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·Original Article·

Voltage-dependent blockade by bupivacaine of cardiac sodium channels expressed in *Xenopus* oocytes

Heng Zhang[3, *], Hui Ji[1, 2, *], Zhirui Liu[3], Yonghua Ji[2, 3], Xinmin You[1, 2], Gang Ding[1, 2], Zhijun Cheng[1, 2]

[1]*Xinhua Hospital (Chongming), Shanghai Jiaotong University School of Medicine, Shanghai 202150, China*

[2]*Xinhua Translational Institute for Cancer Pain, Shanghai 202150, China*

[3]*Lab of Neuropharmacology and Neurotoxicology, Shanghai University, Shanghai 200444, China*

*These authors contributed equally to this work.

Corresponding authors: Zhijun Cheng and Gang Ding. E-mail: zj.cheng@hotmail.com, ddgang@hotmail.com

ABSTRACT

Bupivacaine ranks as the most potent and efficient drug among class I local anesthetics, but its high potential for toxic reactions severely limits its clinical use. Although bupivacaine-induced toxicity is mainly caused by substantial blockade of voltage-gated sodium channels (VGSCs), how these hydrophobic molecules interact with the receptor sites to which they bind remains unclear. $Na_v1.5$ is the dominant isoform of VGSCs expressed in cardiac myocytes, and its dysfunction may be the cause of bupivacaine-triggered arrhythmia. Here, we investigated the effect of bupivacaine on $Na_v1.5$ within the clinical concentration range. The electrophysiological measurements on $Na_v1.5$ expressed in *Xenopus* oocytes showed that bupivacaine induced a voltage- and concentration-dependent blockade on the peak of I_{Na} and the half-maximal inhibitory dose was 4.51 μmol/L. Consistent with other local anesthetics, bupivacaine also induced a use-dependent blockade on $Na_v1.5$ currents. The underlying mechanisms of this blockade may contribute to the fact that bupivacaine not only dose-dependently affected the gating kinetics of $Na_v1.5$ but also accelerated the development of its open-state slow inactivation. These results extend our knowledge of the action of bupivacaine on cardiac sodium channels, and therefore contribute to the safer and more efficient clinical use of bupivacaine.

Keywords: bupivacaine; $Na_v1.5$; voltage-dependent blockade; inactivated state

INTRODUCTION

Bupivacaine is one of the aminoamide drugs belonging to the class I local anesthetics (LAs) which include lidocaine, ropivacaine, and mepivacaine. It is generally used for infiltration and nerve block, as well as epidural and intrathecal anesthesia in clinical management. Despite its long-lasting effect when provided systemically for pain relief, patients still risk adverse drug reactions with accidental intravascular injection, inadvertent intrathecal injection, or an excessive systemic dose[1]. Systemic exposure to excessive bupivacaine mainly results in epilepsy-like syndromes due to central nervous system (CNS) excitation, and arrhythmias or cardiac arrest caused by cardiovascular toxicity[2]. Although efforts have been made to develop a controllable and safer delivery/release system for bupivacaine, a better strategy to avoid the overall toxicity is needed.

It is well-established that the main action of bupivacaine involves the use-dependent blockade of voltage-gated sodium channels (VGSCs) that are responsible for action potential initiation and axonal conduction[3]. Meanwhile, it also has a significant inhibitory effect on K^+ and Ca^{2+} channels that contribute to the repolarization and modulatory shaping of action potentials[4, 5]. Therefore, it is reasonable to infer that membrane depolarization and increased neuronal

excitability associated with these ion channels may account for the systemic toxicity of bupivacaine. However, knowledge about the links between pharmacological interactions and behavioral consequences have still to be worked out.

