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Effects of ropivacaine on membrane potential and voltage-dependent calcium channel current in single guinea-pig ventricular myocytes

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Abstract
Purpose. This study was undertaken to assess the effects of ropivacaine on the membrane action potential and the voltage-dependent L-type calcium channel current (I_{Ca}) in guinea-pig single ventricular myocytes.
Methods. Single ventricular myocytes were prepared by enzymatic dispersion. Whole-cell current and voltage-clamp techniques were used to monitor membrane potentials and I_{Ca}.
Results. Ropivacaine (10^{-5} and 10^{-4} M) reduced the overshoot and shortened the duration of the action potential. Hyperpolarization of the resting membrane potential was observed in the presence of ropivacaine (10^{-4} M). Ropivacaine (10^{-5}–10^{-3} M) reduced I_{Ca} dose-dependently and reversibly, and the 50% inhibitory concentration (IC_{50}) of ropivacaine was estimated as 4.3×10^{-4} M. Furthermore, the inhibition of I_{Ca} was not a use-dependent block.
Conclusion. Ropivacaine has an inhibitory effect on I_{Ca} in the guinea-pig single ventricular myocyte. It is concluded that the mild negative inotropic effect induced by ropivacaine can be attributed in part to shortening of the duration of the action potential, which is caused by inhibition of I_{Ca}.

Key words Ropivacaine · Local anesthetics · Cardiac myocyte · Action potential · Calcium channel

Introduction

Ropivacaine (1-propyl-2′6′-pipecploxylidide hydrochloride monohydrate) is a new, long-acting amide local anesthetic with a structure closely related to bupivacaine and mepivacaine. Whereas both bupivacaine and mepivacaine are used clinically as a racemic mixture, ropivacaine is available only as the hydrochloride of the S-enantiomer [1]. It was developed in response to reports of cardiovascular toxicity from accidental intravenous injection of bupivacaine [2]. Some studies show that ropivacaine has less central nervous and cardiovascular toxicity than bupivacaine [3,4]. These results are supported by the fact that ropivacaine can be readily dissociated from sodium channels, whereas bupivacaine is much more tightly bound in guinea-pig papillary muscle [5].

In addition to sodium channel block, bupivacaine has a potent negative inotropic effect, which may also contribute to its cardiotoxicity [6,7]. It has been established that the voltage-dependent L-type calcium channel current (I_{Ca}) is responsible for the triggering of the contractile apparatus in cardiac muscle, because the calcium-induced calcium release in the sarcoplasmic reticulum that causes the abrupt increase in intracellular calcium level is caused mainly by the calcium ion via calcium channels. Recently we reported that the mechanism of this negative inotropic effect is due to the inhibition of I_{Ca} in the presence of bupivacaine [7]. Ropivacaine is reported to have a mild negative inotropic effect, and its safety margin for cardiovascular system is wider than that of bupivacaine [3,5]. However, the mechanism of the negative inotropic effect induced by ropivacaine has not yet been clarified. The purpose of this study was to examine the effect of ropivacaine on the action potential and on the voltage-dependent L-type calcium channel current, and to clarify the mechanism of the negative inotropic effect induced by ropivacaine.

Materials and methods

Solution

Minimum essential medium (Gibco, Carlsbad, CA, USA) containing (mM) $CaCl_2$ 1.0 or 0.01, taurine 20.0, $MgSO_4$ 0.6, and $NaHCO_3$ 24.0 was used for cell separation. S-MEM solution was saturated with 95% O_2 and

