Effects of Bupivacaine and Ropivacaine on High-voltage–activated Calcium Currents of the Dorsal Horn Neurons in Newborn Rats

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Background: Local anesthetics, such as bupivacaine, have been reported to block calcium currents in primary sensory neurons and to interfere with the release of neurotransmitters in central nervous system neurons. However, it is unknown whether local anesthetics affect the calcium current activity of central nervous system neurons.

Methods: Using a traditional whole cell voltage clamp technique, effects of bupivacaine and ropivacaine on high-voltage-activated calcium currents (HVA-Icα) were investigated in enzymatically dissociated dorsal horn neurons of neonatal rats. Calcium currents were evoked by testing pulses from a holding potential of −90 to 0 mV.

Results: Bupivacaine significantly reduced HVA-Icα in a dose-dependent manner. The peak HVA-Icα decreased by 24.5 ± 2.5, 32.0 ± 6.8, 59.4 ± 6.2, 88.3 ± 1.5, and 91.6 ± 1.1% in response to 10, 30, 50, 100 and 200 μM bupivacaine, respectively. Unlike bupivacaine, ropivacaine markedly increased HVA-Icα at lower concentrations (< 50 μM) but decreased HVA-Icα at higher concentrations (≥ 50 μM). The percent increases in peak HVA-Icα induced by 10 and 30 μM ropivacaine were 95 ± 19.1 and 41.6 ± 8.3%, respectively. The percent decreases in response to 50, 100, and 200 μM ropivacaine were 21.1 ± 2.1, 63.2 ± 6.0 and 79.1 ± 7.6%, respectively. Results indicate that the inhibitory potency of ropivacaine on HVA-Icα was significantly lower than that of bupivacaine at the same concentrations.

Conclusions: The current study showed that bupivacaine inhibited HVA-Icα, recorded from dorsal horn neurons and that ropivacaine increased HVA-Icα at lower concentrations but decreased HVA-Icα at higher concentrations. The inhibitory potency of ropivacaine was lower than that of bupivacaine. Inhibition of calcium currents of central nervous system neurons may be related to the systemic neurotoxic effects of local anesthetics (e.g., convulsions, seizures).

LOCAL anesthetics have been used to provide relief from acute pain associated with surgical procedures or accidental trauma1 and to reduce chronic pain syndromes, such as intractable neuropathic pain.2 One of major adverse effects of local anesthetics is systemic toxicity.3−8 All local anesthetics can cause central nervous system (CNS) toxicity and cardiovascular toxicity if their plasma concentrations are increased by accidental intravenous injection or an absolute overdose. CNS toxicity may be manifested by numbness of the tongue and perioral area, convulsions, and restlessness, which may progress to seizures, respiratory failure, and coma.9 Bupivacaine is the local anesthetic most frequently associated with seizures.9 Although the mechanisms underlying local anesthetic-induced neurotoxicity are not clear, there is evidence that calcium blockers (e.g., diltiazem, verapamil, and bepridil) may significantly decrease the time of latency to obtain neurotoxic effects, such as convulsions.10 It suggests that calcium channels of CNS neurons may be related to local anesthetic-induced systemic effects.

In vertebrate neurons, there are two types of voltage-dependent calcium channels: the low-voltage–activated (LVA-Ica) and the high-voltage–activated calcium channels (HVA-Icα).11−14 Of these, only the HVA-Icα is believed to play a pivotal role in regulating neurotransmitter release.15,16 In rat spinal dorsal horn neurons, three types of Ca2+ current have been identified as low-threshold transient, high-threshold transient, and high-threshold sustained Ca2+ currents that are distinguishable on the basis of their voltage- and time-dependent properties and their response to organic and inorganic Ca2+ channel agonists and antagonists.17

Local anesthetics, such as bupivacaine, have been reported to block both the LVA-Ica and the HVA-Ica in sensory neurons and to interfere with the release of neurotransmitters20 in CNS neurons. However, it is unknown whether local anesthetics affect the calcium current activity of CNS neurons.

