from wild type and W/W^v. Atropine (10^{-4} M) pretreatment did not change the contraction amplitude and frequency of jejunal smooth muscle in wild-type (**A**) and W/W^v (**B**). Atropine (10^{-4} M) significantly inhibited the enhancement of 10^{-6} M AH on jejunal muscle strips in both WT and W/W^v. After pretreatment with nicardipine (10^{-6} M), the basic contraction rhythm of WT (**C**) and W/W^v (**D**) disappeared, and no muscle strip contraction was observed after continuous administration of 10^{-6} M AH. (**E**) The contractile activity of jejunal smooth muscle strips from WT was not significantly enhanced after pretreatment with tetraethylammonium (TEA; 10^{-4} M). TEA (10^{-4} M) could not inhibit the effects of AH (10^{-6} M) on jejunal smooth muscle in WT. (**F**) After pretreatment with TEA (10^{-4} M), the contractions increased significantly, but subsequent addition of AH (10^{-6} M) did not increase contractions in jejunal smooth muscle strips of W/W^v.

Effect of AH on resting membrane potential (RMP) in WT and W/W^v

The results showed that rhythmic slow waves with an RMP of -63.3±1.3 mV, a frequency of 34.2±1.8 cpm and an amplitude of 19.0±0.8 mV were observed in the jejunal smooth muscle of WT (Fig. 3A). After treatment with 10^{-6} M AH, RMP measurements were depolarized to -52.2±0.7 mV (P<0.001, n=12,

Fig. 3Ac, B), slow wave frequency was reduced to 28.5±0.6 cpm (p=0.0012, n=12, Fig. 3Ac, C) and signal amplitude was reduced to 17.2±1.1 mV (*P*=0.1962, n=12, Fig. 3Ac, D).

In jejunal muscle strips from W/W^v, the rhythmic slow waves could be observed, and only a flat and stable resting membrane potential was recorded (Fig. 3E). After perfusion of 10^{-6} M AH, the RMP increased from -51.4±1.0 mV to -43.4±1.5 mV, resulting in obvious depolarization of SMCs (compared with control, *P*<0.001, n=12, Fig. 3Ec, F). These results suggested that the enhancement of jejunal SMC contractions by AH was related to the depolarization of SMC membranes.

We found that AH (10^{-6} M) depolarized the RMP from -61.24±4.23 mV in the control to -45.22±3.31 mV (Fig. 3A, B; *P*<0.05, n=6). Slow wave amplitude was increased from 15.52±3.68 mV to 20.61±4.36 mV (Fig. 3C; *P*<0.05, n=6). It was suggested that the excitatory effect of AH may be related to the depolarization of membrane potential.

