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Methylene Blue-stained Interstitial Cells are Electrically Active in the Myenteric Board Freshly Prepared from the Murine Small Intestine

Kyu Pil Lee, Ju-Hong Jeon, Insuk So, and Ki Whan Kim

Department of Physiology and Biophysics, Seoul National University College of Medicine, Seoul 110-799, Korea

Many gastrointestinal muscles show electrical oscillation, so-called "slow wave", originated from interstitial cells of Cajal (ICCs). Thus, a technique to freshly isolate the cells is indispensable to explore the electrophysiological properties of the ICCs. To apply an enzyme solution on the serosal surface for cell isolation, the intestine was inverted and 0.02% trypsin solution and 0.04% collagenase solution were applied to serosal cavity. After the enzyme treatment, mucosal layer was removed and longitudinal muscle layer was gently separated from the rest of tissue. The thin layer was stretched in the recording chamber and mounted on an inverted microscope. Using β-escine, perforated whole cell patch clamp technique was used. Under a microscope, the tissue showed smooth muscle cells and interstitial cells around the myenteric plexus. Under voltage clamp condition, three types of membrane potential were recorded. One group of interstitial cells, which were positive to methylene blue and CD34, showed spontaneous outward current. These cells had bipolar shape and were considered as fibroblast-like cells because of their peculiar shape and arrangement. Another group, positive to c-kit and methylene blue, showed spontaneous inward current. These cells had more rounded shape and processes and were considered as ICCs. The third, positive to c-kit and had granules containing methylene blue, showed quiet membrane potentials under the voltage-clamp mode. These cells appeared to be resident macrophages. Therefore, in the freshly isolated thin tissue preparation, methylene blue could easily identify three types of cells rather than morphological properties. Using this method, we were able to study electrical properties of fibroblast and residential macrophage as well as myenteric ICCs.

Key Words: Interstitial cells of Cajal, Isolation, Fibroblast-like cell, Methylene blue, Resident macrophage, Pacemaker

INTRODUCTION

It is well known that specialized interstitial cells of Cajal (ICCs) distributed within the tunica muscularis of the gastrointestinal (GI) tract may participate as electrical pacemakers and mediators of enteric neurotransmission (Ward et al, 1994; Huizinga et al, 1995). Signals through Kit receptor tyrosine kinase are essential for development of interstitial cells of Cajal (ICCs), therefore, anti-c-kit antibody has been used as a specific maker of ICC (Huizinga et al, 1995). ICCs generate spontaneous depolarizing inward current and propagate to neighboring smooth muscle by gap junction. Because identification of ICCs is difficult after the procedure for cell isolation, direct ionic mechanisms responsible for pacemaker currents remain largely in discussion.

Previously, several approaches to directly record electrical properties of ICCs have been reported, and they include enzymatic isolation techniques or direct recording from ICC *in situ*; single cell isolation from canine colon (Lee & Sanders, 1993), purification of ICC by fluorescence-activated cell sorting (Ördög et al, 2004), ICCs with their morphological properties after several day's culture (Koh et al, 1998) and direct recording from intact tissue of mouse small intestine (Kito & Suzuki, 2003). However, the use of these approaches is restricted, because they could not evaluate whether the cells are ICCs or not until electrical experiment was done.

In this experiment, we used methylene blue staining technique, which had previously been reported preparation technique by Goto et al (2004), to identify ICCs before electrical experiments. Methylene blue had been widely used before anti-c-kit antibody was employed as specific maker for ICC (Thuneberg et al, 1983). It is nontoxic but become toxic to the cells after exposure to the light. Therefore, under a microscope, we illuminated with dim light during experiments. After incubation of prepared tissues with methylene blue, stained cells were rinsed, and its color was lost, when perfused with warm (37°C) external solution at a constant flow rate (about 2 ml min^{-1}). We could easily

Corresponding to: Insuk So, Department of Physiology and Biophysics, Seoul National University College of Medicine, 28, Yeongeon-dong, Jongno-gu, Seoul 110-799, Korea. (Tel) 82-2-740-8228, (Fax) 82-2-763-9667, (E-mail) insuk@plaza.snu.ac.kr

ABBREVIATIONS: ICCs, interstitial cells of cajal; PE, phycoerythrin; FITC, fluorescein isothiocyanate.

identify the stained cell and patch electrodes were applied to interstitial cells and spontaneous membrane potential changes were recorded. Under voltage clamp condition, two types of currents were recorded, which is identical with previous result. We recorded quiet membrane potentials from the cells, which had morphological properties similar to ICCs and were c-kit positive besides two spontaneous currents. These cells seemed to be resident macrophages located at myenteric plexus. We suggest that this method has worthwhile advantage for the research on direct ionic mechanism responsible for pacemaker current.

