Blockade by mefloquine of intercellular electrical coupling between vascular endothelial cells in the guinea-pig mesenteric arteries

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Abstract

Mefloquine, an antimalarial drug, has been reported to block exogenously transfected gap junctions composed of either Cx36 or Cx50 more potently than those composed of other connexins. Using the conventional whole-cell clamp technique, we investigated the effects of mefloquine on intercellular electrical coupling in vascular endothelial cells of guinea-pig mesenteric arteries, where expressions of Cx40 and Cx43 have been identified. Mefloquine (50 μ M) almost abolished the current required to impose a 10 mV command step, leaving only rapid capacitive currents and some sustained currents after about 3 min. The relaxation of the capacitive current could be well fitted with a single exponential function. The effect of mefloquine was reversible and the time course of the current induced by the voltage step gradually changed back after mefloquine was removed. The mean input resistance and capacitance in the presence of mefloquine were 323 M Ω and 10.1 pF, respectively. While intercellular electrical coupling was well blocked by mefloquine (50 μ M), the membrane hyperpolarized from -24.0 to -32.5 mV. The results indicate that mefloquine effectively blocks gap junctions without producing major side effects in vascular endothelial cells and that this compound is a useful tool in the investigation of gap junctions.

Key words: whole-cell clamp, gap junction blocker, multicellular preparation, connexin

Introduction

Gap junctions are involved in many physiological and pathological events (for reviews, see Mehta, 2007; Meşe *et al.*, 2007; Trosko, 2007). Various gap junction blockers have been used to investigate the possible roles of gap junctions, however, most of them are so called “dirty drugs” having more or less undesirable effects. One of these undesirable effects is membrane depolarization. In fact, 18 β -glycyrrhetic acid depolarizes the membrane in the endothelial cells of guinea-pig mesenteric arterioles (Yamamoto *et al.*, 1998), 18 α - and 18 β -glycyrrhetic

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acids depolarize the membrane in gastrointestinal smooth muscle cells (Takeda *et al.*, 2005), and fenamates including meclofenamic acid, flufenamic acid, niflumic acid and tolafenamic acid block gap junctions and depolarize the membrane (Harks *et al.*, 2001). Less dirty gap junction blockers are needed to further research in this field.

In pairs of cultured neuroblastoma cells transfected with various connexins (Cx26, Cx32, Cx36, Cx43, Cx46 or Cx50), mefloquine, an antimalarial drug, blocks gap junctions composed of either Cx36 or Cx50 more potently than those composed of other connexins (Cruikshank *et al.*, 2004). Mefloquine has only minimal nonspecific actions, one of which is the membrane depolarization of about 1 mV observed in rat neocortical neurons (Cruikshank *et al.*, 2004). Although higher concentrations are required, mefloquine blocks gap junctions composed of connexins other than Cx36 and Cx50. So this drug is a candidate to be a less dirty gap junction blocker.

Cx40 and Cx43 are expressed in endothelial cells of guinea-pig mesenteric arterioles (Yamamoto *et al.*, 2001) and probably also in the upstream arteries. In the present experiments, the effects of mefloquine on vascular endothelial cells were investigated in guinea-pig mesenteric arteries. The results indicate that mefloquine effectively blocks gap junctions in vascular endothelial cells without major side effects and that this compound can be used as a useful tool to investigate gap junctions.

Methods

Preparations

Male guinea pigs, weighing 220–265 g, were anesthetized by sevoflurane inhalation and decapitated with an animal guillotine. The animals were treated ethically according to the rules of the Laboratory Animal Care and Use Committee of Nagoya City University Graduate School of Medical Sciences. Second or third order branches of mesenteric arteries (diameter 300–400 μm) were dissected out from the mesenteric vasculature of the jejunoileal region. Preparations were made as described previously (Yamamoto and Suzuki, 2007). Briefly, a segment of artery was incubated in collagenase solution (nominally Ca^{2+} -free physiological saline containing 0.5 mg/ml collagenase; Wako Pure Chemical Industries, Ltd., Osaka, Japan) for 30 min at 37°C, and the adventitial and smooth muscle layers were then removed mechanically, leaving the endothelial layer surrounded by the internal elastic lamina. The isolated endothelial layer was cut open and placed in a small chamber with the luminal surface upward, then superfused with preheated (30°C) and aerated (100% O_2) bath solution at a constant rate (1 ml/min). The dimensions of a typical preparation were approximately 2.5 mm long and 0.8 mm wide.

