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pH-mediated Regulation of Pacemaker Activity in Cultured Interstitial Cells of Cajal

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Interstitial cells of Cajal (ICCs) are pacemakers in gastrointestinal tracts, regulating rhythmicity by activating nonselective cation channels (NSCCs). In the present study, we investigated the general characteristics and pH-mediated regulation of pacemaker activity in cultured interstitial cells of Cajal. Under voltage clamp mode and at the holding potential of −60 mV, the I-V relationships and difference current showed that there was no reversal potential and voltage-independent inward current. Also, when the holding potentials were changed from +20 mV to −80 mV with intervals of 20 mV, there was little difference in inward current. In pacemaker activity, the resting membrane potential (RMP) was depolarized (In pH 5.5, 23±1.5 mV depolarized) and the amplitude was decreased by a decrease of the extracellular pH. However, in case of increase of extracellular pH, the RMP was slightly hyperpolarized and the amplitude was decreased a little. The melastatin type transient receptor potential (TRPM) channel 7 has been suggested to be required for intestinal pacemaking activity. TRPM7 produced large outward currents and small inward currents by voltage ramps, ranging from +100 to −100 mV from a holding potential of −60 mV. The inward current of TRPM7 was dramatically increased by a decrease in the extracellular pH. At pH 4.0, the average inward current amplitude measured at −100 mV was increased by about 7 fold, compared with the current amplitude at pH 7.4. Changes in the outward current (measured at +100 mV) were much smaller than those of the inward current. These results indicate that the resting membrane potential of pacemaking activity might be depolarized by external acidic pH through TRPM7 that is required for intestinal pacemaking activity.

Key Words: Interstitial cells of cajal (ICCs), Pacemaker activity, Melastatin type transient receptor potential channel 7 (TRPM7)

INTRODUCTION

Interstitial cells of Cajal (ICCs) are specialized cells in the gastrointestinal (GI) tract that are mesenchymal in origin and fundamental to the physiological functions of GI muscles (Huizinga et al, 1997; Sanders et al, 1999). ICCs are present in all of the pacemaking regions of the GI tract, and they initiate slow waves that are propagated to the smooth muscle cells via gap junctions (Horowitz et al, 1999), and also initiate phasic contractions via activation of Ca^{2+} entry through L-type Ca^{2+} channels. The pacemaker activity in the murine small intestine is mainly due to periodic activation of non-selective cation channels (NSCCs) (Koh et al, 2002).

pH-sensitive channels are found in many tissues and cells: For example, TASK channels in ileal and colonic myocytes (Cho et al, 2005). And, the small inward current of TRPM7 is dramatically enhanced by a decrease of extracellular pH (Jiang et al, 2005). Transient receptor potential (TRP) channels have first been cloned from *Drosophila* and constitute a superfamily of proteins encoding a diverse group of Ca^{2+}-permeable cation channels (Montell, 2001). The TRP family is divided into three subfamilies: TRPC, TRPV and TRPM (Hardie, 2001; Clapham, 2003). The eight TRPM family members significantly differ from the aforementioned TRP channels in terms of domain structure, cation selectivity and activation mechanisms (Clapham, 2003; Hofmann et al, 2003). TRPM7 (Nadler et al, 2001; Runnels et al, 2001), a widely expressed member of the TRPM family of ion channels (Harteneck et al, 2000; Montell et al, 2002), is a cation channel that is regulated by intracellular levels of MgATP and is strongly activated when MgATP falls below 1 mM, thereby producing a current designated as MagNuM (for magnesium-nucleotide-regulated metal ion current) (Nadler et al, 2001; Hermosura et al, 2002).

Recently, Kim et al showed that TRPM7 is required for pacemaker activity in ICCs (Kim et al, 2005). In pacemaker activity, however, there has been no study on pH-mediated regulation of pacemaker activity in ICCs and also on the relation between TRPM7 and pacemaker activity when

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ABBREVIATIONS: ICC, interstitial cells of Cajal; TRPM7, melastatin type transient receptor potential channel 7; TASK, TWIK-related Acid-Sensitive K^+.

extracellular pH was changed. Therefore, we investigated that the general characteristics and pH-mediated regulation of pacemaker activity in cultured interstitial cells of Cajal.