METHODS

Tissue preparation

Either sex of ICR mice, aged 7 weeks, was sacrificed by cervical dislocation and small bowel including the stomach was transferred into Ca^{2+}-free external solution. The thin muscle layer was isolated as described previously (Goto et al, 2004). Briefly, to apply an enzyme solution on to the serosal side, the intestine was inverted by suck into the syringe from distal ileum. Stomach was sealed using rubber ring with the end of syringe and gave positive pressure. An intestinal segment of about 40 mm in length was cut and fixed in the digesting tissue chamber. The inverted intestine was perfused with Ca^{2+}-free solution for 5 min while keeping at 30~40 cm hydrostatic pressure. At first, 0.02% trypsin for 5 min, and then 0.04% collagenase (Worthington, USA) for 10 min were perfused. After the enzyme treatment, the segment was moved to Ca^{2+}-free solution in the preparation chamber and cut along the mesenteric board and washed several times. Small pieces (~10×5 mm) of intestine were pinned out on the sylgard-flatted chamber and muscle layer was gently dissected form the rest of the tissue using fine forceps and microscissors. Usually, this muscle preparation contained almost all the longitudinal muscle layer and a part of circular muscle layer. The isolated muscle preparation was stretched and serosal surface face was pinned to the bottom using tungsten pin (0.03 mm) in the recording chamber under a stereoscope and mounted on an inverted microscope. The preparations were perfused with external solution warmed to 37°C.

Methylene blue staining to label ICC

Thin intestine wall prepared was pinned flat on the recording chamber. Before incubation with methylene blue (Sigma Aldrich, USA), tissues should be washed several times with fresh external solution, and then incubated in an external solution containing 50 μM methylene blue, for 25~30 minutes in dark. After incubation, the tissue was washed several times, and mounted on inverted microscope and examined under dim light field. ICCs are stained as dark blue color.

Immunohistochemistry

Isolated thin muscle layer was incubated for 20 min with anti-c-kit antibody [phycoerythrin (PE)-conjugated rat anti-mouse c-kit monoclonal antibody; eBioscience, USA] at a dilution of 1 : 50. After washing twice with the control external solution, the tissues were subsequently incubated with anti-CD34 antibody [fluorescein isothiocyanate (FITC)-conjugated rat anti-mouse CD34 monoclonal antibody; Biosciences pharmingen, USA] at a dilution of 1:50, and washed twice with the control external solution.

Electrophysiological recording

Prepared tissues were transferred to a specialized chamber, four edges were filled with sylgard flat, and the tissues were pinned flat on the stage of an inverted microscope (IMT-2 or IX50, Olympus, Japan) and were constantly superfused with warmed to 37°C normal external solution at a rate of 2~3 ml/min. A glass microelectrode, filled with the pipette solution, with a resistance of 7~10 MW was used to make a gigaohm seal. The conventional perforated-whole-cell patch-clamp technique was used to hold the membrane potential at −60 mV using an Axopatch 200B patch-clamp amplifier (Axon Instrument, Union City, CA, USA). For data acquisition and application of command pulses, pCLAMP software v.9.2 (Axon Instruments) was used. Data were filtered at 5 kHz and recorded to Pentium grade computer for late analysis. Data were analyzed using v.9.2 Clampfit and Origin software (Microcal origin v.7.0, USA).