VGSCs are transmembrane proteins consisting of an ion-conducting α-subunit and one or more auxiliary subunits[6]. Generally, the α-subunit comprises four repeated domains (DI–DIV), each containing six transmembrane α-helixes (S1–S6) and a hairpin-like pore loop lining between S5 and S6[7]. Despite the high structural similarity among VGSC isoforms, they have distinct distributions, gating properties, and functional activities[8]. To date, the bupivacaine-binding site on sodium channels has been located at the intracellular portion[9, 10]. Thereby, bupivacaine blocks Na^+ influx into neurons and prevents depolarization. However, stereoselectivity has been reported in the bupivacaine-induced blockade of the inactivated state of Na^+ channels, but not on the blockade of activated (open-state) Na^+ channels[11]. Point-directed mutagenesis of the rat skeletal muscle $Na_v1.4$ channel revealed that the binding sites of bupivacaine are located in the pore-lining transmembrane segment 6 (S6) of domains 1, 3, and 4 (D1-S6, D3-S6, D4-S6), in which residues L1280 in D3-S6 and N434 in D1-S6 interact directly with bupivacaine and face each other in the ion-conducting pore[9, 10]. Even so, given that a variety of VGSC isoforms are distributed throughout human tissues, thorough investigation of how bupivacaine interacts with other Na^+ channels is still needed.

$Na_v1.5$ is responsible for the upstroke (phase 0) of the action potential in cardiac cells. Opening of the channel leads to a rapid influx of Na^+ (I_{Na}), which depolarizes the membrane potential within tenths of a millisecond[12]. Dysfunction of $Na_v1.5$ channels leads to various arrhythmias, such as long QT syndrome, Brugada syndrome, and cardiac conduction disease (also known as Lev-Lenegre syndrome)[13-15]. In light of this, there may be a relationship between the cardiac toxicity of bupivacaine and its use-dependent blockade of Na^+ channels. But it remains uncertain whether inhibition of VGSCs contributes to the systemic toxic effects of LAs, including the initial CNS excitation and pro-convulsive action[16, 17]. In this study, we investigated the pharmacological kinetics of bupivacaine on $Na_v1.5$ expressed in *Xenopus* oocytes.

MATERIALS AND METHODS

Chemicals and Solutions

Bupivacaine (Sigma-Aldrich, St. Louis, MO) was dissolved at room temperature in the bath solution ND96 (in mmol/L: NaCl 96, KCl 2, $CaCl_2$ 1.8, $MgCl_2$ 2, and HEPES 5, pH 7.4) at 100 mmol/L as stock solution and stored at –20°C. Different doses of bupivacaine were prepared before use and applied to oocytes by continuous perfusion *via* a fast gravity-driven perfusion system. After 10 min of perfusion, step pulses were used to investigate the effect of bupivacaine on $Na_v1.5$ channels. The rate of perfusion with blank or test solution was adjusted to 0.1 drop per second to minimize changes in the flow rate.

Plasmid

The gene encoding the rat Na^+ channel α-subunit of $Na_v1.5$ in pcDNA 3.1 vector was a generous gift from Dr. Kaoru Yamaoka (Hiroshima International University, Higashi-Hiroshima, Hiroshima, Japan) and was sub-cloned into pSP64 Poly(A) vector (Promega, Madison, WI) with SP6 promoter to ensure robust expression in *Xenopus* oocytes.

RNA Transcription and Expression in *Xenopus* Oocytes

The $Na_v1.5$ cRNA was synthesized from an EcoR I linearized DNA template and was transcribed *in vitro* using SP6 RNA-polymerase and the mMESSAGE mMACHINE™ system (Ambion, Austin, TX). The quality of mRNA produced was checked by running on a 1% agarose gel and Nanodrop 2000 (Thermo Scientific, Waltham, MA).

Xenopus laevis oocytes were injected with 10–20 ng of $Na_v1.5$ cRNA. Oocytes were incubated at 20°C for 2–4 days in ND96 solution supplemented with 5 mmol/L pyruvate and 0.1 mg/mL gentamycin.

Xenopus oocytes that expressed $Na_v1.5$ were clamped at −100 mV before electrophysiological recordings. Robust Na^+ currents were induced in oocytes when depolarized by a series of step stimuli ranging from −100 mV to +70 mV. To minimize individual difference between samples, only oocytes with peak I_{Na} currents elicited at −20 mV or −30 mV were chosen for subsequent tests[18, 19].