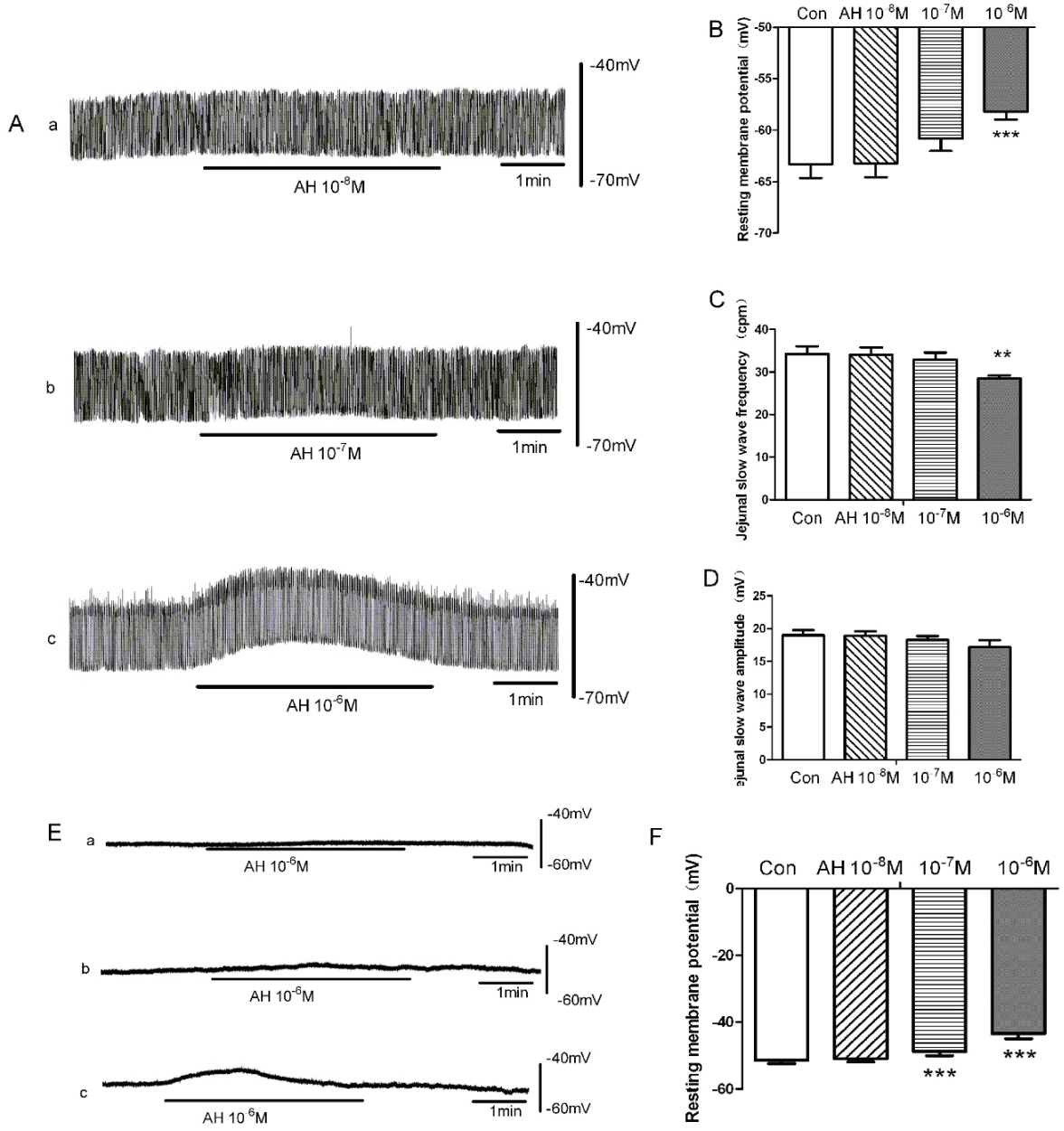

Fig. 3. Effects of arecoline hydrobromide on jejunal slow waves in wild-type and W/W^v. (**A**) Representative trace of changes in jejunal smooth muscle slow waves induced by 10^{-8} M (**a**), 10^{-7} M (**b**) and 10^{-6} M (**c**) of arecoline hydrobromide (AH) for wild-type (WT). Column chart comparing resting membrane potential (RMP, (**B**), frequency (**C**) and amplitude (**D**) of jejunum slow wave activity in WT. (**E**) Representative trace of changes in jejunal smooth muscle RMP induced by 10^{-8} M (**a**), 10^{-7} M (**b**) and 10^{-6} M (**c**) AH for W/W^v. Column chart comparing RMP of jejunum slow wave activity in W/W^v. ** *P*<0.01 and *** *P*<0.001, vs. the control. (WT: n=12, W/W^v: n=13).

The inhibitory effect of TEA on depolarization of RMP by AH

Pretreatment with TEA (10^{-4} M) depolarized the jejunal SMCs of WT, increasing the RMP from -63.7±1.7 mV to -52.1±1.0 mV and slow wave amplitude from 17.3±0.7 mV to 18.0±0.6 mV, while decreasing signal frequency from 34.9±1.9 cpm to 31.4±1.2 cpm (Fig. 4). After reperfusion of 10^{-6} M AH, there were no further changes to RMP or slow wave frequency, but slow wave amplitude was significantly increased to 19.4±0.5 mV (P=0.0012, n=6, compared with TEA pre-treatment).

Similarly, pretreatment with TEA (10^{-4} M) could depolarize the RMP of jejunal SMCs of W/W^v from -51.1±0.8 mV to -45.0±1.4, while subsequent AH (10^{-6} M) perfusion could not further depolarize the RMP in W/W^v (Fig. 4).