METHODS

Preparation of cells and cell cultures

Balb/c mice (8 ~ 13 days old) of either sex were anaesthetized with ether and killed by cervical dislocation. The small intestines from 1cm below the pyloric ring to the caecum were removed and opened along the mesenteric border. Luminal contents were washed away with Krebs-Ringer bicarbonate solution. The tissues were pinned to the base of a Sylgard dish and the mucosa removed by sharp dissection. Small tissue stripes of intestine muscle (consisting of both circular and longitudinal muscles) were equilibrated in Ca^{2+}-free Hank's solution (containing in mM: KCl 5.36, NaCl 125, NaOH 0.34, Na_2HCO_3 0.44, glucose 10, sucrose 2.9 and HEPES 11) for 30 min. Then, the cells were dispersed with an enzyme solution containing 1.3 mg/ml collagenase (Worthington Biochemical Co, Lakewood, NJ, USA), 2 mg/ml bovine serum albumin (Sigma Chemical Co., St Louis, MO, USA), 2 mg/ml trypsin inhibitor (Sigma) and 0.27 mg/ml ATP. Cells were plated onto sterile glass coverslips coated with murine collagen (2.5 μg/ml, Falcon/BD) in a 35 mm culture dish. The cells were then cultured at 37°C in a 95% O_2 ~ 5% CO_2 incubator in a smooth muscle growth medium (SMGM, Clonetics Corp., San Diego, CA, USA) supplemented with 2% antibiotics/antimycotics (Gibco, Grand Island, NY, USA) and murine stem cell factor (SCF, 5 ng/ml, Sigma). ICCs were identified immunologically with anti-c-kit antibody [phycoerythrin (PE)-conjugated rat anti-mouse c-kit monoclonal antibody; eBioscience, San Diego, CA, USA] at a dilution of 1 : 50 for 20 min (Goto et al, 2004). Since morphologies of ICC were distinct from other cell types in the culture, it was possible to identify the cells with phase contrast microscopy, once the cells had been verified with anti-c-kit antibody.

TRPM7 expression in human embryonic kidney 293 cells

HEK-293 cells transfected with the Flag-murineLTRPC7/pCDNA4-TO construct were grown on glass coverslips in DMEM supplemented with 10% fetal bovine serum, blasticidin (5 μgml^{-1}), and zeocin (0.4 $mgml^{-1}$). LTRPC7 expression was induced by adding 1 μgml^{-1} tetracycline to the culture medium. Whole-cell patch-clamp experiments were performed 18 ~ 24 h after induction at 21 ~ 25°C, using cells grown on glass coverslips.

Patch-clamp experiments

The whole-cell configuration of the patch-clamp techniques was used to record membrane currents (voltage clamp) and potentials (current clamp) of cultured ICC. Axopatch I-D (Axon Instruments, Foster, CA, USA) was used to amplify membrane currents and potentials. The command pulse was applied using an IBM-compatible personal computer and pClamp software (version 6.1; Axon Instruments). The data were filtered at 5 kHz and displayed on an oscilloscope, a computer monitor and with a pen recorder (Gould 2200, Gould, Valley view, OH, USA). Results were analysed using pClamp and Origin (version) software. All experiments were performed at 30°C.

The internal pipette solution for whole cell current recordings in ICCs contained (in mM) KCl 140, $MgCl_2$ 5, K_2ATP 2.7, NaGTP 0.1, creatine phosphate disodium 2.5, HEPES 5 and EGTA 0.1, adjusted to pH 7.2 with KOH. The internal pipette solution for whole cell current recordings in HEK-293 cells which express TRPM7 contained (in mM) Cs-glutamate 145, NaCl 8, MgCl2 1, Cs-BAPTA 10, HEPES-CsOH 10, pH 7.2.

The standard extracellular Tyrode's solution contained (in mM) NaCl 145, KCl 3.6, $CaCl_2$ 2, glucose 10, HEPES 20, pH 7.4 with NaOH. HEPES (20 mM) was used for pH 7.0 and 7.4 solutions, and it was replaced by 10 mM HEPES and 10 mM MES for pH ≤ 6 solutions (Jordt et al, 2000; Askwith et al, 2004; Yermolaieva et al, 2004).