Electrophysiological Recording

Two-electrode voltage-clamp recordings were performed using an Axon 900A amplifier (Molecular Devices,

Sunnyvale, CA) and pClamp 10.0 software (MDC). Data were acquired using Clampfit 10.3 (MDC) and analyzed with Origin 7.5 (Northampton, MA). The voltage and the current electrodes were filled with 3 mol/L KCl. Currents were filtered at 1.3 kHz and sampled at 10 kHz with a four-pole Bessel filter. The bath solution contained (in mmol/L): NaCl 96, KCl 2, $CaCl_2$ 1.8, $MgCl_2$ 2, and HEPES 5 (pH 7.4).

Data Analysis

Mean conductance (G) was calculated using the equation $G = I/(V-V_r)$, where I is the peak current elicited upon depolarization, V is the membrane potential, and V_r is the reversal potential. The voltage-dependence for the activation was fit with the Boltzmann relation, $G/G_{max} = 1/[1+\exp(V-V_m)/k_m]$, where V_m is the voltage for half-maximum activation and k_m is the slope factor. Current decays were fit with a double exponential equation: $I = A_{fast}*\exp[-(t-K)/\tau_{fast}]+A_{slow}*\exp[-(t-K)/\tau_{slow}]+I_{SS}$, where I is the current, A_{fast} and A_{slow} represent the percentage of channels inactivating with time constants τ_{fast} and τ_{slow}, K is the time shift, and I_{SS} is the steady-state asymptote.

The Hill formula was used to fit the dose-response relationship of bupivacaine: $I_{drug}/I_{control} = A*[\text{Bupivacaine}]\text{^}n/(EC_{50}\text{^}n+[\text{Bupivacaine}]\text{^}n)$, where [Bupivacaine] is the concentration of bupivacaine, and n is the Hill coefficient. A is a constant representing the maximum reduction rate of Na^+ currents by bupivacaine. The value of $I_{drug}/I_{control}$ provides the maximal value of normalized inhibition of Na^+ currents at each concentration of bupivacaine. EC_{50} is the concentration of half-maximal inhibition of Na^+ currents by bupivacaine.

The voltage-dependence of fast inactivation and slow inactivation was described by the two-state Boltzmann equation: $I/I_{max} = A+(1-A)/\{1+\exp[(V-V_{1/2})/k]\}$, where A reflects the fraction of channels that are resistant to slow inactivation, V is the membrane potential of the conditioning step, $V_{1/2}$ is the membrane potential at which half-maximal inactivation is achieved, and k is the slope factor. The parameters for fast inactivation were characterized by the half-maximal voltage V_f and the slope factor k_f; and for slow inactivation were V_s and k_s.

Current decays were fitted with a double-exponential equation: $I = A_{fast}*\exp[-(t-K)/\tau_{fast}]+A_{slow}*\exp[-(t-K)/\tau_{slow}]+C$, where I is the current, A_{fast} and A_{slow} represent the percentage of channels inactivating with time constants τ_{fast} and τ_{slow}, t is the conditioning pulse duration, and K is the time shift.

The time constants for the development of slow inactivation were determined by fitting the data with a double-exponential equation: $I/I_{max} = A_{fast}*\exp(-t/\tau_{fast})+A_{slow}*\exp(-t/\tau_{slow})+C$. The normalized currents I/I_{max} provide information about how many channels entered slow inactivation during the conditioning pulse.

In each testing sample, control and bupivacaine data were acquired from the same oocyte. Only recordings with leakage <0.08 μA and fluctuation within 0.05 μA were selected for statistical analysis. Data are presented as mean ± SEM. Statistically significant differences between parameters of currents measured in control and drug-containing solutions were assessed with one-way ANOVA followed by Dunnett's multiple comparison test in GraphPad Prism 6.0 (GraphPad Software, Inc., La Jolla, CA) or Student's *t*-test in Origin 7.5.

RESULTS

Voltage- and Use-dependent Blockade by Bupivacaine

Although it has been reported that blockade of the Na^+ channel by LAs is differentially modulated by β1 and β3 subunits[20], this was not of concern in the present study in that we intended to provide a direct assessment of the pharmacological effects of bupivacaine on the $Na_v1.5$ channel. Hence, *Xenopus* oocytes were chosen as an expression system of $Na_v1.5$ alone.