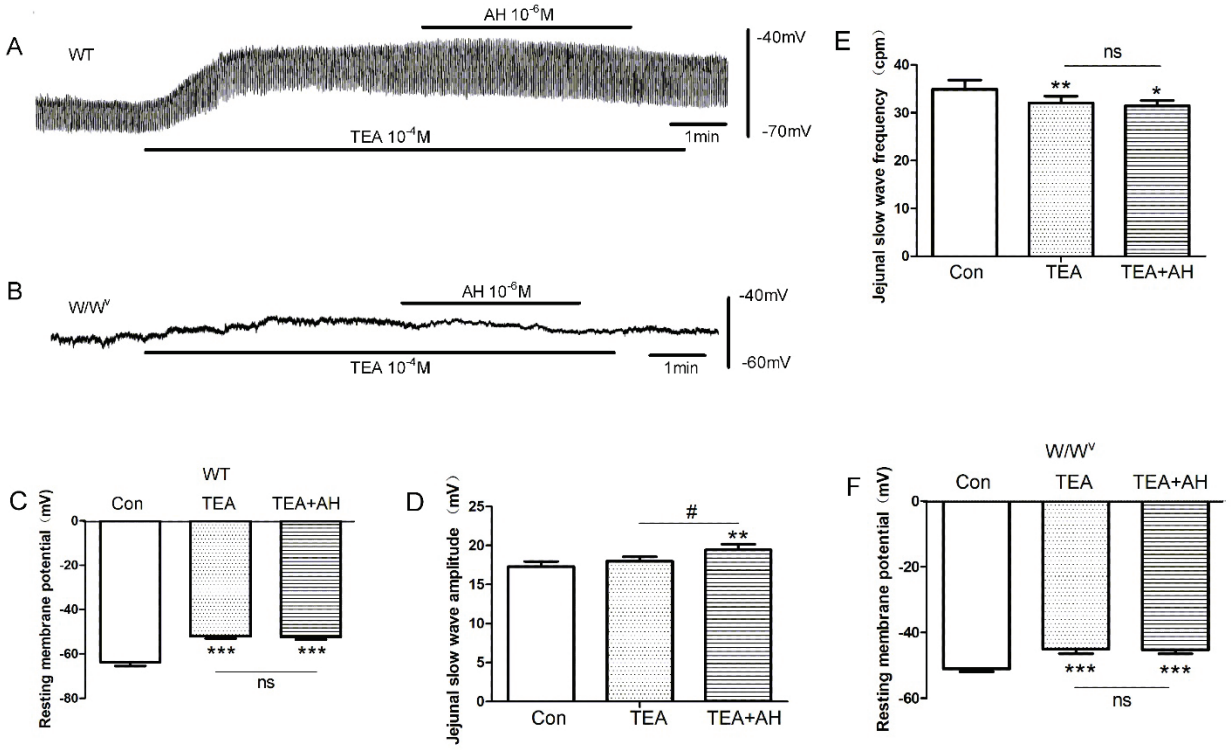

Fig. 4. TEA blocked arecoline hydrobromide-induced resting membrane potential depolarization in jejunal smooth muscle in wild-type and W/W^v. (**A**) Representative trace of TEA blocking arecoline hydrobromide- (AH) induced resting membrane potential depolarization in jejunal smooth muscle from wild type (WT). Jejunal slow wave RMP was depolarized after pretreatment with TEA (10^{-4} M). The subsequent perfusion of 10^{-6} M AH did not cause the RMP to depolarize further, although the amplitude of slow wave activity increased. Representative trace of TEA blocking AH-induced RMP depolarization in jejunal smooth muscle from W/W^v (**B**) and WT (**C**). As in WT, TEA inhibits AH-induced depolarization of RMP in W/W^v. After pretreatment with TEA (10^{-4} M) alone or treatment with both TEA (10^{-4} M) and AH (10^{-6} M), the RMP depolarized significantly in WT. There was no significant difference in RMP between TEA and TEA+AH treatments. Column chart of the effects of TEA and AH on slow wave amplitude (**D**) and frequency (**E**) in WT. (**F**) Column chart of TEA block of AH-induced RMP depolarization in the jejunum of W/W^v. TEA (10^{-4} M) depolarized resting membrane potential and blocked the effect of the subsequent AH (10^{-6} M). Data are expressed as mean ± SE, WT, n=6; W/W^v, n=7. * P<0.05, ** P<0.01, *** P<0.001 vs. the control; compared with TEA and TEA+AH, $^{\#}$ P<0.05, ns P>0.05.