Statistics

All data are expressed as meanS.E. Student's t-test for unpaired data was used to compare the control and the experimental groups. *P* value of less than 0.05 was considered to indicate statistically significant differences.

RESULTS

General characteristics of pacemaker activity in cultured ICCs

Under voltage clamp mode and at the holding potential of −60 mV, the ramp pulse, ranging from +100 mV to −100 mV, was applied at both the peak current and the resting current (Fig. 1A). The I-V relationships and difference current showed no reversal potential and voltage-independent inward current (Fig. 1B). Furthermore, when the holding potentials were changed from +20 mV to −80 mV with intervals of 20 mV, there was little difference in inward current (n=7; Fig. 2A). There is a space clamp problem in cultured ICCs, therefore, current clamp mode was used throughout the study, instead of voltage clamp, and membrane potential was recorded (Fig. 2B).

pH-mediated regulation of pacemaker activity in cultured ICCs

To investigate the effect pH on pacemaker activity, we changed the pH of external solution to 5.5, 6.5, 8.5, and 10. When the pH of external solution was decreased, the resting membrane potential (RMP) was depolarized and the amplitude was decreased (pH 5.5, n=3, 23 ± 1.5 mV; pH 6.5, n=4, 12 ± 0.8 mV; Fig. 3Aa, B). On the other hand, when the pH of external solution was increased, the RMP was slightly hyperpolarized and the amplitude was decreased a little (pH 8.5, n=3, −1.8 ± 0.16 mV; pH 10, n=3, −2.7 ± 0.3 mV; Fig. 3Ab, B). Therefore, the above result indicates that the resting membrane potential (RMP) of pacemaking activity was depolarized by external acidic pH.

Concentration-dependent effects of protons on TRPM7 currents

TRPM7 currents were elicited by voltage ramps, ranging from +100 to −100 mV, from a holding potential of −60

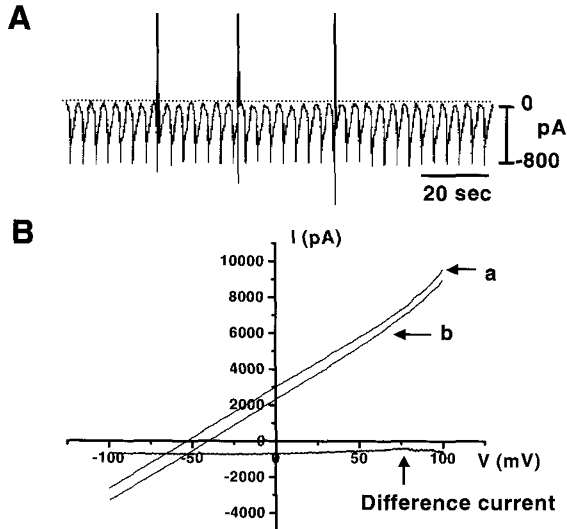

Fig. 1. I-V relationship of spontaneous inward currents. (A) Under a voltage clamp, we applied ramp pulse, ranging from +100 mV to 100 mV, at both the peak current and the resting current (HP= −60 mV). (B) The I-V relationships and difference current. There was voltage-independent inward current.

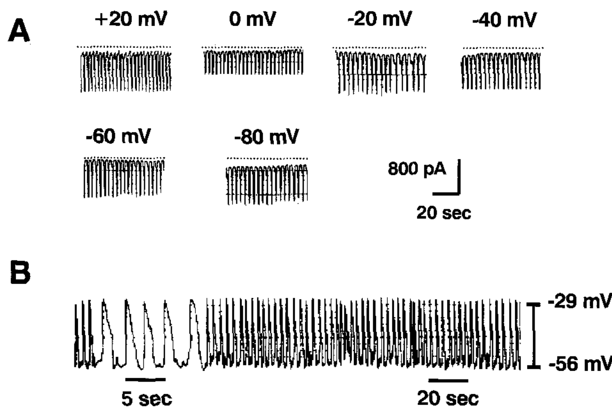

Fig. 2. Little difference in inward current and Current clamp. (A) There was no difference in inward current, although we changed the holding potential from +20 mV to 80 mV with intervals of 20 mV. (B) Under a current clamp mode, ICCs produced electrical pacemaker potential.

