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The Effect of External Divalent Cations on Intestinal Pacemaking Activity

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Electrical rhythmicity in the gastrointestinal (GI) muscles is generated by pacemaker cells, known as interstitial cells of Cajal (ICC). In the present study, we investigated the effect of external divalent cations on pacemaking activity in cultured ICC from murine small intestine by using whole-cell patch clamp techniques. ICC generated pacemaker currents under a voltage clamp or electrical pacemaker potentials under a current clamp, and showed a mean amplitude of -500 ± 50 pA or 30 ± 1 mV and the frequency of 18 ± 2 cycles/min. Treatments of the cells with external 0 mM Ca^{2+} stopped pacemaking activity of ICC. In the presence of 2 mM Ca^{2+}, 0 mM external Mg^{2+} depolarized the resting membrane potential, and there was no change in the frequency of pacemaking activity. However, 10 mM external Mg^{2+} decreased the frequency of pacemaking activity (6.75 ± 1 cycles/min, n=5). We replaced external 2 mM Ca^{2+} with equimolar Ba^{2+}, Mn^{2+} and Sr^{2+}, and they all developed inward current in the sequence of $Ba^{2+}>Mn^{2+}>Sr^{2+}$. Also the frequency of the pacemaking activity was stopped or irregulated. We investigated the effect of 10 mM Ba^{2+}, Mn^{2+} and Sr^{2+} on pacemaking activity of ICC in the presence of external 0 mM Mg^{2+}, and found that 10 mM Ba^{2+} and Mn^{2+} induced large inward current and stopped the pacemaking activity of ICC (n=5). Interestingly, 10 mM Sr^{2+} induced small inward current and potentiated the amplitude of pacemaking activity of ICC (n=5). These results indicate that extracellular Ca^{2+} and Mg^{2+} are requisite for the pacemaking activity of ICC.

Key Words: Divalent cations, Interstitial cells of Cajal (ICC), Pacemaking activity

INTRODUCTION

Spontaneous electrical depolarizations, termed slow waves, are generated within the tunica muscularis in many regions of the gastrointestinal (GI) tract (Szurszewski, 1987). These events time the phasic contractions of the gut (Tomita, 1981; Sanders, 1992). Many studies indicate that interstitial cells of Cajal (ICC) are the pacemaker cells that generate pacemaker potential (Langton et al, 1989; Ward et al, 1994, 2000; Huizinga et al, 1995; Torihashi et al, 1995; Sanders, 1996; Dickens et al, 1999). From ICC, pacemaker potentials spread passively via gap junctions to neighbouring smooth muscle cells where depolarization activates voltage-dependent, dihydropyridine-sensitive Ca^{2+} channels (Horowitz et al, 2000). The smooth muscle response to pacemaker potential can manifest either as Ca^{2+} action potentials or a sustained 'plateau' depolarization depending on the voltage-dependent K^+ channels available in smooth muscle cells. Influx of Ca^{2+} during slow waves activates phasic contractions (Ozaki et al, 1991). Recent work has suggested that the pacemaker current that generates slow waves is due to a voltage-independent, Ca^{2+}-inhibited, non-selective cationic conductance in ICC (Thomsen et al, 1998; Koh et al, 1998, 2002). This conductance is activated by release of Ca^{2+} from intracellular stores via IP_3 receptors, followed by Ca^{2+}-stimulated uptake of Ca^{2+} by mitochondria (Suzuki et al, 2000; van Helden et al, 2000; Ward et al, 2000).

The trace elements including divalent cations, are absolutely essential to life, usually because they are essential to the function of specific enzymes. For example, Mn^{2+} has the function of cofactor of arginase and other enzymes.