Robust Na^+ currents of $Na_v1.5$ were elicited by step stimuli from a holding potential of −100 mV to +70 mV with 100 ms duration (Fig. 1A). To reach equilibrium for each recording, oocytes were perfused with the external solution of ND96 for 10 min prior to the subsequent protocols. After application of 50 μmol/L bupivacaine for 10 min, the peak $Na_v1.5$ current was inhibited by ~70%. The I–V curves showed that the blockade of I_{Na} occurred at quite depolarized potentials (about −40 mV for 10–100 μmol/L) (Fig. 3C). To quantify the dose-dependent blockade potency, clinical-range concentrations were selected to determine the IC_{50}. The results showed that bupivacaine blocked I_{Na} in a dose-dependent and "slow-out" (in that the effect was hard to be abolished during washing step) manner (Fig. 1B). The highest concentration (200 μmol/L) almost completely blocked I_{Na}, and the remaining currents were

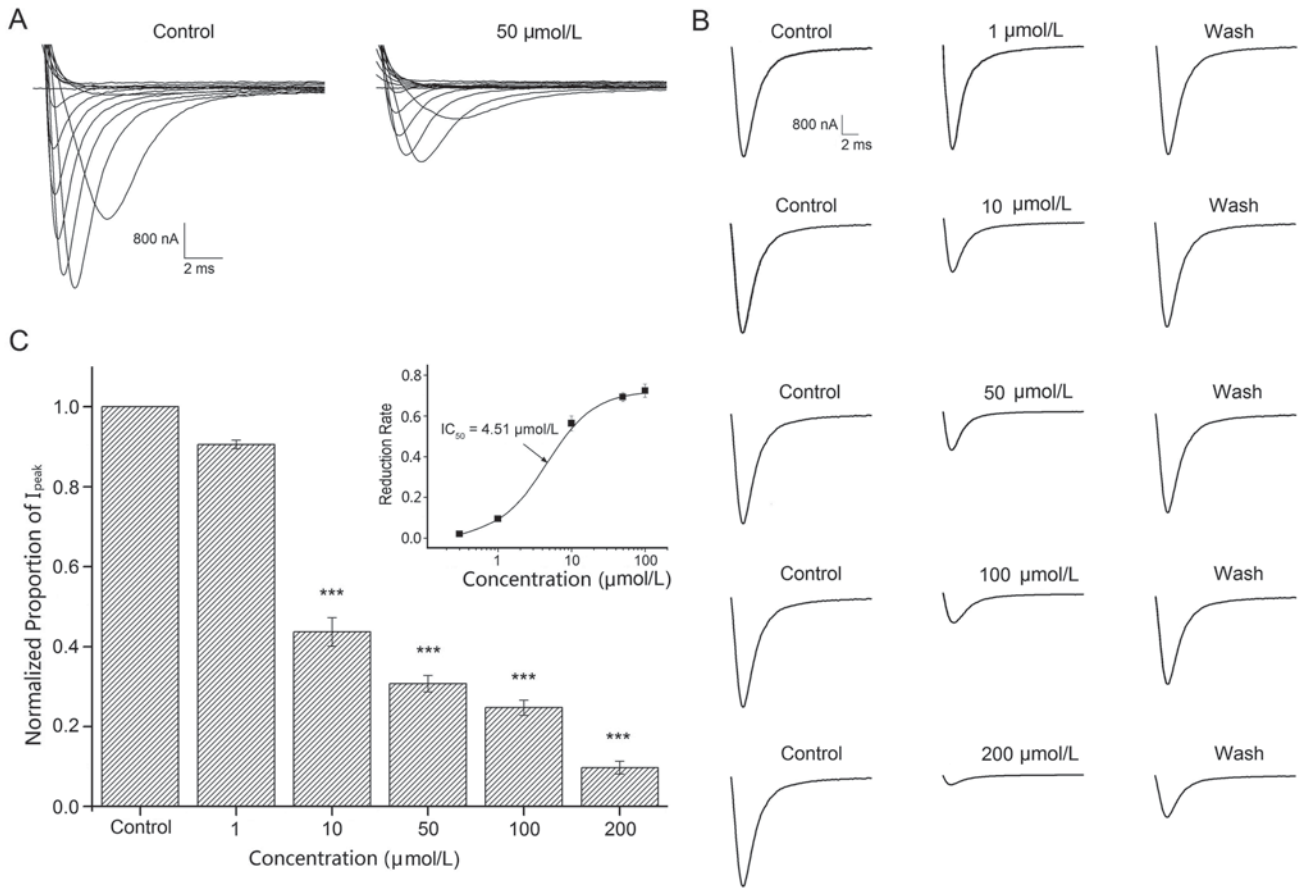

Fig. 1. Voltage and dose-dependent blockade of $Na_v1.5$ channels by bupivacaine. A: Representative traces of I_{Na} in blank (Control, left) and 50 μmol/L bupivacaine (right). B: Representative traces of I_{Na} illustrating the blocking potency of bupivacaine on $Na_v1.5$ at different concentrations at −20 mV; C: Dose-dependent blockade of $Na_v1.5$ by bupivacaine. Mean ± SEM. ***P <0.001 *vs* control; one-way ANOVA, n = 6 for each. Inset: dose-response curve for I_{Na} reduction and bupivacaine concentration.