Arecoline hydrobromide reduced voltage-gated potassium current in jejunal SMCs

As shown previously, AH depolarized the RMP. Therefore, we further tested the IKv for the effect of AH. Freshly dispersed intestinal SMCs were used for whole-cell patch clamp IKv recording. The mean peak current decreased from 1425.8±50.6 pA in the control to 1289.6±32.5 pA, 1019.1±40.8 pA, and 855.0±37.2 pA after treatment with 10^{-7}, 10^{-6}, and 10^{-5} M AH, respectively (Fig. 5A, B; all P<0.01, n=9). A step voltage command was used to determine the effect of 10^{-6} M AH on the current-voltage (I-V) relationship of IKv. Treatment with 10^{-6} M AH significantly decreased the I-V curve of IKv at each tested (Fig. 5C). The IKv at +40 mV was decreased by 23.9±5.5 % (Fig. 5C, P<0.05, n=9) with 10^{-6} M AH. It suggested that IKv may be involved in AH-induced jejunal smooth muscle RMP depolarization in mice.

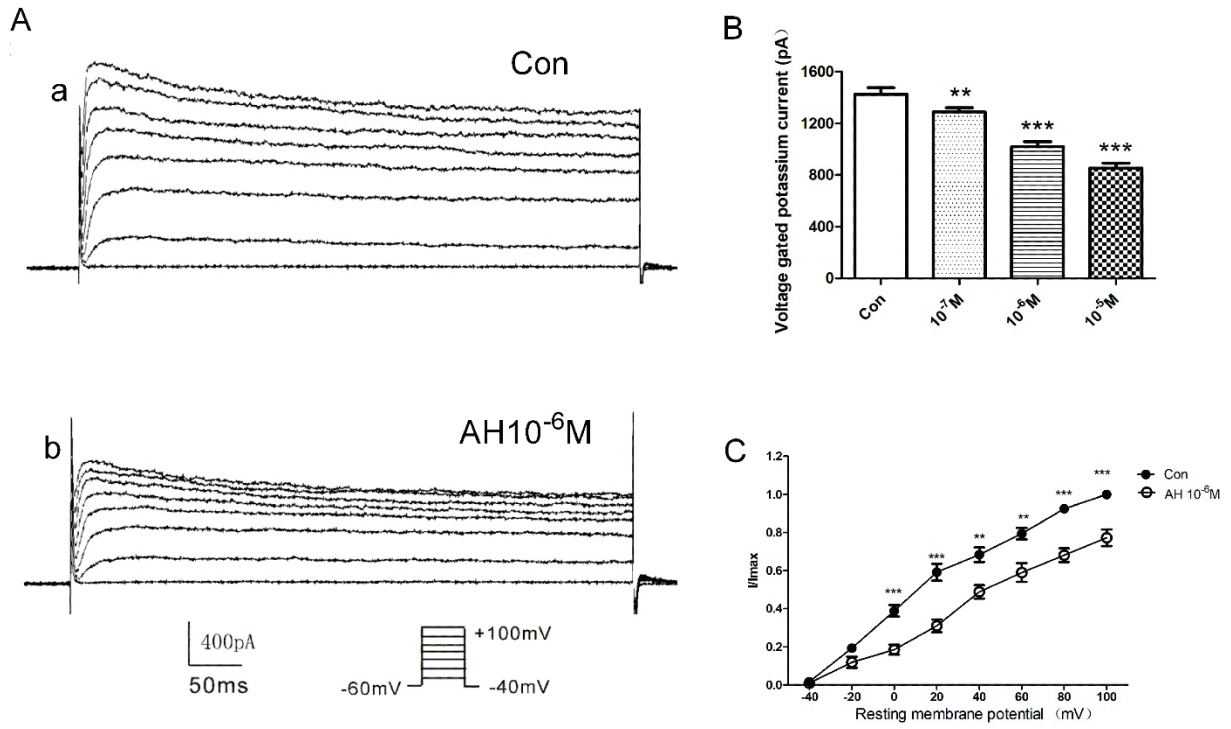

Fig. 5. Effects of arecoline hydrobromide on voltage-gated potassium currents in murine jejunal smooth muscle cells. (**A**) Representative trace of arecoline hydrobromide (AH) reducing activity of voltage gated potassium currents (IKv). (**a**) Control, (**b**) AH (10^{-6} M). (**B**) 10^{-7}, 10^{-6} and 10^{-5} M AH dose-dependently decreased IKv. (**C**) The I-V relationship curve of the AH-induced change in IKv. Data are expressed as mean ± SE, ** P<0.01, *** P<0.001, n=9, vs. the control.