Recently, a plethora of papers have been published on pacemaking activity of ICC. For example, the effect of mibefradil on sodium and calcium currents in human (Strege et al, 2005), purinergic modulation of pacemaker Ca^{2+} activity (Furuzono et al, 2005), involvement of ryanodine receptors in pacemaker Ca^{2+} oscillation in murine gastric ICC (Liu et al, 2005), inhibition of pacemaker currents by deoxycholic acid by activating ATP-dependent K^+ channels through prostaglandin E2 (Jun et al, 2005), co-contribution of IP_3R and Ca^{2+} influx pathways to pacemaker Ca^{2+} activity in stomach ICC (Liu et al, 2005) and so on. Nevertheless, the effect of external divalent cations on pacemaking activity of ICC has not yet been investigated. Therefore, we investigated the effect of external divalent cations on pacemaking activity of ICC.

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ABBREVIATIONS: ICC, 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 collagenase (Worthington Biochemical Co, Lakewood, NJ, USA) 1.3 mg/ml, bovine serum albumin (Sigma Chemical Co., St Louis, MO, USA) 2 mg/ml, trypsin inhibitor (Sigma) 2 mg/ml and ATP 0.27 mg/ml. 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). The morphologies of ICC were distinct from other cell types in the culture, so it was possible to identify the cells with phase contrast microscopy once the cells had been verified with anti-c-kit antibody.

Patch-clamp experiments

The physiological salt solution used to bathe cells (Na^+-Tyrode) contained (mM): KCl 5, NaCl 135, $CaCl_2$ 2, glucose 10, $MgCl_2$ 1.2 and HEPES 10, adjusted to pH 7.4 with NaOH. The pipette solution contained (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 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) amplified 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 5kHz and displayed on an oscilloscope, a computer monitor and with a pen recorder (Gould 2200, Gould, Valley view, OF, USA). Results were analysed using pClamp and Origin (version) software. All experiments were performed at 30°C.

Statistics

All data are expressed as mean±S.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

Characteristics of pacemaker currents and pacemaker potentials in ICC

Under a voltage clamp and at a holding potential of −60 mV, cultured ICC showed spontaneous inward currents, which are referred to as pacemaker currents. The frequency of the pacemaker currents was 18±2 cycles/min and the amplitude was −500±50 pA (n=136; Fig. 1A). Under current clamp mode, the cultured ICC had a mean resting membrane potential of −58±3 mV and produced electrical pacemaker potential (n=136). The frequency of this pacemaker potential was 18±2 cycles/min with the amplitude of 30±1 mV (n=136; Fig. 1B). There is no qualitative difference between pacemaker currents and pacemaker potentials.

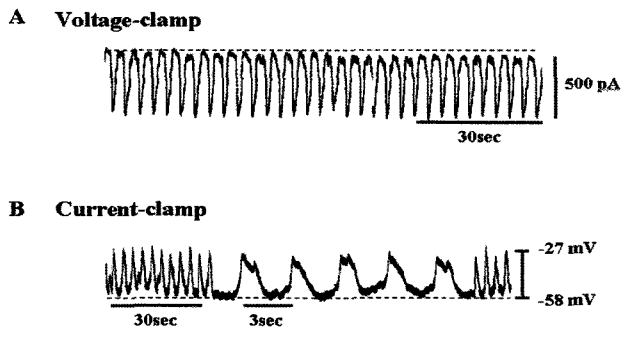

Fig. 1. Characteristics of pacemaker currents and pacemaker potentials in cultured ICC of the murine small intestine. (A) Under a voltage clamp and at a holding potential of −60 mV, cultured ICC showed spontaneous inward currents, which are referred to as pacemaker currents. (B) Under current clamp mode, cultured ICC produced electrical pacemaker potential from the same cell.

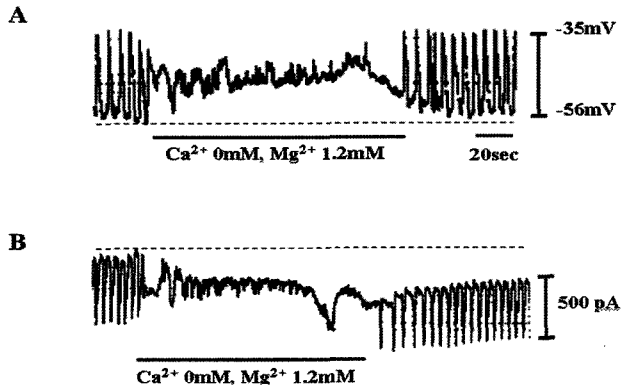

Fig. 2. Effect of extracellular Ca^{2+} on intestinal pacemaking activity We applied 1.2 mM Mg^{2+} externally in the absence of external Ca^{2+}. (A), (B) This treatment stopped pacemaking activity. Also the membrane potential became depolarized (A) and the membrane inward current was developed (B).