Solution and drugs

The control external solution contained (mM) 140 NaCl, 5.4 KCl, 0.33 Na_2HPO_4, 1.8 $CaCl_2$, 0.5 $MgCl_2$, 5.5 glucose, 5 Na-pyruvate, and 5 HEPES, and the pH was adjusted to 7.4 with NaOH. In the nominal Ca^{2+}-free solution, $CaCl_2$ was omitted from the external solution. The pipette solution contained (mM), 110 K-aspartate, 20 KCl, 1 $MgCl_2$, 2 KH_2PO_4, 0.2 EGTA and 5 HEPES, and the pH was adjusted to 7.2 with KOH. For perforated patch clamp experiment, 50 μM b-escine (Sigma, USA) was added into pipette solution. 1 μM nifedipine (Sigma, USA) was added to the external solution for preventing the movement of tissue.

RESULTS

Cell identification by methylene blue and direct immunohistochemistry

Methylene blue stained cells in the tissue preparation: Mounted preparations were observed under an inverted microscope with dim light. As shown in Fig. 1, the tissue had smooth muscle cells and the interstitial cells around the myenteric plexus. Smooth muscle (black arrow in the upper panel of Fig. 1) was contracted and round. In a part of smooth muscle, however, spontaneous contraction remained and it made the whole tissue move during experiment. To prevent the movement, 1 μM nifedipine, which has no effect on spontaneous electrical activity, was used to record membrane potential (Suzuki & Hirst, 1999). Methylene blue stained even ganglia (G) and some spindle-shape interstitial cells. Stained spindle- shape interstitial cells have processes branching to neighboring cells. Almost all the methylene blue positive cells have rounded cell body and located near the ganglia. However, there are stained cells, which have bipolar and peculiar- shape and are considered to be fibroblast-like cells (white arrow in below panel of Fig. 1).

Both of these methylene blue-positive cells showed elec-

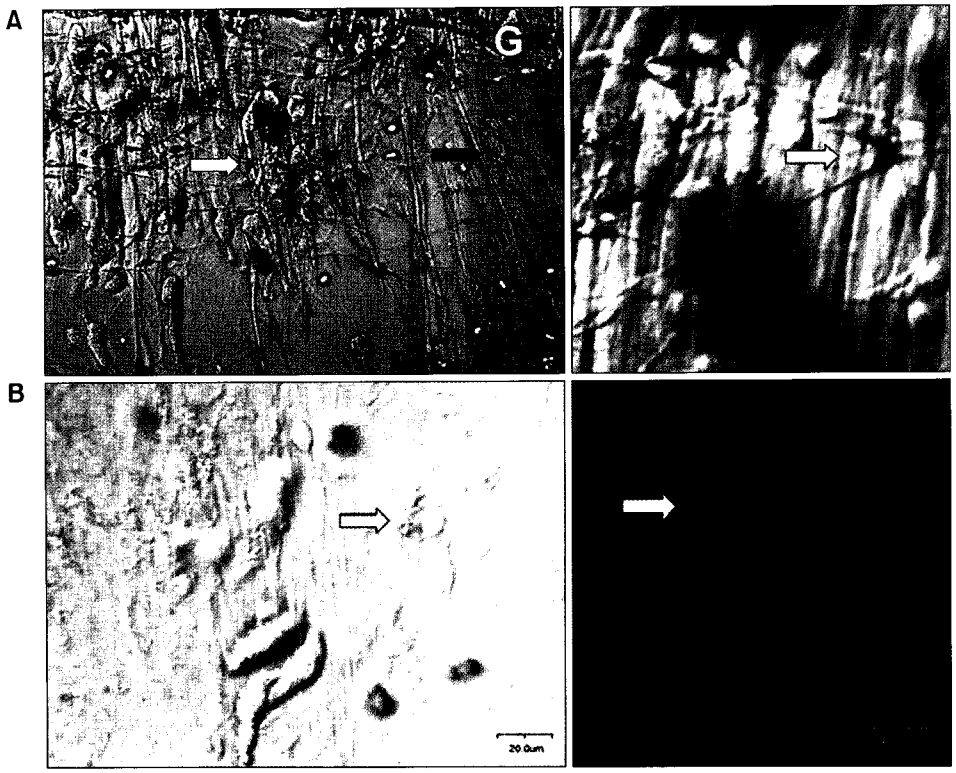

Fig. 1. Immunohistochemical reaction for methylene blue, c-kit and CD34 antibodies in the freshly isolated muscle layer. (A) Enzymatically obtained thin muscle layer was stained with 50 μM methylene blue for 25 min. Upper panel is a conventional phase contrast image. Ganglia (G), round ICC-like cell (white arrow) stained by methylene blue, and smooth muscle contracted to round (black arrow) are shown. Below panel shows an enlarged view around an ICC-like cell. (B) Double immunostaining with c-kit antibody and CD34 antibody (right panel). Left panel shows a conventional DIC image. The c-kit positive cell was a round cell in red color (white arrow). CD34-positive cells formed a network over the field.