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5% CO_2, at 37°C. Kraft-bruhe (KB) medium [8] containing (mM) glutamic acid 70.0, taurine 15.0, KCl 30.0, KH_2PO_4 10.0, $MgCl_2$ 0.5, glucose 11.0, hydroxyethylpiperazine ethanesulfonic acid (HEPES) 10.0, and ethyleneglycoltetraacetic acid (EGTA) 0.5 (pH 7.3 with KOH) was used for cell storage. Standard Tyrode's solution contained (mM) NaCl 135.0, KCl 5.4, NaH_2PO_4 0.33, $MgCl_2$ 1.0, $CaCl_2$ 2.0, HEPES 5.0, and glucose 5.5 (pH 7.4 with NaOH). To minimize the influence of background K^+ current and to characterize I_{Ca}, 10.0 mM of tetraethylammonium chloride was added to the bathing solution when I_{Ca} was examined. The pipette solution contained (mM) L-aspartic acid (potassium salt) 100.0, KCl 30.0, $MgCl_2$ 1.0, HEPES 5.0, EGTA 5.0, ATP (Na salt) 3.0, and guanosine triphosphate (GTP) 0.1 (pH 7.2 with KOH). All chemicals were purchased from Sigma (St. Louis, MO, USA) or Wako (Tokyo, Japan). Ropivacaine hydrochloride (AstraZeneca, Tokyo, Japan) was dissolved in Tyrode's solution. The bathing solution was saturated with 100% oxygen.

Cell isolation

The technique for isolating ventricular myocytes from guinea-pig heart has been previously described [9]. After permission had been obtained from the local animal committee, a guinea pig of either sex (weight, 200–250 g) was anesthetized by intraperitoneal injection of sodium pentobarbital (40 mg). The heart was then excised and a dull-edged needle was cannulated from the ascending aorta. To wash out the blood, S-MEM solution was circulated for 10 min via the coronary artery, and then low-calcium S-MEM solution was circulated for 10 min to wash out excessive calcium. After these treatments, a low-calcium S-MEM solution containing 0.02% collagenase (type II) and 0.003% trypsin (Sigma) was circulated for 10 min. Then the ventricular part of the heart was cut out, minced into small pieces in low-calcium S-MEM solution containing bovine serum albumin for 5 min, transferred into KB solution, and stored at 5°C for 1 h. After storage, single ventricular myocytes were obtained by applying gentle pipetting with a wide-bored pipette.

Recording method

The membrane potential and the ionic currents were recorded with low-resistance glass microelectrodes (3–5 Mς pipette resistance) filled with the previously described pipette solution. Glass microelectrodes were fabricated with a horizontal microelectrode puller (P-87, Sutter, Novato, CA, USA) from glass microtubes (G-1.5, Narishige, Tokyo, Japan). The whole-cell current-clamp method was used to monitor membrane potentials, and the whole-cell voltage-clamp method was applied to examine ionic currents [10]. A patch-clamp amplifier (EPC-9, Heka, Lambrecht, Germany) was used to monitor the membrane potentials and ionic current. Data were stored in an Apple Macintosh computer. Acquisition and analysis of the data were performed by Pulse and Pulsefit software (Heka). The action potential was elicited by current injection (100 mV, 2 ms). The resting membrane potential, overshoot, upstroke velocity of the rapid phase of depolarization (V_{max}), and duration at 30% (APD_{30}) and 95% (APD_{95}) of repolarization (from plateau height) were examined. It was assumed that APD_{30} reflected the plateau phase of the action potential, which was carried by the calcium influx, and that APD_{95} reflected the repolarization phase, which was carried by the outward potassium current. I_{Ca} was evoked by applying depolarizing pulses (from −30 to +60 mV, 10-mV step, 300- or 100-ms duration) from a holding potential of −30 mV, which avoided interference from the voltage-dependent sodium channel current. I_{Ca} was identified after the current was blocked in the presence of nifedipine (10^{-6} M). All of the experiments were carried out at room temperature (21°–23°C).

Statistical analysis

Statistical analysis was performed first by one-way analysis of variance (ANOVA), and, if indicated, multiple comparison tests (Bonferroni) were employed to test for significant differences between the groups. A P value less than 0.05 was considered significant.