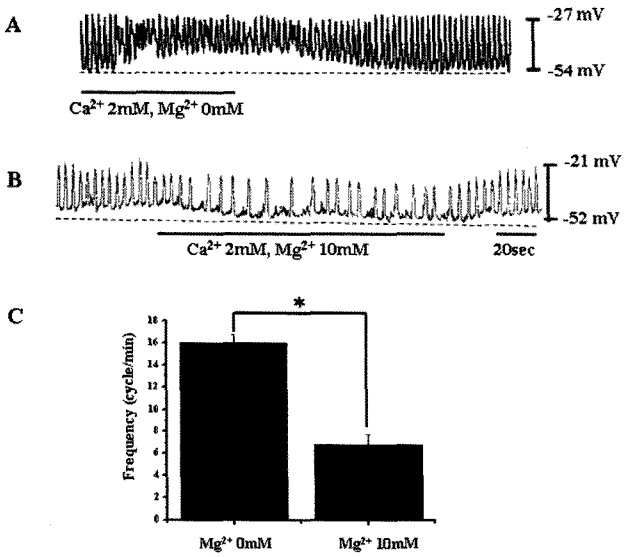

Fig. 3. Effect of extracellular Mg^{2+} on intestinal pacemaking activity (A) In the presence of 2 mM Ca^{2+}, 0 mM external Mg^{2+} depolarized the base line, but there was no change in the frequency of pacemaking activity. (B) In the presence of 2 mM Ca^{2+}, 10 mM external Mg^{2+} decreased the frequency of pacemaking activity (6.75 $\pm$1 cycles/min). (C) The summary of results was plotted. Asterisk (*) represents P value is less than 0.05.

Effect of extracellular Ca^{2+} on intestinal pacemaking activity

To investigate the effect of extracellular Ca^{2+} on intestinal pacemaking activity of ICC, we applied 1.2 mM Mg^{2+} externally in the absence of external Ca^{2+}. This treatment stopped the pacemaking activity. Also, the membrane potential became depolarized (n=5; Fig. 2A) and the membrane inward current was developed (n=5; Fig. 2B). Extracellular Ca^{2+} appears to have very important role in pacemaking activity of ICC.

Effect of extracellular Mg^{2+} on intestinal pacemaking activity

In the presence of 2 mM Ca^{2+}, 0 mM external Mg^{2+} depolarized the base line, however, there was no change in the frequency of pacemaking activity (n=6; Fig. 3A). In the presence of 2 mM Ca^{2+}, 10 mM external Mg^{2+} decreased the frequency of pacemaking activity (6.75$\pm$1 cycles/min, n=5; Fig. 3B and 3C). Therefore, the pacemaking activity of ICC appears to be regulated by external Mg^{2+} concentration. These results indicate that extracellular Mg^{2+} is requisite for pacemaking activity of ICC.

Effect of external divalent cations on intestinal pacemaking activity

To investigate the effect of external divalent cation on pacemaking activity, we replaced external 2 mM Ca^{2+} with equimolar concentration of Ba^{2+}, Mn^{2+} and Sr^{2+}. All these