only 9.75 ± 1.61% (P <0.001, n = 6) of control, while 1 μmol/L had little effect on I_{Na}, the remaining currents being 90.58 ± 1.06% of control (P >0.05, n = 6). The I_{Na} reduction rates induced by bupivacaine were 56.31 ± 3.60% at 10 μmol/L (P <0.001, n = 6); 69.24 ± 2.08% at 50 μmol/L (P <0.001, n = 5); and 72.37 ± 3.24% at 100 μmol/L (P <0.001, n = 5) (Fig. 1C). Accordingly, the dose-response relationship fit the Hill equation well, giving an IC_{50} of 4.51 μmol/L with a Hill coefficient of 1.33 (Fig. 1C, inset).

Since the hallmark of most class I LAs is the induction of a use-dependent blockade of Na^+ channels, the kinetic properties of bupivacaine blockade was characterized in $Na_v1.5$ with steps of depolarizing stimuli from −100 mV to +10 mV at 0.5, 1, and 2 Hz for 60 pulses each. Each peak Na^+ current was normalized to the peak current during the first pulse. Under control conditions, there was hardly any reduction in peak I_{Na} (Fig. 2). After treatment with bupivacaine at different concentrations, the potency of blockade was progressively enhanced. Notably, the blockade by bupivacaine at 1 μmol/L tested at 2 Hz was more efficient than that tested at 1 Hz, while this relationship was reversed at higher concentrations (50 and 100 μmol/L) (P <0.001, n = 5–6, Fig. 2C). The development of blockade was accelerated with increasing bupivacaine concentration at all frequencies tested (Fig. 2B, D). Almost all the use-dependent blockade at different concentrations was achieved within the first 15 pulses, indicating a “fast-in” (in that the inhibition rate was fast) manner. When fitted to the first order exponential equation, the resultant time constants for entry into the steady-state blockade

