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RAPID COMMUNICATION

Involvement of mitochondrial Na+–Ca2+ exchange in intestinal pacemaking activity

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Abstract

AIM: Interstitial cells of Cajal (ICCs) are the pacemaker cells that generate slow waves in the gastrointestinal (GI) tract. We have aimed to investigate the involvement of mitochondrial Na^+-Ca^{2+} exchange in intestinal pacemaking activity in cultured interstitial cells of Cajal.

METHODS: Enzymatic digestions were used to dissociate ICCs from the small intestine of a mouse. The whole-cell patch-clamp configuration was used to record membrane currents (voltage clamp) and potentials (current clamp) from cultured ICCs.

RESULTS: Clonazepam and CGP37157 inhibited the pacemaking activity of ICCs in a dose-dependent manner. Clonazepam from 20 to 60 μmol/L and CGP37157 from 10 to 30 μmol/L effectively inhibited Ca^{2+} efflux from mitochondria in pacemaking activity of ICCs. The IC_{50}s of clonazepam and CGP37157 were 37.1 and 18.2 μmol/L, respectively. The addition of 20 μmol/L $NiCl_2$ to the internal solution caused a "wax and wane" phenomenon of pacemaking activity of ICCs.

CONCLUSION: These results suggest that mitochondrial Na^+-Ca^{2+} exchange has an important role in intestinal pacemaking activity.

Key words: Mitochondrial Na^+-Ca^{2+} exchange; Interstitial cells of Cajal

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INTRODUCTION

The interstitial cells of Cajal (ICCs) produce spontaneous rhythmic inward currents that are critical for the generation of slow waves in intestinal smooth muscle[1-3]. Pacemaker currents in ICCs result from the activation of a voltage-independent, non-selective cation conductance[4,5]. Pacemaking activity in ICCs is dependent upon metabolic activity[6] and Ca^{2+} release from intracellular stores[7]. Recent findings suggested that the pacemaker conductance in ICC is regulated by intracellular Ca^{2+} modulation[8]. The close association between IP_3 receptor-dependent Ca^{2+} stores, mitochondria, and ion channels in the plasma membrane creates a basic cellular structure[9,10]. Release of Ca^{2+} from IP_3 receptors does not directly initiate pacemaker currents in ICC, but rather, initiates Ca^{2+} uptake into the mitochondria. It was found that mitochondria in ICCs experience Ca^{2+} oscillations at the same frequency as pacemaker currents and that a rise in mitochondrial Ca^{2+} slightly precedes the activation of pacemaker currents[8]. This implies that pacemaker channels in the plasma membrane are activated by the falling phase of localized Ca^{2+} transients.

In isolated mitochondria, Ca^{2+} influx occurs via a Ca^{2+} uniporter driven by the membrane potential[11]. Ca^{2+} efflux occurs via Na^+-Ca^{2+} exchange and can be inhibited by diltiazem, clonazepam, CGP37157, and by high external Ca^{2+}[11-14]. However, the effect of inhibiting mitochondrial efflux, by using inhibitors of the Na^+-Ca^{2+} exchange, on pacemaking activity of ICCs has not yet been investigated. Therefore, we undertook to investigate the involvement of mitochondrial Na^+-Ca^{2+} exchange in pacemaking activity of ICCs.

MATERIALS AND METHODS

Preparation of cells and cell cultures

Balb/c mice (8-13 days old) of either sex were anesthetized with ether and killed by cervical dislocation. The small intestines from 1 cm below the pyloric ring to the cecum were removed and opened along the mesenteric border. Luminal contents were removed by washing with Krebs-Ringer bicarbonate solution. The tissues were pinned to