The purpose of the current study was twofold. The first purpose was to measure the HVA-Icα of spinal dorsal horn neurons with and without the presence of local anesthetics. The second was to compare the potency of ropivacaine and bupivacaine to block calcium currents.

Methods

Experiments were performed on dorsal horn neurons of newborn Sprague-Dawley rats (2−9 days old) after a procedure as described previously.14,21 Briefly, with approval of the Institutional Animal Care Committee (Shanghai First People’s Hospital, Shanghai, China), the rats were killed, and the lumbar enlargement of the...
spinal cord was dissected and incubated in ice-cold, oxygenated (95% O2 and 5% CO2), buffered saline (in mM: NaCl, 124; KCl, 5; KH2PO4, 1.2; MgSO4, 1.3; CaCl2, 2.4; NaHCO3, 26; glucose, 10). After removal of the pial membrane with No. 5 Dumont forceps, transverse slices (500 μm) of the spinal cord were cut as described14 and incubated for 7–20 min at 31°C in pronase (0.25–0.5 mg/ml; Calbiochem, San Diego, CA)-containing buffered saline. The incubating solution was then replaced with thermolysin (0.25–0.5 mg/ml)-containing solution and allowed to incubate for another 7–20 min at 31°C. After being transferred into HEPEST solution (in mM: NaCl, 150; KCl, 5; CaCl2, 2; MgCl2, 1; HEPES, 10; glucose, 10), the region of laminae I–III or IV–VI was dissected out using small hand-held needles. The tissue was mechanically dissociated under a dissecting microscope using fire-polished glass pipettes. Spinal dorsal horns neurons selected in the study were bright and had a three-dimensional appearance under phase-contrast optics as described previously.21 During electrophysiologic recording, spinal dorsal horn neurons were continuously perfused at 1–2 ml/min with oxygenated HEPES solution at room temperature (23–24°C).

GΩ seals were achieved using glass microelectrodes with impedance of 2–4 MΩ. Whole-cell recordings were performed using an Axopatch 200B amplifier (Axon Instruments, Inc., Foster City, CA) operated at 15–20 kHz. Capacity compensation and series resistance compensation (> 80%) were used to minimize voltage errors after the start of whole cell recording. Leak subtraction of the current through the leak conductance was performed on-line using a P/4 protocol described by Bezanilla and Armstrong.22

HVA-IcA was recorded under voltage clamp mode. The external solution used to isolate calcium currents contained (in mM): NaCl, 107.3; BaCl2, 2.0; CsCl, 2.0; MgCl2, 1.3; KH2PO4, 1.2; glucose, 10; NaHCO3, 26; tetraethylammonium, 20; tetrodotoxin, 0.001 (pH 7.4 when oxygenated with 95% O2 and 5% CO2). The pipette solution contained (in mM): CsCl, 10; CsAsp, 120; MgCl2, 2; EGTA, 1.1; Na-ATP, 5; HEPES, 10 (pH 7.2, adjusted with 1 N NaOH). Ba2+ was used as a charge-carrier for Ca2+-dependent currents. Cs+ and tetraethylammonium were used to minimize voltage-dependent potassium currents. Tetrodotoxin was initially dissolved in acetate solution to a stock concentration of 0.1 mM and further diluted to final concentration of 1 μM, and was used to block tetrodotoxin-sensitive sodium currents. Drugs were applied via gravity using a rapid perfusion system as described previously.23 HVA-IcA was first recorded in the absence of bupivacaine or ropivacaine (10, 30, 50, 100, and 200 μM) and was recorded again after perfusing the neurons with bupivacaine or ropivacaine for 5 min.

