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The Role of Mitochondrial ATP-sensitive Potassium Channel on Intestinal Pacemaking Activity

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Interstitial cells of Cajal (ICCs) are the pacemaker cells that generate slow waves in the gastrointestinal (GI) tract. In the present study, we investigated the effect of mitochondrial ATP-sensitive potassium (mitoKATP) channel on pacemaking activity in cultured ICCs from murine small intestine by using whole-cell patch clamp techniques. Under current clamp mode, at 10 μM glibenclamide, there was no change in pacemaking activity of ICCs. At 30 μM glibenclamide, an inhibitor of the ATP sensitive K^+ channels, we could find two examples. If pacemaking activity of ICCs was irregulating, pacemaking activity of ICCs was changed into regulating and if in normal conditions, membrane potential amplitude was increased. At 50 μM glibenclamide, the resting membrane potential was depolarized. At 3mM 5-HDA, an inhibitor of the mitoKATP channels, inhibited the pacemaking activity of ICCs. Both the amplitude and the frequency were decreased. At 5 mM 5-HDA, both the amplitude and the frequency were completely abolished. Diazoxide, an opener of the mitoKATP channels, was applied to examine its effect on pacemaking activity of ICCs. At 50 μM concentration, the pacemaking activity of ICCs was inhibited. Both the amplitude and the frequency were decreased. At 1 mM concentration, both the amplitude and the frequency were completely abolished and the resting membrane potential was shaked. These results indicate that mitoKATP channel has an important role in pacemaking activity of ICCs.

Key Words: Interstitial cells of Cajal (ICCs), Mitochondrial ATP-sensitive potassium channel, Pacemaking activity

INTRODUCTION

ATP-sensitive potassium (KATP) channels are inhibited by intracellular ATP, and so these channels play a role in linking cell metabolic state to membrane potential. KATP channels are associated with diverse cellular functions, such as shortening of action potential duration in cardiac myocytes (Noma, 1983), insulin release in pancreatic β-cells (Cook & Hales, 1984), regulation of excitability in skeletal muscle (Spruce et al, 1985), and regulation of vascular smooth muscle contractility (Standen et al, 1989). KATP channels are heteromultimers composed of inwardly rectifying K^+ channel subunits (Kir6.χ) and sulfonylurea receptors (SURs) that associate in a 4 : 4 stoichiometry to form an octameric KATP channel. Various combinations of these two subunits convey the heterogeneity in channel properties observed in native cells such as Kir6.2-SUR1 in pancreatic β-cells, Kir6.2-SUR2A in cardiac and skeletal muscles, and Kir6.1-SUR2B or Kir6.2-SUR2B in smooth muscle (Seino, 1999).

Since early 1990s, K^+ selective transport has been widely observed in the mitochondria. A K^+ channel activity, with characteristics similar to those of surface KATP channel, was discovered in 1991 (Inoue et al, 1991). This mitochondrial ATP-sensitive potassium (mitoKATP) channel is modulated by a variety of K^+ channel openers and inhibitors (Grover & Garlid, 2000). Furthermore, these modulators significantly influence mitochondrial function and cell survival, suggesting a link between mitoKATP and protection against ischemic injury. This protective effect of mitoKATP has been demonstrated in several tissues besides the heart, including liver, brain, kidney and gut (Gross & Fryer, 1999; O'Rourke, 2000; Oldenburg et al, 2002; McCully & Levitsky, 2003).

Many regions of the tunica muscularis of the gastrointestinal tract display spontaneous contraction, and these spontaneous contractions are mediated by periodic generation of electrical slow waves (Szurszewski, 1987). Recent studies have shown that the interstitial cells of Cajal act as pacemakers and conductors of electrical slow waves in gastrointestinal smooth muscles (Langton et al, 1989; Ward et al, 1994; Huizinga et al, 1995; Sanders, 1996; Ordog et al, 1999). Although the exact mechanisms for these events remain still unclear, several studies suggest that mitochondria modulates pacemaking activity of ICCs.

In this study, therefore, we investigated the possibility that mitoKATP channel might affect electrical properties of cultured ICCs.

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ABBREVIATIONS: ICCs, Interstitial cells of Cajal; mitoKATP, mitochondrial ATP-sensitive potassium.