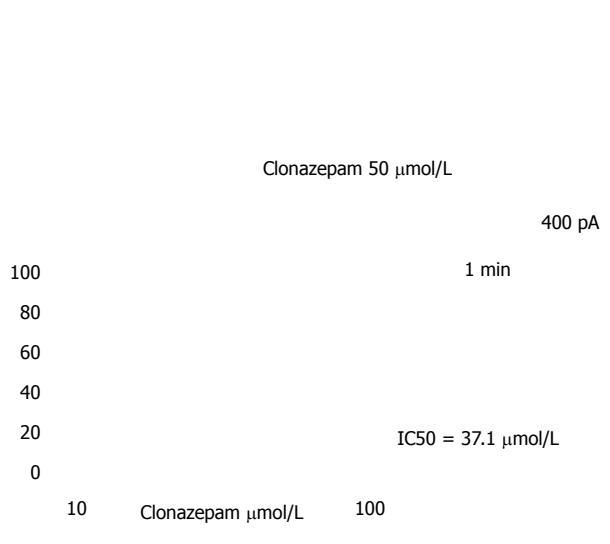

Figure 1 The effect of clonazepam on the pacemaking activity of ICCs. Clonazepam was applied to examine its effect on the pacemaking activity of ICCs. A: Under a voltage clamp at a holding potential of –60 mV, 50 μmol/L clonazepam inhibited the pacemaking currents of ICCs (n = 4). B: Clonazepam from 20 to 60 μmol/L effectively inhibited Ca^{2+} efflux from mitochondria in the pacemaking activity of ICCs. The IC_{50} of clonazepam was 37.1 μmol/L.

the base of a Sylgard dish and the mucosa removed by sharp dissection. Small tissue strips of the intestine muscle (consisting of both circular and longitudinal muscles) were equilibrated in Ca^{2+}-free Hanks solution (containing in mmol/L: 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 using 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, Franklin Lakes, NJ, USA) in a 35-mm culture dish and then cultured at 37 °C in a 95% O_2, 50 mL/L CO_2 incubator in a smooth muscle growth medium (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-conjugated rat anti-mouse c-kit monoclonal antibody; eBioscience, San Diego, CA, USA) at a dilution of 1:50 for 20 min[15]. ICCs were morphologically distinct from other cell types in the culture and thus it was possible to identify the cells by phase contrast microscopy once they had been verified with anti-c-kit antibody.

Patch-clamp experiments

The whole-cell patch-clamp configuration was used to record membrane currents (voltage clamp) and potentials (current clamp) from cultured ICCs. An axopatch ID (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). Data obtained were filtered at 5 kHz and displayed on an oscilloscope, a computer monitor, and using a pen recorder (Gould 2200, Gould, Valley View, OH, USA).

Results were analyzed using pClamp and Origin (version 6.0) software. All experiments were performed at 30-32 °C.

Solutions and drugs

The physiological salt solution used to bathe cells (Na^+-Tyrode) contained (mmol/L): 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 (mmol/L): 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.

Before the development of CGP37157, several benzodiazepines (except clonazepam) were used as mitochondrial Na^+-Ca^{2+} exchange inhibitors[12]. Clonazepam and CGP37157 were dissolved in dimethyl sulfoxide (DMSO) for 100 and 50 mmol/L stock solution, respectively and added (1 000 times dilution) to the bathing solution at the day of the experiment. The final concentration of DMSO in the bath solution was always <0.1%, and we confirmed that this concentration of DMSO did not affect the results that were recorded. Nickel chloride was directly added to the pipette solutions at the day of the experiment. CGP37157 was purchased from Tocris Cookson (Ellisville, MO, USA). The rest of the drugs were obtained from Sigma (Sigma Chemical Co., USA), unless otherwise stated. Diltiazem was not used because of its known effects on the cell membrane Ca^{2+} channels.

RESULTS

Effect of clonazepam on the pacemaking activity of ICCs

Under a voltage clamp at a holding potential of -60 mV, clonazepam 50 μmol/L inhibited the pacemaking currents of ICCs (n = 4, Figure 1A). Clonazepam from 20 to 60 μmol/L effectively inhibited Ca^{2+} efflux from mitochondria on the pacemaking activity of ICCs. Concentrations of clonazepam of >100 μmol/L produced no further inhibition. The IC_{50} of clonazepam was 37.1 μmol/L (Figure 1B).