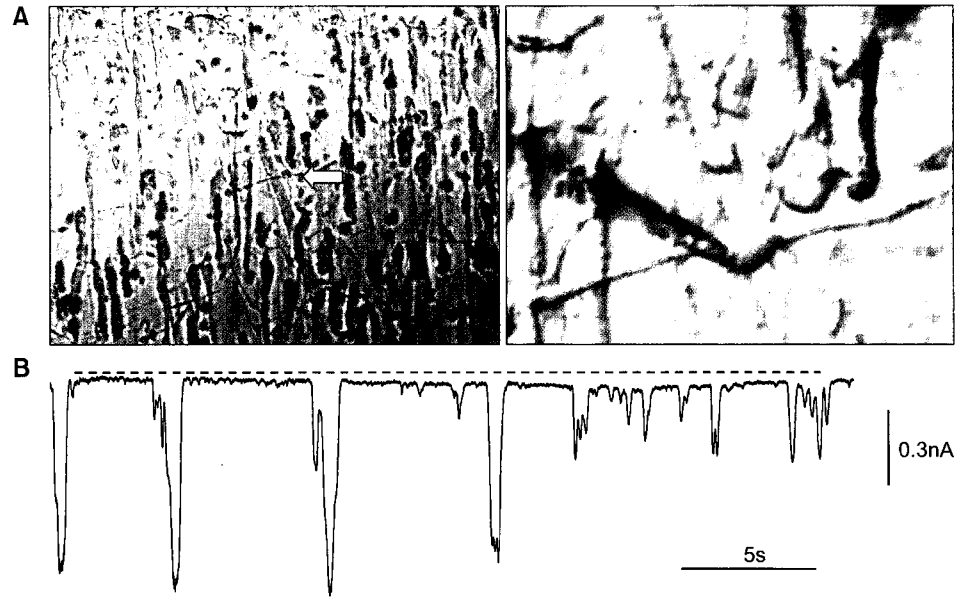

Fig. 2. Electrical activity recorded from an ICC-like cell. (A) Conventional phase contrast image is shown. White arrow indicates an individual cell, in which recording was made. Right panel shows an enlarged view of an ICC-like cell. (B) This cell showed a spontaneous inward current at a holding potential of −40 mV. Dotted line indicates 0 pA.