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 ICCs 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, K2ATP 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 potentials (current clamp) from cultured ICCs. 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 5 kHz 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

Cultured interstitial cells of Cajal cluster preparation

We started the approach of isolating ICC from the musculature to investigate the characteristics of pacemaker potential of the small intestine. However, ICCs from the small intestine are difficult to identify in cell suspension. Therefore, we cultured small intestinal ICCs. These cells grew into well-defined networks within 1~3 days that are morphologically similar to the myenteric plexus network of ICCs in the intact small intestine. We could see the spontaneous rhythmic contraction that was seen in most cell clusters and lasted up to several days. Fig. 1 shows an example of such ICCs cluster. ICCs within networks had a more robust electrical rhythmicity and tissue-like spontaneous pacemaker potentials were recorded from these cells.

Effect of glibenclamide on intestinal pacemaking activity

Under current clamp mode, to investigate the effect of glibenclamide on intestinal pacemaking activity of ICCs, first we applied 10 μM glibenclamide. But there was no change in pacemaking activity of ICCs (Fig. 2A). When we applied 30 μM glibenclamide, we could find two examples. If pacemaking activity of ICCs was irregulating, pacemaking activity of ICCs was changed into regulating (control frequency, 8±1 cycles/min, n=5; in glibenclamide, 13±1 cycles/min, n=5; Fig. 2B) and if in normal conditions, membrane potentiation amplitude was increased (control, 30±2 mV, n=4; in glibenclamide, 25±1 mV, n=4; Fig. 2C). When we applied 50 μM glibenclamide, the resting membrane potential was depolarized (control, −65±3 mV, n=5; in glibenclamide, −60±2 mV, n=5; Fig. 2D).

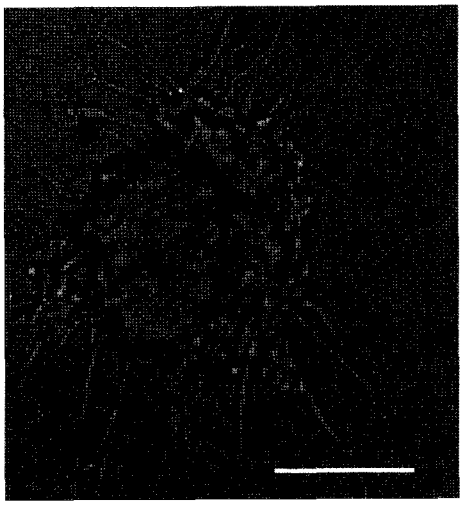

Fig. 1. Cultured interstitial cells of Cajal cluster preparation isolated from murine small intestine. Transmission image of an example cell cluster that shows spontaneous contraction under a light microscope (phase-contrast). Scale bar: 100 μm.

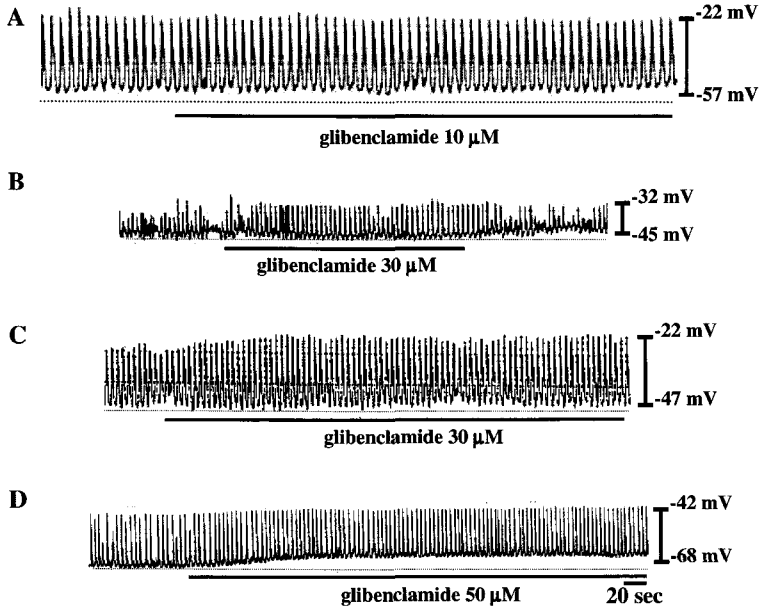

Fig. 2. Effect of glibenclamide on intestinal pacemaking activity. First, we applied 10 μM glibenclamide. But there was no change in pacemaking activity of ICCs (A). When we applied 30 μM glibenclamide, we could find two examples. If pacemaking activity of ICCs was irregulating, pacemaking activity of ICCs was changed into regulating (B) and if in normal conditions, membrane potentiation amplitude was increased (C). When we applied 50 μM glibenclamide, the resting membrane potential was depolarized (D).