Effect of CGP37157 on the pacemaking activity of ICCs

The benzodiazepine CGP37157 has been shown to be a more potent inhibitor than either clonazepam or diltiazem in terms of Ca^{2+} efflux measured in isolated mitochondria[12]. Thus, CGP37157 was applied to examine its effect on the pacemaking activity of ICCs. Under a current clamp (I = 0), CGP37157 was found to inhibit pacemaking potentials in a dose-dependent manner (n = 15, Figures 2A-2C). CGP37157 from 10 to 30 μmol/L effectively inhibited Ca^{2+} efflux from mitochondria on the pacemaking activity of ICCs. Concentrations of CGP37157 of >50 μmol/L produced no further inhibition. The IC_{50} of CGP37157 was 18.2 μmol/L (Figure 2D).

Effect of internal Ni^{2+} on the pacemaking activity of ICCs

Ni^{2+} is a competitive inhibitor of the Ca^{2+} carrier, but it is not transported into the mitochondria[16]. Micromolar concentrations of nickel (Ni^{2+}) chloride have been reported

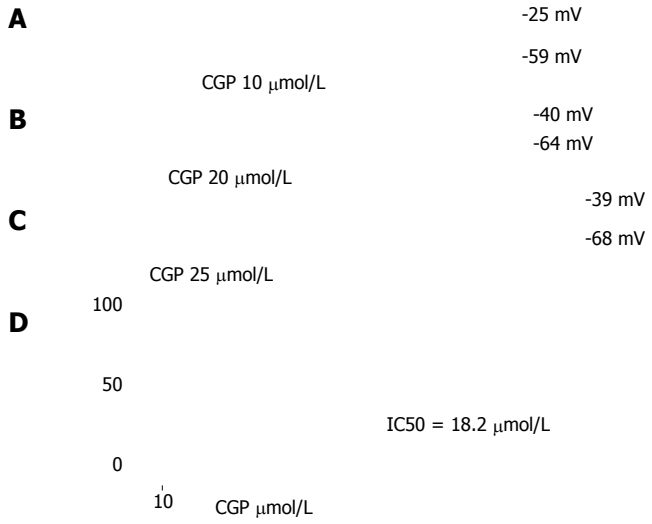

Figure 2 The effect of CGP37157 in the pacemaking activity of the ICCs. CGP37157 was applied to examine its effect on the pacemaking activity of ICCs. **A, B**, and **C:** Under a current clamp ($I=0$), CGP37157 inhibited the pacemaking potentials in a dose-dependent manner ($n=15$). **D:** CGP37157 from 10 to 30 μmol/L effectively inhibited Ca^{2+} efflux from mitochondria in the pacemaking activity of ICCs. The IC_{50} of CGP37157 was 18.2 μmol/L.

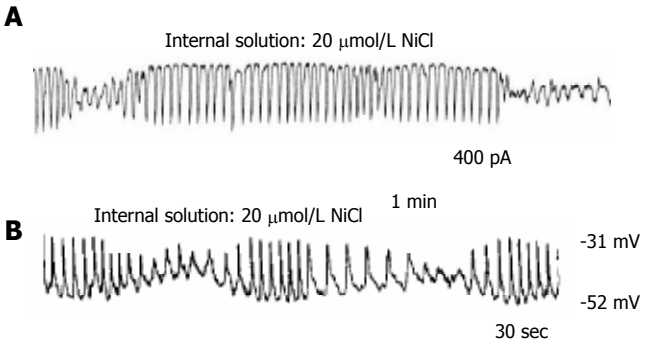

Figure 3 Effect of internal Ni^{2+} on the pacemaking activity of ICCs. In order to investigate the effect of $NiCl_2$ on the pacemaking activity of ICCs, we added 20 μmol/L of $NiCl_2$ to the internal solution. A: Under a voltage clamp at a holding potential of -60 mV, the pacemaking activity of ICCs showed a "wax and wane" phenomenon ($n=6$). B: Current clamp mode ($I=0$) showed the same phenomenon ($n=3$).

to inhibit Na^+-Ca^{2+} exchange in both vascular and non-vascular cells[17]. In order to investigate the effect of $NiCl_2$ on the pacemaking activity of ICCs, we added 20 μmol/L $NiCl_2$ to the internal solution. Under a voltage clamp mode at a holding potential of -60 mV, the pacemaking activity of ICCs showed a "wax and wane" phenomenon ($n=6$, Figure 3A). Also in current clamp mode ($I=0$), the same phenomenon was shown ($n=3$, Figure 3B). In case of 100 μmol/L $NiCl_2$, the pacemaking activity of ICCs stopped (data not shown).