mV. As previously been reported, TRPM7 produced large outward currents and small inward currents (n=35; Fig. 4) (Nadler et al, 2001; Runnels et al, 2001; Schmitz et al, 2003). After break-in, 3∼5 min was allowed to let TRPM7 current amplitude reach a steady state before changing external solutions. As shown in Fig. 4, the inward current of TRPM7 was dramatically increased by a decrease of the extracellular pH (n=35). The concentration-dependent increase of TRPM7 inward currents from the same cell is shown in Fig. 4A. A small increase in the inward current was seen at pH 7.0 (n=5, 10±2% increase; Fig. 3B), and the increase was significant at pH 6.0 (n=5, 110±5% increase), reaching maximum at pH 4.0 (n=5, 550±20% increase; Fig. 3C∼E). At pH 4.0, the average inward

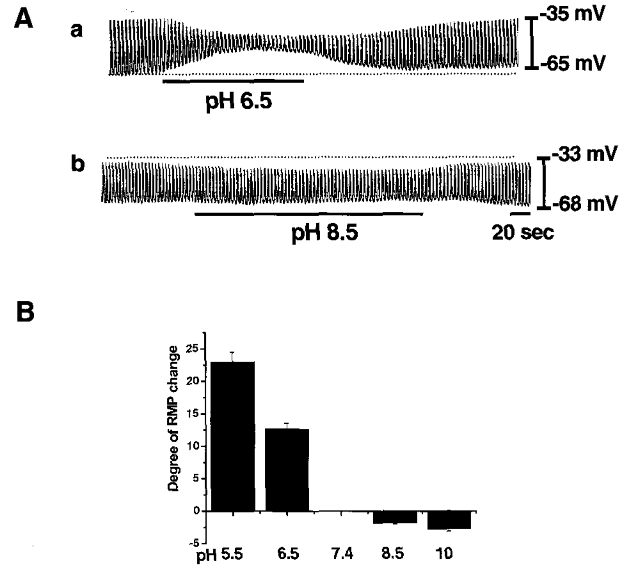

Fig. 3. pH-mediated regulation in pacemaker activity. (A-a) To investigate the effect of pH on pacemaker activity, we changed the pH of external solution to 6.5. The resting membrane potential was depolarized and the amplitude was decreased. (A-b) In increased extracellular pH, the resting membrane potential was slightly hyperpolarized and the amplitude was decreased a little. (B) The histograms summarize the change of resting membrane potential.

current amplitude measured at −100 mV was increased by about 10 fold, compared with the current amplitude at pH 7.4. Changes in the outward current (measured at +100 mV) were much smaller than those of the inward current.

DISCUSSION

Electrical slow waves are a fundamental property of phasic GI muscles, and these events activate periodic Ca^{2+} entry and regulate contractions in electrically coupled smooth muscle cells. ICCs are responsible for generating slow waves in gastrointestinal tract. Initially, a Ca^{2}-inhibited, nonselective cation conductance contributes to the pacemaker current, initiating slow wave activity (Koh et al, 2002), and TRPC4 (Walker et al, 2002) or TRPV6 (Yue et al, 2002) has been suggested as a molecular candidate for a Ca^{2+}-inhibited, nonselective cation conductance. However, we suggest TRPM7 as an another candidate for pacemaker channels.

TRPM7 is a ubiquitously distributed ion channel that belongs to the long or melastatin-related transient receptor potential (TRPM) ion channel subfamily (Harteneck et al., 2000; Montell, 2001; Clapham, 2003; Fleig and Penner, 2004). It is unique, because it is an ion channel and also a protein kinase. Although the physiological functions of the protein kinase are not well understood, recent studies suggested that TRPM7 plays important roles in cellular Mg^{2+} homeostasis (Schmitz et al, 2003), anoxic neuronal cell death (Aarts et al, 2003), cell proliferation and viability (Nadler et al, 2001; Hanano et al, 2004), diseases caused by abnormal magnesium absorption (Schlingmann et al, 2002; Walder et al, 2002; Chubanov et al, 2004) and intes-