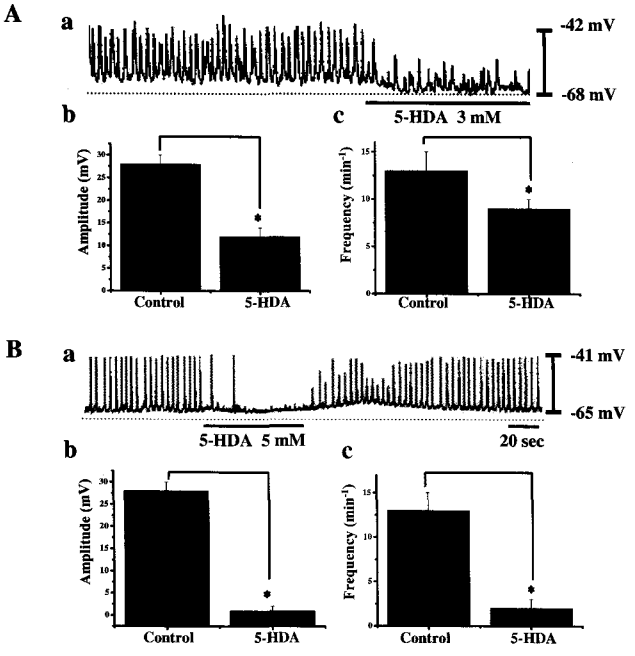

Fig. 3. Effect of 5-HDA on intestinal pacemaking activity. At 5-HDA 3 mM concentration, the pacemaking activity of ICCs was inhibited (Aa). The amplitude was decreasd (Ab) and also the frequency was decreased (Ac). At 5-HDA 5 mM concentration, the pacemaking activity of ICCs was inhibited (Ba). Both the amplitude and the frequency were completely abolished (Bb, c). Asterisk(*) represents p value is less than 0.05.

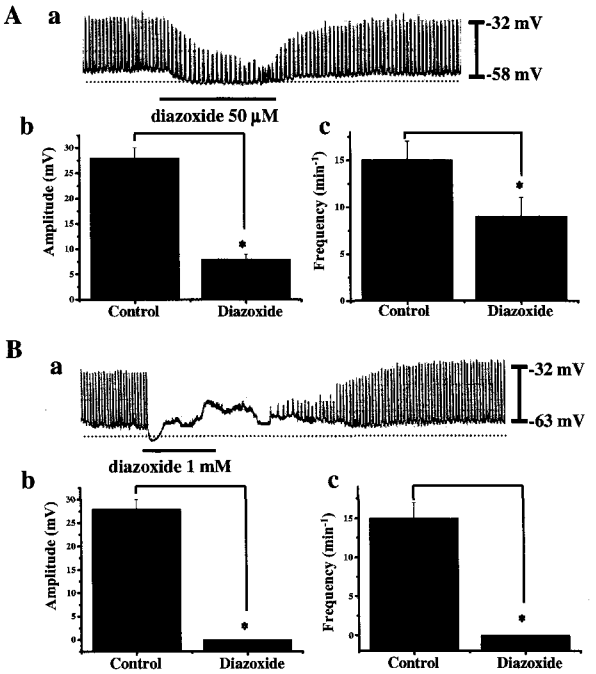

Fig. 4. Effect of diazoxide on intestinal pacemaking activity. Diazoxide was applied to examine its effect on pacemaking activity of ICCs. At 50 μM concentration, the pacemaking activity of ICCs was inhibited (Aa). The amplitude was decreased (Ab), and the frequency was also decreased (Ac). At 1mM concentration, the pacemaking activity of ICCs was inhibited (Ba). Both the amplitude and the frequency were completely abolished (Bb, c). Asterisk(*) represents p value is less than 0.05.

Effect of 5-HDA on intestinal pacemaking activity

5-HDA, an inhibitor of the mitoKchondrial ATP-sensitive K^+ channels (Grover & Garlid, 2000), at 3 mM concentration, inhibited the pacemaking activity of ICCs (Fig. 3Aa). The amplitude was decreasd (control, 28±2 mV, n=4; in 5-HAD, 12±2 mV, n=4; Fig. 3Ab) and also the frequency was decreased (control, 13±2 cycles/min, n=3; in 5-HDA, 9±1 cycles/min, n=3; Fig. 3Ac). At 5 mM concentration, the pacemaking activity of ICCs was also inhibited (Fig. 3Ba). Both the amplitude and the frequency were completely abolished (Fig. 3Bb, c).