Electrophysiological techniques

A patch electrode was applied to one of the endothelial cells, and conventional whole-cell clamp experiments were performed. Current and voltage signals were acquired using a MultiClamp 700A system (Molecular Devices, Sunnyvale, CA, USA). These signals were digitized with a Digidata 1322A data acquisition system (Molecular Devices). The sampling rate was always five times higher than the cutoff frequency (–3 dB) of a low-pass Bessel filter.

Voltage clamp protocols were controlled with pCLAMP 10 software (Molecular Devices). The liquid junction potential calculated as the potential of the bath solution with respect to the pipette was +15.5 mV (Barry and Lynch, 1991) and compensated manually after each experiment.

Solutions and chemicals

The composition of the standard bath solution was (mM) NaCl, 146; KCl, 5.4; CaCl₂, 1.8; MgCl₂, 1; HEPES, 10; glucose, 5. The pH was adjusted to 7.3 using NaOH. The pipette solution contained (mM) KCl, 20; K-aspartate, 128; MgCl₂, 1; HEPES, 10; glucose, 5; EGTA, 1; ATP-2Na, 2. The pH was adjusted to 6.9 using KOH. Mefloquine was obtained from Sigma (St. Louis, MO, USA) and dissolved in DMSO to make a stock solution of 10⁻¹ M.

Statistics

Numerical data are represented as the mean $\pm$ SD, with n indicating the number of measurements. For statistical evaluation, a *P* value <0.05 was considered to be significant.

Results

The preparations used in the present experiments were sheets of freshly isolated endothelium, which consisted of many electrically coupled endothelial cells. The conventional whole-cell clamp method was applied to one of the endothelial cells, and changes in the time course of the current required to impose a 10 mV voltage clamp step were used to determine the extent of coupling between cells (de Roos *et al.*, 1996, Yamamoto *et al.*, 1998). It can be seen in Fig. 1 that mefloquine (50 μ M) almost abolished the current required to impose a 10 mV command step after about 3 min (Fig. 1A). In the presence of mefloquine, only rapid capacitive currents and some sustained currents were observed. The effect of mefloquine was reversible and the time course of the current induced by the voltage step gradually changed back after mefloquine was removed (Fig. 1B). The process of recovery was rather slow and it took more than 10 min to get the current response comparable to the control.

The relaxation of capacitive current induced by a step depolarization in the presence of mefloquine (50 μ M) could be well fitted with a single exponential function (Fig. 2B). The input resistance (R_{in}) and capacitance (C_{in}) were calculated according to the following equations:

$$R_{in} = (V_P - R_a I_{ss}) / I_{ss} \quad \text{and} \quad C_{in} = \tau / R_a$$

where V_P is the amplitude of voltage step, I_0 the membrane current at the beginning of voltage step, I_{ss} the steady-state current, R_a the access resistance ($R_a = V_P / I_0$) and τ the time constant of capacitive current relaxation (Fig. 2A). R_{in} and C_{in} in the presence of mefloquine were 323 ± 94 M Ω and 10.1 ± 1.5 pF, respectively (n=13).

Mefloquine hyperpolarized the membrane. At the time when the intercellular electrical coupling was well blocked by mefloquine (50 μ M), the membrane had hyperpolarized in all 13 preparations examined (Fig. 3A). The membrane potentials in the absence and the presence of mefloquine were -24.0 ± 2.9 mV and -32.5 ± 2.8 mV, respectively (n=13), and these were

Fig. 1. Effect of mefloquine ($50 \mu\text{M}$) on membrane currents induced by step depolarizations (top traces). Holding potential was -15.5 mV . Mefloquine was applied at time 0 in A and almost abolished the current required to impose a 10 mV command step after about 3 min. The time of mefloquine application was about 24 min. Washout commenced at time 0 in B and required more than 10 min to restore to control levels. Signals were filtered at a cut-off frequency of 10 kHz . Time scale shown in the bottom traces applies to all.