Data were collected and analyzed using Clampex 8 software (Axon Instruments, Inc.). The peak HVA-IcA was measured as the lowest point of the recorded traces minus half of the noise (typically 50/2 = 25 pA). The percent reduction in peak HVA-IcA at a given concentration of bupivacaine was used to generate the dose-response curve. Each data point represented an average of values obtained from at least five different neurons. Dose–response was fitted with a nonlinear least-squares method with a modified Hill equation: 1 = Imax/(1 − [C]/IC50n + [C]n)), where C is the concentration used, IC50 is the concentration of bupivacaine at which half-maximum channel inhibition was obtained, n is the Hill coefficient, and Imax is the peak calcium current. Data were expressed as mean ± SEM. Statistical comparison was performed for the current–voltage curve comparisons with and without the presence of bupivacaine using one-way analysis of variance followed by all pairwise comparisons. Student t tests were used to compare the inhibitory potency of bupivacaine and ropivacaine. P values less than 0.05 were considered to be statistically significant.

Results

A total of 54 neurons from 20 newborn rats were studied. Because Ca2+ was replaced with Ba2+ in the external solution, there was no rundown of calcium currents during voltage clamp recording. HVA-IcA was recorded after a stabilization period of at least 10 min. Perfusion of the spinal neurons with bupivacaine significantly inhibited the HVA-IcA, particularly the transient component. The percent reductions in peak HVA-IcA in response to 10, 30, 50, 100, and 200 μM bupivacaine were 24.5 ± 2.5, 32.0 ± 6.8, 59.4 ± 6.2, 88.3 ± 1.5, and 91.6 ± 1.1%, respectively. The inhibitory effect of bupivacaine on HVA-IcA was dose dependent, with an IC50 of 47.9 ± 10.3 μM. The effect of bupivacaine was reversible and repeatable. The HVA-IcA typically returned to previous level after a 30-min washout period with HEPES solution alone. Figure 1 shows the representative whole cell HVA-IcA traces (fig. 1A) and the dose–response curve in the absence (control) and presence of bupivacaine (fig. 1B).

Current–voltage relations of dorsal horn neurons were elicited by applying depolarizing pulses with a duration of 200 ms over the voltage range of −40 to 60 mV under a holding potential of −90 mV in 10-mV increments. In four neurons, the current–voltage curves were compared before and after applications of 30 μM bupivacaine. Bupivacaine significantly reduced the peak HVA-IcA. The mean peak amplitude of total HVA-IcA measured at 10 mV was −268.63 pA in the absence of bupivacaine and −185.29 pA in the presence of bupivacaine (P < 0.05, one-way analysis of variance). However, there was no kinetic difference in terms of the voltage threshold of

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A biphasic effect on HVA-Ica was produced by ropivacaine when concentrations were varied. Ropivacaine markedly increased HVA-Ica at lower concentrations (10 and 30 \( \mu M \)) in all five neurons tested. As shown in figure 2A, both of the transient and sustained components of high threshold Ca\(^{2+} \) currents were enhanced after topical application of 10 \( \mu M \) ropivacaine. The percent increases in peak HVA-Ica induced by 10 and 30 \( \mu M \) ropivacaine were 95 \( \pm \) 19.1 and 41.6 \( \pm \) 8.3\%, respectively. However, ropivacaine suppressed HVA-Ica at higher concentrations (50, 100, and 200 \( \mu M \)) (fig. 2B). The percent decreases in response to 50, 100, and 200 \( \mu M \) ropivacaine were 21.1 \( \pm \) 2.1, 63.2 \( \pm \) 6.1, and 79.2 \( \pm \) 7.6\%, respectively. The inhibitory potency of ropivacaine on HVA-Ica was significantly lower than that of bupivacaine at the same concentrations (fig. 2C).