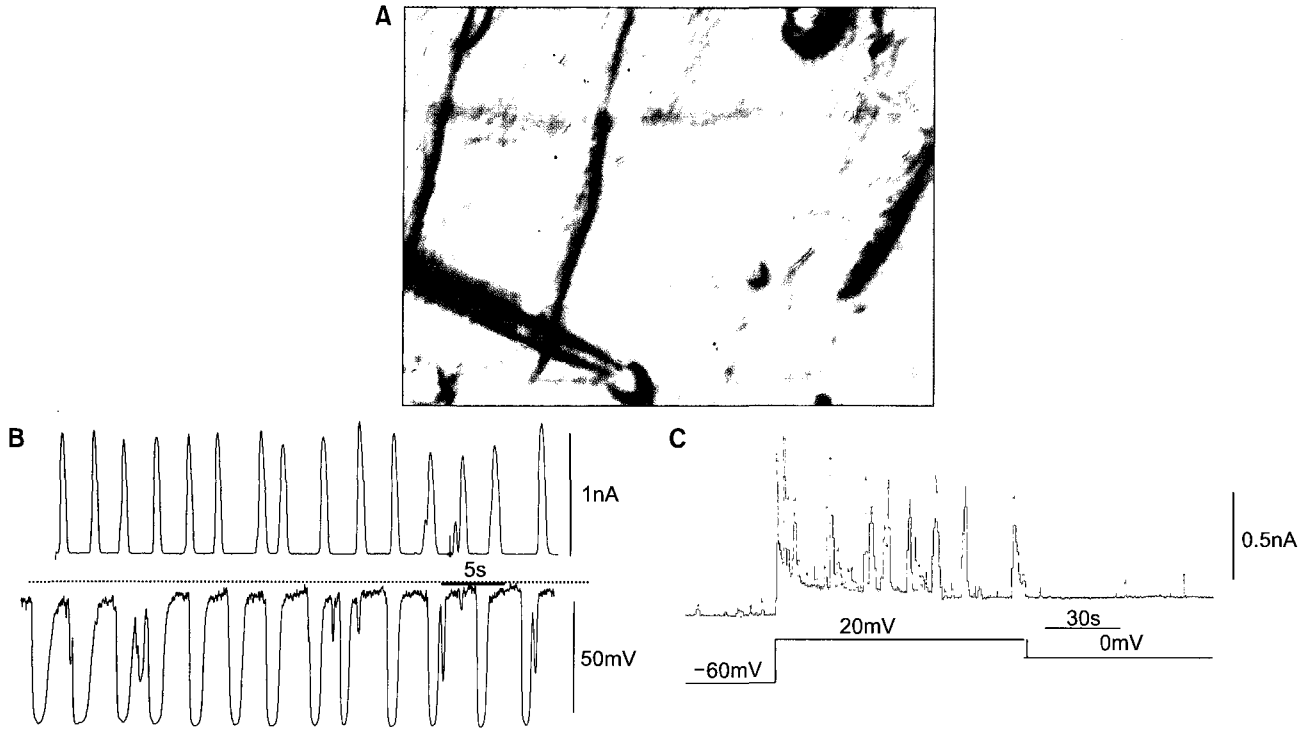

Fig. 3. Electrical activity recorded from a fibroblast-like cell. (A) A conventional phase contrast image of fibroblast-like cell. (B) Spontaneous membrane potential and outward current from two different individual cells are shown. (C) The outward current increase in proportion to the depolarization of the holding membrane potential.

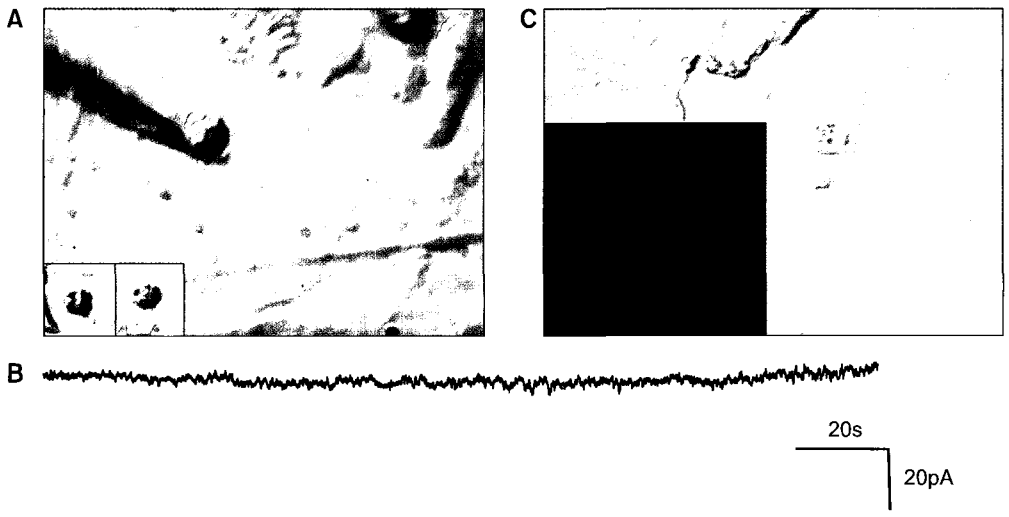

Fig. 4. Electrical activity recorded from a resident macrophage. (A) A conventional phase contrast image of resident macrophage. (B) There was no electrical activity in resident macrophage. (C) Immunostaining with c-kit antibody. Macrophage contains large granules.

trical activity. However, the round-shaped cells showed transient inward currents, whereas the other bipolar cells showed outward currents.