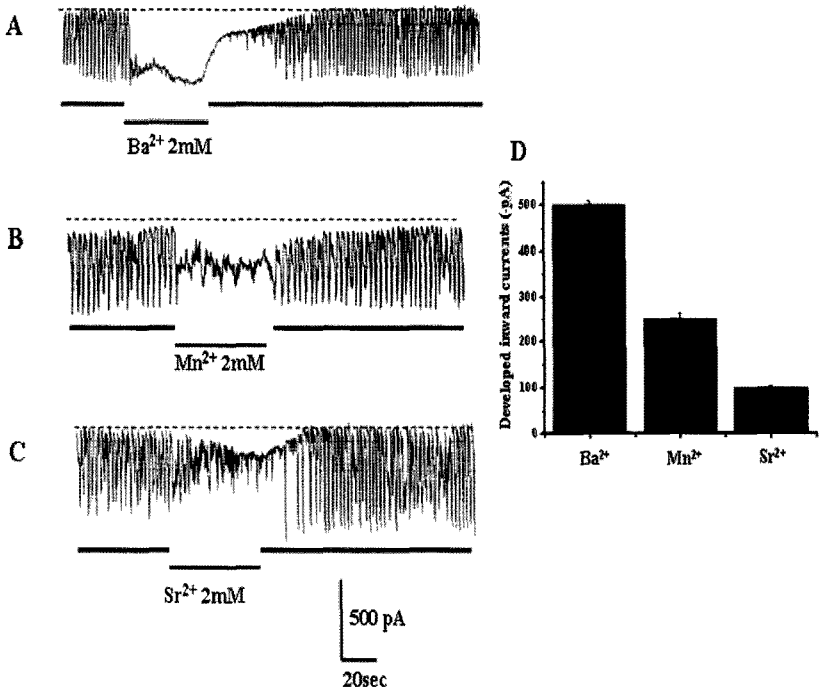

Fig. 4. Equimolar substitution of Ba^{2+}, Mn^{2+}, Sr^{2+} for 2 mM extracellular Ca^{2+}. (A), (B) and (C) We replaced external Ca^{2+} 2 mM with equimolar concentrations of Ba^{2+}, Mn^{2+} and Sr^{2+}, and recorded the membrane inward currents. All these divalent cations induced inward currents. (D) The summary of developed inward currents. These cations developed inward currents in the sequence of $Ba^{2+} > Mn^{2+} > Sr^{2+}$.

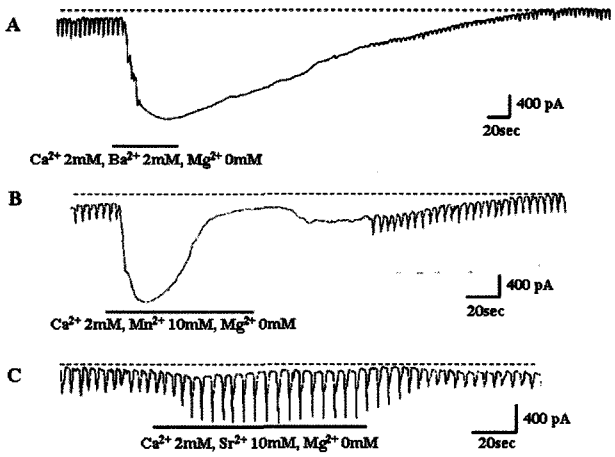

Fig. 5. Effect of external divalent cations on intestinal pacemaking activity In the presence of external 2 mM Ca^{2+} and 0 mM Mg^{2+}, we investigated the effect of 10 mM Ba^{2+}, Mn^{2+} and Sr^{2+} on pacemaking activity of ICC. (A) 10 mM Ba^{2+} induced a large inward current and stopped the pacemaking activity of ICC. (B) 10 mM Mn^{2+} also induced inward current and stopped the pacemaking activity of ICC. Mn^{2+} induced inward current came back spontaneously to basal current. (C) Sr^{2+} at 10 mM induced a small inward current and potentiated the amplitude of pacemaking activity of ICC.