Results

Effects of ropivacaine on membrane potentials

Ropivacaine (10^{-4} M) hyperpolarized the resting membrane potential by about 1.5 mV. This effect was reversible after washout of ropivacaine. Overshoot of the action potential was reduced markedly in the presence of 10^{-5} and 10^{-4} M ropivacaine. In addition, ropivacaine (10^{-5} and 10^{-4} M) reduced V_{max}. Both APD_{30} and APD_{95} were shortened in the presence of 10^{-5} and 10^{-4} M ropivacaine (Table 1, Fig. 1). Application of a high concentration of ropivacaine (10^{-3} M) completely abolished the development of an action potential. These effects were reversible after washout of ropivacaine.

Effects of ropivacaine on I_{Ca}

Ropivacaine inhibited I_{Ca} within the range between 10^{-5} and 10^{-3} M (10^{-6} M: 98.5% ± 6.5%, 10^{-5} M: 84.6% ± 7.6%, 10^{-4} M: 66.2% ± 8.0%, 10^{-3} M: 5.7% ± 6.6% of control, mean ± SEM, n = 7) in a dose-dependent

Table 1. Effects of ropivacaine on action potential

Effect	Control	Ropivacaine 10^{-5} M	Ropivacaine 10^{-4} M
RMP (mV)	−70.1 ± 0.4	−71.1 ± 0.8	−71.6 ± 0.3*
Overshoot (mV)	156 ± 12	107 ± 15*	65 ± 12*,**
APD_{30} (ms)	1208 ± 41	963 ± 27*	753 ± 35*,**
APD_{95} (ms)	1350 ± 44	1161 ± 28*	1011 ± 40*,**
V_{max} ($V \cdot s^{-1}$)	3.11 ± 0.02	1.49 ± 0.09*	1.32 ± 0.03*,**

Data shown as mean ± SD
RMP, Resting membrane potential; Overshoot, initial spike, which is carried by sodium current; APD_{30} and APD_{95}, duration of action potential at 30% and 95% of repolarization from plateau height, respectively; V_{max}, upstroke velocity of initial spike
* $P < 0.05$ vs control ($n = 10$)
** $P < 0.05$ vs 10^{-5} M ($n = 10$)

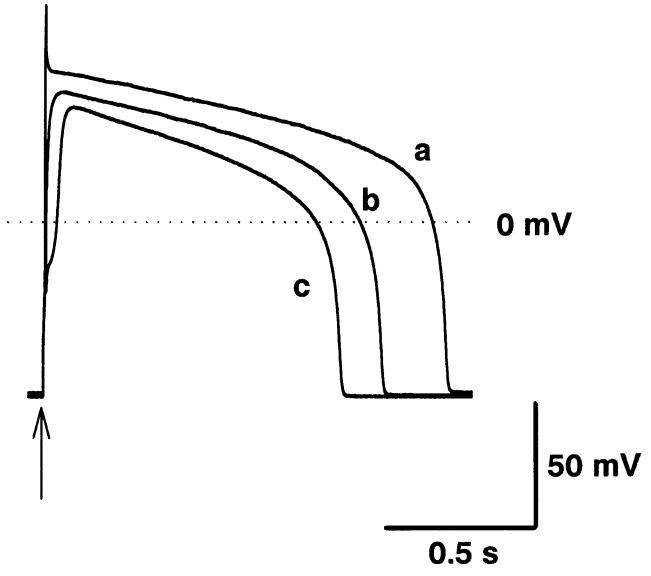

Fig. 1. Effect of ropivacaine on action potential. Typical traces of action potential are shown (*a*, control; *b*, 10^{-5} M; *c*, 10^{-4} M ropivacaine). Action potential was elicited by current injection (0.33 Hz, 2 ms, 100 mV) in whole-cell current-clamp mode