DISCUSSION

Intracellular Ca^{2+} plays an important role in the regulation of various cellular functions including exocytosis, metabolic activity, contractile activity, and gene expression in excitable cells. In these cells, the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) is lowered by various mechanisms such as Ca^{2+} extrusion due to the actions of Na^+-Ca^{2+} exchangers and Ca^{2+} pumps in the plasma membrane, Ca^{2+} uptake by Ca^{2+} pumps in the endoplasmic reticulum, and by Ca^{2+} uniporters in the mitochondria[17]. Sequestered Ca^{2+} in mitochondria is, in turn, released to the cytoplasma via various mechanisms[11]. Na^+-Ca^{2+} exchangers and/or permeability transition pores are proposed to be involved in Ca^{2+} efflux from the mitochondria[11].

In case of ICCs, pacemaking activity is associated with mitochondrial Ca^{2+} transients. Pacemaker currents and rhythmic mitochondrial Ca^{2+} uptake by ICCs are blocked by inhibitors of IP_3dependent Ca^{2+} release from the endoplasmic reticulum and by inhibitors of endoplasmic reticulum Ca^{2+} reuptake. Therefore, integrated Ca^{2+} management by endoplasmic reticulum and mitochondria is a prerequisite of electrical pacemaking in the gastrointestinal tract[8].

CGP37157 is a benzodiazepine derivative that inhibits electroneutral mitochondrial Na^+-Ca^{2+} exchanger with submicromolar potency. In the heart, for example, this transporter is inhibited by CGP37157 at 400 nmol/L[12,14]. Before the development of CGP37157, several related benzodiazepines (e.g., clonazepam and diltiazem) were used as mitochondrial Na^+-Ca^{2+} exchange inhibitors[14]. In general, few have reported that these compounds inhibit the cardiac plasmalemmal Na^+-Ca^{2+} exchanger[18,19]. Mitochondrial Na^+-Ca^{2+} exchanger protein participates in Ca^{2+} efflux and operates in opposition to a Ca^{2+} uniporter within the inner mitochondrial membrane. Thus, the inhibition of mitochondrial Na^+-Ca^{2+} exchanger leads to an increase in Ca^{2+} levels within the mitochondria[14]. Calcium within the mitochondria serves as an important regulator of several key enzymes involved in energy metabolism. For example, the upregulation of the steady-state level of mitochondrial $[Ca^{2+}]$ ($[Ca^{2+}]_m$) result increases NADH production and stimulates oxidative phosphorylation[14].

The benzodiazepine CGP37157 has been shown to be a more potent inhibitor than either clonazepam or diltiazem on Ca^{2+} efflux, as measured in isolated mitochondria[12]. In ICCs, the IC_{50}s of clonazepam and CGP37157 were found to be 37.1 and 18.2 μmol/L, respectively. Thus, CGP37157 was about twofold more potent than clonazepam.

Ni^{2+} is a potent inhibitor of mitochondrial Ca^{2+} transport[20] and a competitive inhibitor of Ca^{2+} carrier[16]. Also micromolar concentrations of nickel (Ni^{2+}) chloride were found to inhibit Na^+-Ca^{2+} exchanger in both vascular and non-vascular cells[21]. Therefore, we have investigated the effect of $NiCl_2$ added to the internal solution. At 20 μmol/L, we observed a "wax and wane" phenomenon and at 100 μmol/L, the pacemaking activity of ICCs stopped.

Our data indicate that both clonazepam and CGP37157 inhibit the pacemaking activity of ICCs in a dose-dependent manner. The IC_{50}s of clonazepam and CGP37157 were 37.1 and 18.2 μmol/L, respectively. When 20 μmol/L $NiCl_2$ was added to the internal solution, the pacemaking activity of ICCs showed a "wax and wane" phenomenon.

We conclude that mitochondrial Na^+-Ca^{2+} exchange has an important role in intestinal pacemaking activity.

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