Discussion

The current study showed that local anesthetics (e.g., bupivacaine and ropivacaine) inhibited HVA-Ica in dorsal horn neurons. Similar effects of local anesthetics on calcium currents were also observed in dorsal root ganglion neurons.\textsuperscript{18,19,24} Although the mechanisms underlying calcium channel blockade by local anesthetic are not clear, previous studies by different investigators suggested that local anesthetics may directly interact with the neuronal dihydropyridine binding sites.\textsuperscript{25} Although it is uncertain if the concentrations (10, 30, 50, 100, and 200 \( \mu M \)) of bupivacaine and ropivacaine...
used in the current study are clinically relevant, same concentrations have been used previously by various investigators\(^{39,26,27}\) to study local anesthetic-induced blockade of sodium, potassium, or calcium currents. Meyer \textit{et al}.\(^{28}\) reported that after injection of a total of 15 mg bupivacaine in adult patients, the maximal concentration in cerebrospinal fluid averaged 284 μg/ml (875 μM). It is reasonable to believe that the concentration of bupivacaine at individual spinal neurons (located under the surface of the spinal cord) is lower than the cerebrospinal fluid concentration because of the tissue and sheaths for topical bupivacaine to diffuse. However, we hesitate to conclude that the concentrations used in the current study reflect the actual clinical concentrations that reach each spinal neuron because of lack of data.

Ropivacaine is a pure \(\delta\) enantiomer and is structurally similar to bupivacaine but less toxic to the heart\(^{29}\) and CNS.\(^{6}\) In the current study, a biphasic effect on HVA-Ica was evoked by ropivacaine in the spinal neurons. A similar biphasic effect of ropivacaine has been reported previously in isolated human arteries.\(^{30}\) Excitatory effects of local anesthetics, such as procaine, have also been observed when applied to sensory neurons.\(^{31}\) Apparently, different mechanisms are involved in the biphasic effects of ropivacaine on the Ca\(^{2+}\) currents. Although there is no clinical report of ropivacaine-induced excitatory effect, theoretically, this could occur at some point as a block diminishes after spinal or epidural anesthesia with ropivacaine.

Although we have tried to study dorsal horn neurons from the region of laminae I-III or IV-VI, it is possible that some of the neurons we recorded may not be sensory neurons, but rather interneurons. It would have been useful to have the sensory neurons labeled in our electrophysiologic experiments.

The alteration in signal/noise ratio as shown in figure 1A suggests that the access resistance or capacitance compensation may have changed during the recording of a single cell, which could be a possible limitation of the current study. However, we believe that these minor changes do not influence our results significantly because results from the same study also show that 10 μM bupivacaine markedly inhibited calcium currents without any change in noise level.

Our results have shown that bupivacaine has more blocking potency on the HVA-Ica than does ropivacaine. Clinical studies indicate that ropivacaine is as effective as bupivacaine in maintaining analgesia during labor and delivery,\(^{32}\) suggesting that the blocking effects of ropivacaine and bupivacaine on calcium currents may not be related to local anesthetic-induced spinal analgesia and anesthesia. Because Ca\(^{2+}\) influx through high-voltage-activated calcium channel is essential for neurotransmitter release in the CNS,\(^{35}\) results from our study suggest that both Ca\(^{2+}\) influx block through HVA-Ica and subsequent block of neurotransmitter release by local anesthetics may contribute to local anesthetic-induced systemic neurotoxicity. This is supported by the fact that calcium channel blockers shorten the time of latency to obtain toxic effects, such as convulsions, after an overdose of bupivacaine. The result that ropivacaine is less potent than bupivacaine in blocking calcium currents is also consistent with clinical finding that ropivacaine is generally less toxic than bupivacaine to the CNS. However, because the current study does not provide direct evidence regarding the mechanisms underlying local anesthetic-induced systemic toxicity, further investigation is necessary to ascertain the correlation between calcium channel block and systemic toxicity.

**Conclusion**

Bupivacaine inhibited the HVA-Ica. The effect of ropivacaine on HVA-Ica was biphasic, dependent on concentration. The inhibitory potency of bupivacaine was greater than that of ropivacaine at same concentrations.

**References**