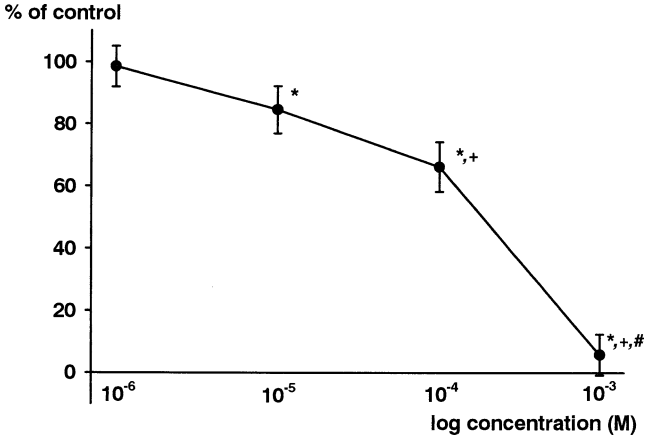

Fig. 2. Dose-dependent inhibition of I_{Ca}. Ropivacaine inhibited I_{Ca} between 10^{-5} and 10^{-3} M. *, +, #$P < 0.05$ vs 10^{-6}, 10^{-5}, 10^{-4} M, respectively. Data are shown as mean ± SEM ($n = 7$)

manner (Figs. 2, 3). This inhibitory effect was reversed after 10 min of washout. Peak I_{Ca} was observed between the test pulses of +10 and +20 mV (Fig. 3B). A high concentration of ropivacaine (10^{-3} M) blocked I_{Ca} completely (Fig. 3B). The 50% inhibitory concentration of peak I_{Ca} (IC_{50}) was estimated as 4.3×10^{-4} M from these results.

To further examine the mode of blockade of L-type calcium channels, we examined whether ropivacaine caused use-dependent block of I_{Ca}. Cells were held at −30 mV, and test depolarization pulses to +10 mV were applied every 5 s. After the control currents had been recorded, the test pulses were stopped and ropivacaine (10^{-4} M) was applied for 10 min. Then the test pulses were resumed and the amplitude of I_{Ca} was recorded. Ropivacaine (10^{-4} M) did not cause use-dependent block of I_{Ca} in five cells (Fig. 4).

Discussion

The mechanisms of cardiovascular effects of local anesthetics are complex, involving direct effects on the myocardium [11], on vascular tissue [12], and on the central innervation of the heart [13,14]. The primary cardiac electrophysiologic effect of local anesthetics is a decrease in the rate of depolarization in the fast conducting tissues of Purkinje fibers and ventricular muscle [15,16]. Our data showed marked reduction of the overshoot and V_{max} of action potentials. Arlock reported about 55% reduction of V_{max} in the presence of 10^{-5} M ropivacaine in guinea-pig papillary muscle [5], which was consistent with our data (52% reduction). Because the reduction of V_{max} induced by 10^{-5} M bupivacaine was reported to be about 74% in guinea-pig and canine papillary muscle [5,17], this inhibitory effect on V_{max} seemed less in the presence of ropivacaine. Because the overshoot and V_{max} are supposed to be regulated by the availability of fast Na^+ channels in cardiac membranes, a smaller inhibitory effect of ropivacaine on V_{max} is beneficial to avoid cardiotoxicity. It has been reported