Discussion

ICCs are located in the gastrointestinal tract in many mammals and produce a rhythmic depolarization potential and slow wave activity (Hyun *et al.* 2009, Wang *et al.* 2018, Drumm *et al.* 2018). ICCs respond to stimulation by exogenous nerves or gastrointestinal hormones and generate slow waves through the activity of CaCC channels (Hwang *et al.* 2019). SMCs receive slow waves produced by ICCs through gap junctions and, in response, activate voltage-dependent L-Ca^{2+} channels (Kim *et al.* 2008, Jin *et al.* 2002). These currents generate action potentials, which allow calcium influx and support the contraction of SMCs (Thornbury *et al.* 2011). In this study, AH did not significantly alter the contraction frequencies of slow wave and mechanical activity in wild mice. In contrast, high concentrations of AH increased the contraction frequency of jejunal smooth muscle in W/W^v. Therefore, the frequency of mechanical contraction is the same as that of slow wave activity in WT. However, as there were no slow waves in W/W^v jejunal cells, the triggering of action potentials was determined only by the excitability of the SMCs themselves. After the excitation of SMCs, the probability of calcium channels opening was increased.

Voltage-dependent potassium channels play a key role in establishing the resting membrane potential of gastrointestinal smooth muscle, and it is the main ion channel responsible for maintaining a stable resting membrane potential (Nagasaki *et al.* 1993, Hashitani *et al.* 2005). Resting membrane potential depolarization of SMCs can increase the opening probability of Ca^{2+} channels (Rich *et al.* 1993, Gibbons *et al.* 2009). A small depolarization can re-stimulate Ca^{2+} channels, which can then generate an action potential. The number and frequency of action potentials are determined by K^+ channels (Koh *et al.* 1999). Therefore, we observed the effects of IKv-specific blocker TEA on AH treatment of jejunal smooth muscle in wild and W/W^v (Fig. 2). These results showed that TEA could block the effect of AH on jejunal smooth muscle of W/W^v, but not on that of WT. Subsequent experiments showed that AH can depolarize RMP of jejunal SMCs, and inhibit the voltage-gated potassium currents in SMCs. Rhythmic slow waves exist in jejunal smooth muscle of wild mice and are essential for excitation-contraction coupling (Kito 2011). L-Ca^{2+} channels open at the slow wave plateau stage, which allows extracellular Ca^{2+} to enter cells and participate in the excitation-contraction process (Rich *et al.* 1993, Kovac *et al.* 2005). Therefore, the excitability

of jejunum SMCs in WT is mainly determined by the normal ICC network structure and slow wave activity. In contrast, slow waves were not found in the jejunum of W/W^v, where the excitability of SMCs is closely related to their resting membrane potential. TEA blocks potassium channels, which resulted in depolarization of SMCs and slightly increased contraction of muscle strips. However, it also reduced the opening of potassium channels, further limited the recovery of excitability in SMCs, and eventually abolished the excitatory effect of AH on contractions of jejunal smooth muscle in W/W^v.

The mechanical digestion of food depends on the motility of the gastrointestinal tract, including peristalsis and segmental movement. As smooth muscle is the main participant in gastrointestinal motility, the excitability of smooth muscle itself is the basis of gastrointestinal motility (Huizinga *et al.* 2014). SMCs make up to final link of excitation-contraction coupling, such that the change in SMC tension depends on the total influence of external factors (Sanders 2008). AH promotes jejunal smooth muscle contraction by inhibiting voltage-gated potassium channels and increasing the excitability of SMCs in W/W^v, which lack ICCs. Many digestive tract diseases have been associate with structural damage of the ICC network, which leads to abnormal slow wave activity and gastrointestinal motility disorders (Cipriani *et al.* 2018, Jang *et al.* 2018, Gamage *et al.* 2018). The regulation of smooth muscle motion by nerves and ICCs through gap junction is greatly weakened when the ICC network structure is destroyed. In this state, the contractile function of smooth muscle itself becomes more critical. Therefore, understanding the excitability of resting membrane potential in SMCs may be helpful for the treatment of ICCs abnormalities, especially neuromuscular dysfunction.

In conclusion, we observed that AH depolarized the resting membrane potential of jejunum muscle cells in W/W^v by acting through voltage-gated potassium channels, thereby enhancing the jejunal motility of mice.

Conflict of Interest

There is no conflict of interest.

Acknowledgements

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