divalent cations were found to induce inward current and the pacemaking activity stop or irregulate. The degree of inward current was 500±10 pA with Ba^{2+} (n=5, Fig. 4A), 250±12 pA with Mn^{2+} (n=6, Fig. 4B) and 100±5 mV with Sr^{2+} (n=5, Fig. 4C). Thus, these cations developed inward current in the sequence: $Ba^{2+} > Mn^{2+} > Sr^{2+}$ (Fig. 4D). There are two possible mechanisms for inward current development: an increase of inward current or a decrease of outward current. To assess whether these divalent cations permeate the pacemaking NSCC, we performed ratiometric calcium imaging. In each case, we found an increased ratio of F340/F380. The change of ratio indicates elevated intracellular divalent cation concentration even in the absence of external Ca^{2+}, suggesting that these cations do indeed carry current. In the presence of external Ca^{2+}, the divalent cations inhibited inward currents, thus acting as blockers of pacemaking activity (Jun et al, 2004). These results indicate that these divalent cations can carry NSCC in pacemaking activity of ICC instead of Ca^{2+}, and only extracellular Ca^{2+} can regularly develop pacemaking activity of ICC.

In the presence of external 2 mM Ca^{2+} and 0 mM Mg^{2+}, we investigated the effect of 10 mM Ba^{2+}, Mn^{2+} and Sr^{2+} on pacemaking activity of ICC. Ba^{2+} at 10 mM induced a large inward current and stopped the pacemaking activity of ICC (n=5, Fig. 5A). Mn^{2+} at 10 mM also induced inward current and stopped the pacemaking activity of ICC (n=5, Fig. 5B). Mn^{2+} induced inward current came back spontaneously to basal current. Interestingly, 10 mM Sr^{2+} induced a small inward current and potentiated the amplitude of the pacemaking activity of ICC (n=5, Fig. 5C). Sr^{2+} had the potentiation effect on the amplitude of the pacemaking activity of ICC in the presence of external 0 mM Mg^{2+}.

DISCUSSION

Interstitial cells of Cajal generate spontaneous pacemaker currents that depolarize membrane, which then spreads to smooth muscle via gap junctions and results in the depolarization of smooth muscle cell membrane. This membrane depolarization leads to smooth muscle contraction by generating an action potential via the activation of voltage dependent Ca^{2+} channels. It has been suggested that the pacemaker currents of interstitial cells of Cajal are mediated by the activation of voltage-independent nonselective cation channels (Koh et al, 1998; Thomsen et al, 1998), allowing net inward current predominantly by Na^{+} under physiological condition and leading to excitatory action in gastrointestinal smooth muscles.

The generation of pacemaker currents is initiated by the release of Ca^{2+} from the endoplasmic reticulum. Cyclopiazonic acid, a Ca^{2+} ATPase inhibitor in the endoplasmic reticulum, or xestospongin C, an inhibitor of inositol (1,4,5)-triphosphate receptor, in the endoplasmic reticulum abolished generation of pacemaker current. These findings suggest that inositol (1,4,5)-triphosphate-mediated Ca^{2+} release from the endoplasmic reticulum is essentially required for pacemaker current generation.

In this study, we investigated the external divalent cations on pacemaking activity of ICC. We recorded the pacemaking activity of ICC in normal condition (external 2 mM Ca^{2+} and 1.2 mM Mg^{2+}). In case of 0 mM Ca^{2+}, pacemaking activity was stopped. In case of 0 mM Mg^{2+}, there was no change in pacemaking frequency, but membrane potential was depolarized and membrane inward current was developed. In case of 10 mM Mg^{2+}, pacemaking frequency was decreased and membrane potential was hyperpolarized. Therefore external Ca^{2+} and Mg^{2+} have very important role in pacemaking activity of ICC. In case of 0 mM Mg^{2+}, the effect of 10 mM Ba^{2+}, Sr^{2+} and Mn^{2+} was studied. Interestingly, 10mM Sr^{2+} had the potentiation effect on the amplitude of pacemaking activity. Both Ba^{2+} and Mn^{2+} might not have the role in pacemaking activity of ICC by intracellular pathway, however, Sr^{2+} has the potentiation role in pacemaking activity of ICC by intracellular pathway. More detailed studies are needed in the future.

In conclusion, extracellular divalent cations play a critical role in pacemaking activity of ICC.

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