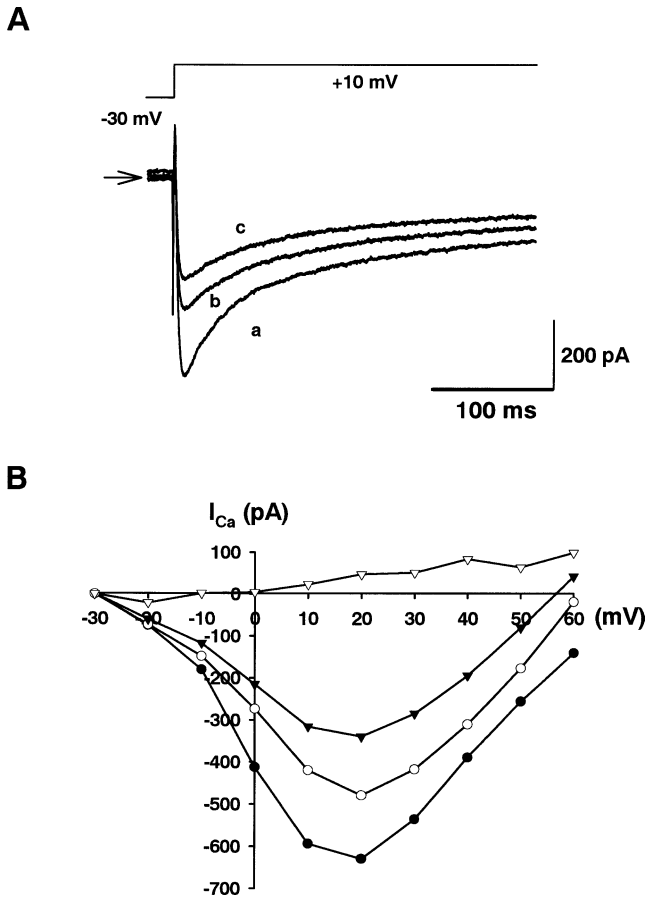

Fig. 3A,B. Inhibitory effect of ropivacaine on I_{Ca}. **A** Typical traces of I_{Ca} and its inhibition in the presence of ropivacaine (*a*, control; *b*, 10^{-5} M; *c*, 10^{-4} M ropivacaine). I_{Ca} was elicited by a test pulse (from −30 to +10 mV, 300 ms duration). *Arrow* shows the zero current level. **B** A typical current-voltage relationship of I_{Ca} (*closed circles*, control; *open circles*, 10^{-5} M; *closed triangles*, 10^{-4} M; *open triangles*, 10^{-3} M ropivacaine). I_{Ca} was elicited by applying a test pulse from a holding potential of −30 mV by 10-mV increments to +60 mV

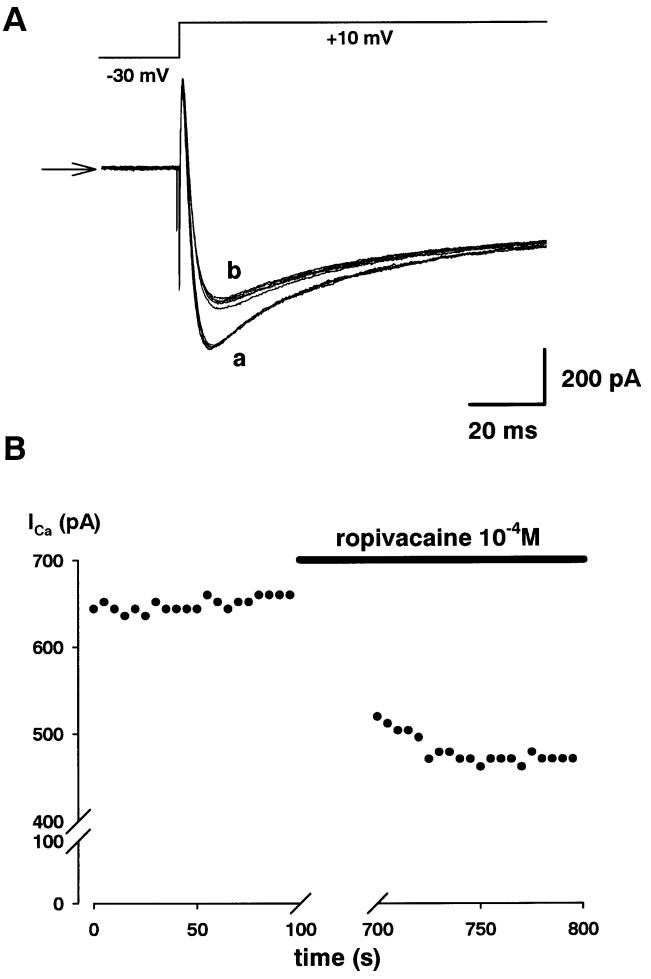

Fig. 4A,B. Lack of use-dependent block of I_{Ca} in the presence of ropivacaine. **A** Traces of I_{Ca} are shown (*a*, last five traces of control; *b*, first five traces after the test pulse was restored). Cell was held at −30 mV, and test pulse to +10 mV was applied every 5 s for 100 ms. *Arrow* shows zero current level. Note that I_{Ca} was inhibited from the first trace in the presence of 10^{-4} M ropivacaine. **B** Plot of peak current amplitude of I_{Ca}. On resumption of the test pulse, I_{Ca} showed direct inhibition without use-dependent block in the presence of 10^{-4} M ropivacaine

that a direct cardiodepressant action is induced by the very slow reversal of Na^+ channel blockade after the cardiac action potential, and this reversal is considerably faster with ropivacaine than with bupivacaine [5]. Therefore, it is suggested that ropivacaine has a smaller inhibitory effect on cardiac Na^+ channel than bupivacaine.