Effect of diazoxide on intestinal pacemaking activity

Diazoxide, an opener of the mitoKATP channels (Garlid et al, 1997), was applied to examine its effect on pacemaking activity of ICCs. At 50 μM concentration, the pacemaking activity of ICCs was inhibited (Fig. 4Aa). The amplitude was decreased (control, 28±2 mV, n=5; in diazoxide, 8±1 mV, n=5; Fig. 4Ab), and the frequency was also decreased (control, 15±2 cycles/min; in diazoxide, 9±2 cycles/min; Fig. 4Ac). At 1mM concentration, the pacemaking activity of ICCs was inhibited (Fig. 4Ba) and both the amplitude and the frequency were completely abolished (Fig. 4Bb, c). The resting membrane potential was shaked.

DISCUSSION

There are two KATP channels that have been identified in the heart, brain, liver, skeletal muscle and the kidneys: the cell surface (surface KATP) and the mitochondrial (mitoKATP) channels (Gross & Fryer, 1999; O'Rourke, 2000; Debska et al, 2002; Oldenburg et al, 2002; McCully & Levitsky, 2003). Early studies suggested that opening of surface KATP channels may mediate the cardioprotective effects of ischemic preconditioning (IPC). The opening of the surface KATP channel leads to shortening of phase 3 of the action potential and hyperpolarizing cell membrane (Noma, 1983). This, in turn, would result in a reduction in the intracellular Ca^{2+} levels, which initially was thought to produce the cardioprotective effects. However, studies published as early as 1994 suggested that surface KATP channels did not play an important role in IPC. Yao & Gross (1994) showed that a low dose of bimakalin produced cardioprotective effects, even though it did not shorten action potential duration (APD) at that low dose. Subsequently, Grover et al (1996) studied the effect of the class III antiarrhythmic agent dofetilide on preconditioning at a dose that prevented APD shortening during ischemia. Their results showed that dofetilide abolished the APD shortening of ischemia, but did not alter the protective effects of preconditioning. In 1991, Inoue et al (1991) reported the identification of a KATP channel in the mitochondria. They patch clamped mitoplasts (mitochondria stripped of their outer membranes) from rat liver and identified K^+ selective channels, sensitive to inhibition by ATP, 4-aminopyridine and glibenclamide. The conductance of these channels was lower than that of surface KATP channels (about 10 pS in 100 mM matrix K^+ and 33 mM cytosolic K^+). Subsequent studies by several groups have demonstrated that mitoKATP may be the key player in IPC.

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), which allows net inward current predominantly by Na^+ under physiological condition, and which leads to excitatory action in gastrointestinal smooth muscles.

Activation of pacemaker currents depends upon periodic release of Ca^{2+} from IP3 receptors. Ca^{2+} release from IP3 receptors triggers Ca^{2+} uptake by mitochondria, possibly by gating the rapid uptake mode of the uniporter (Sparagna et al, 1995; Litsky and Pfeiffer, 1997). Mitochondrial Ca^{2+} uptake is linked in an unknown way to activation of pacemaker currents. The pacemaker cycle may be completed and reset by uptake of Ca^{2+} into the ER (i.e. via a thapsigargin-sensitive Ca^{2+} pump). Uptake and periodic release of Ca^{2+} from IP3 receptor-operated stores appears to be the main oscillatory process responsible for GI autorhythmicity. Thus the pacemaker mechanism is a highly integrated process requiring physical proximity and coordination between ion channels and transport proteins in the ER, mitochondria and plasma membrane (Ward et al, 2000).

In the present study, glibenclamide 10 μM had no effect on pacemaking activity of ICCs. Therefore, surface KATP in ICCs have no functions on pacemaking activity. But, interestingly, glibenclamide 30 μM had the augmentation effect on pacemaking activity of ICCs. We thought that these effects were generated by inhibition of mitoKATP channels (Fukuta et al, 2002; Ardehali, 2004). 5-HDA, an inhibitor of the mitoKATP channels (Grover & Garlid, 2000), and diazoxide, an opener of the mitoKATP channels (Garlid et al, 1997) inhibited the pacemaking activity of ICCs. Thus, disordered mitochondrial Ca^{2+} handling may prevent the rhythmic pacemaking activity of ICCs.

In conclusion, mitochondrial ATP-sensitive potassium channel has an important role in pacemaking activity of ICCs.

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