Direct immunohistochemistry: PE (phycoerythrin)- conjugated c-kit antibody positive cells (red color in the right panel of Figure 1B) have round cell body and the processes were blunted. These cells are located near the ganglia in myenteric plexus. FITC (fluorescein isothiocyanate)-conjugated CD34 antibody positive cells (green color in the right panel of Fig. 1B) form a network with fusiform cells

in the preparation. CD34 positive cells are abundant through the whole thin muscle layer.

Electrophysiological properties of methylene blue-positive cells: In electrophysiological experiment, we attempted to record only methylene blue-positive cells in the conventional phase contrast field. We could detect the depolarization current, and the success rate was one in three (10 : 23) and the rest of the cells showed spontaneous hyperpolarization and outward current.

Methylene blue-positive round cell showed a spontaneous and repetitive depolarization-inducing inward current under the voltage-clamp mode (Fig. 2). Membrane potential was hold at −40 mV, And frequency and amplitude of depolarization-inducing inward current was 20.6±2.49 cycle/min (n=6) and −808±10.72 pA (n=9 events), respectively.

Methylene blue-positive bipolar cells showed a spontaneous rapid hyperpolarization and outward current (Fig. 3). Resting membrane potential and peak hyperpolarized potential were −10.05±8.39 mV and -56.96±4.61 mV (n=8 events), respectively. Membrane potential was hold at 0 mV. Current amplitude was 918.3±123.65 pA (n=8 events), and frequency was 17.25±3.56 cycle/min (n=12 cells).

Moreover, there are c-kit positive cells that are not active electrophysiologically. These cells have round and blunted cell bodies. They also contain large granules within the cytoplasm, which are shown as methylene blue-positive spots in the cell (Fig. 4).

DISCUSSION

Since it has been revealed that ICCs generate pacemaker current in the gastrointestinal tract, various approaches have been tested to record pacemaker currents from ICCs; These approaches employed completely dissociated individual cells (Lee et al, 1993), purification by fluorescence-activated cell sorting (Ördög et al, 2004), ICCs cultured from small intestine of newborn mice (Koh et al, 1998) and direct recording from intact tissue (Kito & Suzuki, 2004). However, significance of these of approaches is restricted, because they cannot distinguish whether the cells are ICCs or not until electrophysiological experiment was done.

Goto et al (2004) developed a cell isolation method, by which tissues were treated with collagenase and out a transparent sheet of tissue was dissected from the intestine wall instead of completely dissociating individual cells. The key factor for well-digested muscle layer is to give hydrostatic pressure (about 30~40 cm) to the inverted intestine during collagenase is applying. With adequate hydrostatic pressure given, fine dissecting skill and optimal stretching thin tissue sheet on recording chamber with tungsten pin are essential to prepare available tissue preparation.

In the dissected thin muscle sheet, many interstitial cells were observed, and patch electrode was successfully approached to the individual interstitial cells. However, since identification of the ICCs is difficult after cells are isolated, the nature of direct ionic mechanisms responsible for pacemaker currents remains in dispute.

Methylene blue had been widely used before anti-c-kit antibody was employed as specific maker for ICC. In this study, we applied methylene blue to identify ICCs after they were enzymatically isolated. Methylene blue is a vital and nontoxic stain, but becomes toxic to cells after exposure to light. Thuneberg et al (1983) showed that slow waves recorded in the mouse small intestine were abolished, when methylene blue-stained preparations were exposed to high intensity of light. Previous reports did not discuss that muscularis fibroblast can be stained by methylene blue, however, we observed in our preparation that methylene blue stained both c-kit positive cells and fibroblast-like cells. It is quite possible that the enzymatic procedure used for cell digestion resulted in some effects, by which fibroblasts absorbed methylene blue or passively permeated to its membrane.

In the summary, this ICC-like cell showed spontaneous depolarization inducing inward current, in accordance with previous reports; in the same methods by Goto et al (2004) and from *in situ* by Kito and Suzuki (2003). Methylene blue enhanced the possibility to make successive recording from ICCs, compared with the method to distinguish ICCs by morphology only. Additionally, using this presently described method, we can study electrophysiological properties of myenteric ICCs, fibroblast and resident macrophage.

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