Ropivacaine hyperpolarized the resting membrane potential at 10^{-4} M in a reversible manner. Because the resting membrane potential in the cardiac myocyte is regulated by the inward-rectifying K^+ channel current [18], there is a possibility that ropivacaine has an inhibitory effect on this K^+ channel. However, there may be an effect on some other pump currents, such as the Na^+-K^+ pump mechanism, which influence the resting membrane potential. Further investigation is obviously required.

Local anesthetics may depress myocardial contractility by inhibiting calcium influx and calcium-induced calcium release [19]. Ropivacaine at doses between 10^{-5} and 10^{-3} M inhibited I_{Ca} dose-dependently and reversibly. Particularly, I_{Ca} was blocked in the presence of 10^{-3} M ropivacaine. General anesthetics, such as isoflurane and sevoflurane, have a negative inotropic effect on the heart via inhibition of calcium influx [20,21]. Our results suggest that ropivacaine may also have a negative inotropic effect by the same mechanism. Because I_{Ca} forms the plateau phase of the action potential and plays the major role in the determination of the duration of action potential [20], these results are also responsible for the shortening of the duration of

the action potential at 10^{-5} and 10^{-4} M ropivacaine and the lack of action potentials at a higher concentration (10^{-3} M). On the other hand, some general anesthetics, such as ketamine and thiopental, prolonged the repolarization phase of the action potential and the total duration of the action potential [22,23], and it was suggested that ropivacaine might have a different effect from those anesthetics. Moreover, ropivacaine (10^{-5} and 10^{-4} M) reduced APD_{30} (20.3% and 37.7% reduction, respectively) more than APD_{95} (14% and 25.2% reduction, respectively). APD_{95} reflected the repolarization phase of the action potential, which is carried mainly by the delayed rectified K^+ channel current [24]. This result suggested that ropivacaine also has an effect on the delayed rectified K^+ channel current. Although it has been reported that ropivacaine seems to cause a use-dependent block [25] of Na^+ channel current [5], our data show the lack of a use-dependent block of I_{Ca}. These results suggest that ropivacaine acts as an open-channel blocker on Na^+ channels but that ropivacaine affects Ca^{2+} channels by a different mechanism. Because the blockade of inactivated channels by ropivacaine may be particularly important for the mechanism of the inotropic effect on the heart, further investigation is required.

In clinical use for epidural anesthesia, the total plasma concentration of ropivacaine varies between 5.8×10^{-7} and 7.5×10^{-6} M [26]. During intravascular injection of ropivacaine during axillary brachial plexus blockade, the reported total plasma concentration of ropivacaine varies between 4.3×10^{-6} and 1.6×10^{-5} M, and the unbound plasma concentration varies between 4.9×10^{-7} and 2.1×10^{-6} M [27]. Because the unbound plasma concentration, even in accidental intravascular injection, is considerably lower than in our experimental protocol, it is supposed that ropivacaine is a safe local anesthetic for the cardiovascular system. However, we must pay attention to the patient whose total plasma protein level is low, because the unbound plasma concentration of ropivacaine may rise.

In conclusion, ropivacaine decreased the duration of the action potential, and this effect is mediated by the inhibitory effect on I_{Ca} in guinea-pig single ventricular myocytes. Moreover, this inhibitory effect on I_{Ca} was less than that of bupivacaine. It is suggested that this mechanism might play a role in the mild negative inotropic effect induced by ropivacaine.

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