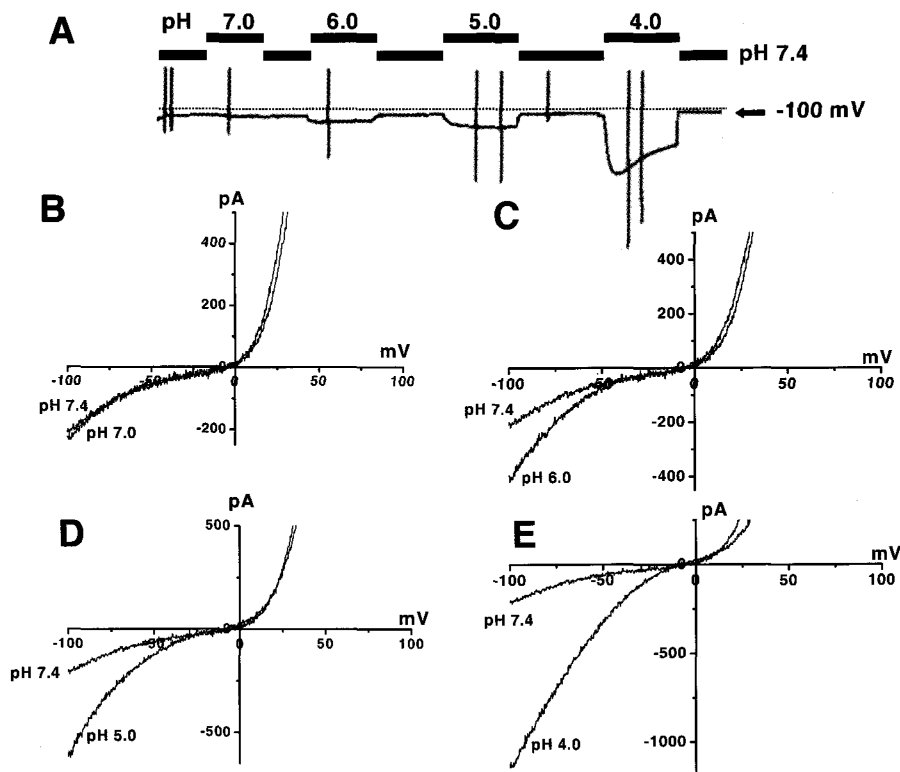

Fig. 4. Concentration-dependent effects of protons on TRPM7 currents. (A) Concentration-dependent changes in inward current amplitude measured at -120 mV from the recordings elicited by voltage ramps at pH indicated. The dashed line represents zero current levels. (B-E) Potentiation of TRPM7 inward currents by protons at pH 7.0 (B), pH 6.0 (C), pH 5.0 (D), and pH 4.0 (E).

tinal pacemaking activity (Kim et al, 2005). TRPM7 produces pronounced outward currents at nonphysiological voltages, ranging from $+50$ to $+100$ mV, and small inward currents at negative potentials between -100 to -40 mV when expressed heterologously in mammalian cells (Nadler et al, 2001; Runnels et al, 2001; Monteilh-Zoller et al, 2003; Schmitz et al, 2003). The basal activity of TRPM7 is regulated by millimolar levels of intracellular MgATP and Mg^{2+}, therefore, TRPM7 is activated by depletion of intracellular MgATP and Mg^{2+} and is inhibited by high concentrations of MgATP and Mg^{2+} with an IC_{50} of 0.6 mM (Nadler et al, 2001). The present study extended our understanding about TRPM7 by showing that TRPM7 is also a pH-sensitive ion channel.

We investigated the characteristics and pH-mediated regulation of pacemaker activity. In cultured ICCs, there was a space clamp problem, therefore, we used current clamp mode instead of voltage clamp throughout the study, and recorded the membrane potential. Similar to Jiang et al (2005), we also identified the potentiation of TRPM7 inward currents by protons: In pacemaker activity, acidic pH developed depolarization of resting membrane potential, and this phenomenon might have been due to influx of proton in ICCs by TRPM7. This point might constitute one of the important proofs that TRPM7 is a candidate for pacemaker channels.

In conclusion, the resting membrane potential of pacemaking activity might be depolarized by external acidic pH through TRPM7 that is required for intestinal pacemaking activity.

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