Fig. 2. A: Current required to impose a 10 mV voltage clamp step (top trace) in the presence of mefloquine ($50 \mu\text{M}$). Holding potential was -15.5 mV . I_0 is membrane current at the beginning of voltage step and I_{ss} is steady-state current. Broken line represents the holding current level. B: The decay of the capacitive current during the period indicated by the box in A was plotted in an expanded time-scale ($\circ$, the number of data points was reduced for clarity). It could be well fitted with a single exponential function (continuous curve).

Fig. 3. Effect of mefloquine ($50 \mu\text{M}$) on membrane potentials. A: Change in the membrane potentials of 13 preparations before and after the application of mefloquine. In two preparations the recording was continued after the washout of mefloquine. Each symbol represents data from a separate experiment. B: Average membrane potentials in the absence and presence of mefloquine. Error bars represent the SD. *, significant difference (Student's paired t test).

significantly different statistically (Fig. 3B). While continuous recordings for more than 30 min was not easy, recovery from the effect of mefloquine was observed in two preparations where the membrane potential returned to the control level after the washout (Fig. 3A).

Discussion

Mefloquine reversibly blocked gap junctions in vascular endothelial cells of the guinea-pig mesenteric arteries in the present experiments. The input resistance was more than $300 \text{ M}\Omega$ in the presence of $50 \mu\text{M}$ mefloquine. Although no data are available about the input resistance while gap junctions were completely blocked in multicellular preparations, this value might be lower than expected, possibly indicating that some gap junctions might be still functioning at this concentration. The value of $1.7 \text{ G}\Omega$ reported in cultured guinea-pig coronary endothelial cells (Daut *et al.*, 1988) may serve as a reference. Even if the blocking action of mefloquine at this concentration may not be perfect, intercellular electrical coupling seemed to be effectively blocked because, in the presence of mefloquine, the relaxation of the capacitive current could be well fitted with a single exponential function indicating a simple RC-network load (Lindau and Neher, 1988). As the input resistance is $500 \text{ M}\Omega$ in the presence of 18β -glycyrrhetic acid (40

μM) in endothelial cells of guinea-pig mesenteric arterioles (Yamamoto *et al.*, 1998), mefloquine seems to be as potent as 18β -glycyrrhetic acid as a gap junction blocker.

Mefloquine blocks exogenously transfected gap junctions composed of either Cx36 or Cx50 more potently than those composed of other connexins, yet higher concentration (25 μM) is required to block the natural gap junctions composed of Cx36 in brain slices from the rat neocortex (Cruikshank *et al.*, 2004). The authors claim that concentrations of the drug at the recording site may be lower than in the bath solution as a result of slow drug diffusion through the slices (Cruikshank *et al.*, 2004). Alternatively exogenously expressed Cx36 and Cx50 may be somewhat different from their natural form in terms of their sensitivity to mefloquine.

Mefloquine hyperpolarized the membrane in the present experiments. Although this compound may have a side effect of modifying the activities of ionic channels which determine the resting membrane potential, there is another possibility that should be considered. During the preparation process a proportion of the cells, particularly those at the cut edges, will have been damaged making the final preparation a mosaic of healthy and damaged cells. Giga seals cannot be made on the damaged cells, so only the healthy cells can be patched and recorded. So mefloquine might merely allow patched cells to restore their own resting membrane potentials by isolating them electrically from the neighboring cells which may have been damaged and depolarized. The hyperpolarizing effect was also reversible and the potential returned to the control level after washing out mefloquine.

Ca^{2+} -activated channels including K^+ channels are activated by acetylcholine causing the membrane to become hyperpolarized in endothelial cells (Yamamoto *et al.*, 1999; Yamamoto and Suzuki, 2005; Yamamoto and Suzuki, 2007). This response was well preserved after intercellular electrical coupling had been blocked by mefloquine (Y. Yamamoto, unpublished observation). Although more research is required to draw a conclusion, the results presented in this paper indicate that mefloquine effectively blocks gap junctions without producing major side effects in vascular endothelial cells and that this compound is a useful tool in the investigation of gap junctions.

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