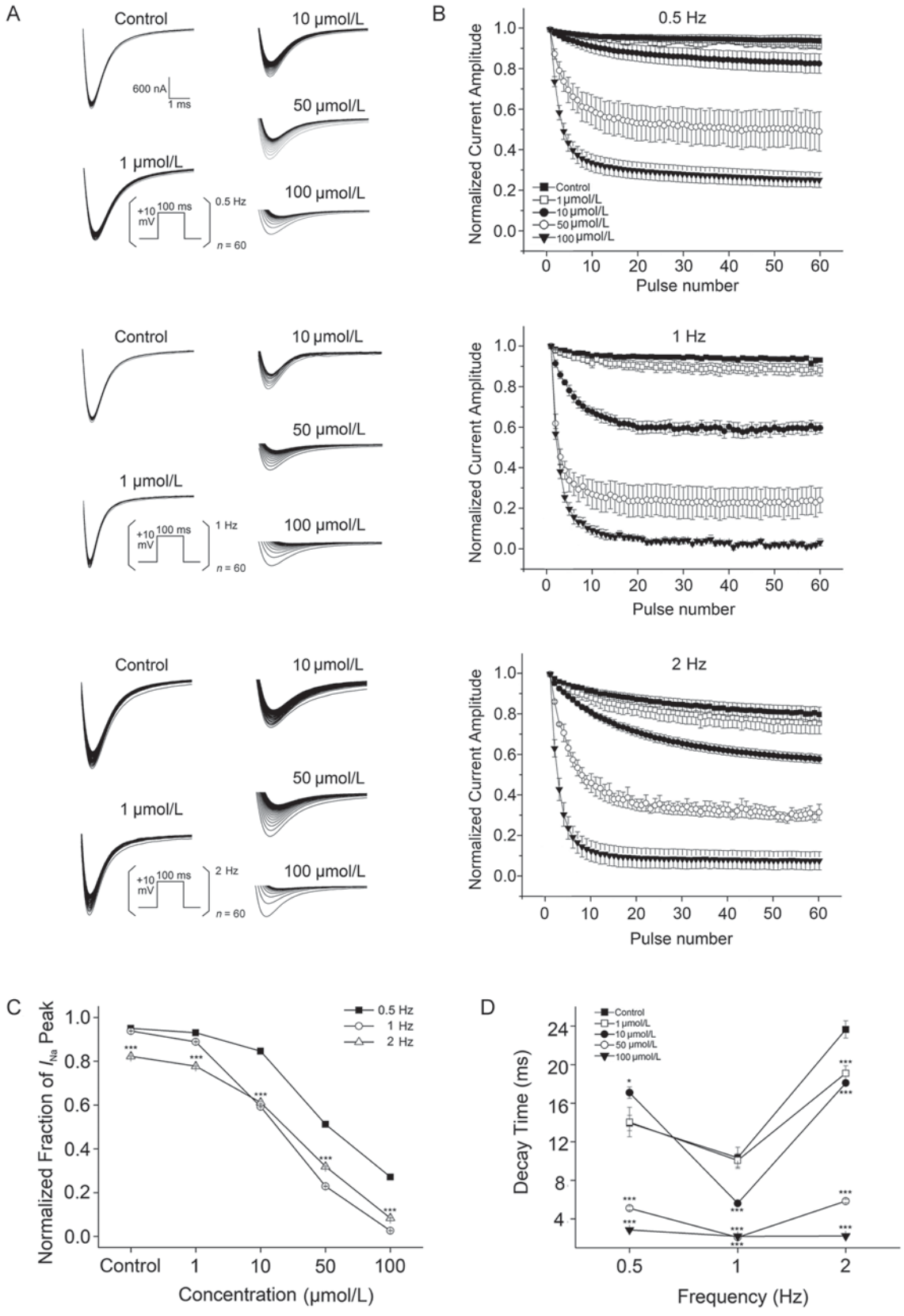
A
Control
10 μmol/L
600 nA
1 ms
50 μmol/L
1 μmol/L
100 μmol/L
+10 mV
100 ms
0.5 Hz
n = 60
1 Hz
2 Hz
B
0.5 Hz
1 Hz
2 Hz
Normalized Current Amplitude
Pulse number
Control
1 μmol/L
10 μmol/L
50 μmol/L
100 μmol/L
C
Normalized Fraction of I_{Na} Peak
Concentration (μmol/L)
D
Decay Time (ms)
Frequency (Hz)

Fig. 2. Use-dependent blockade of $Na_v1.5$ by bupivacaine at different concentrations. Oocytes were held at –100 mV and a train of sixty 100-ms pulses was applied to +10 mV at three frequencies (0.5, 1, and 2 Hz, inset). The peak currents elicited by each pulse (A) were normalized to the current of the first pulse (P_n-P_1, where n = 1–60) and were then plotted *versus* pulse number (B). Values represent mean ± SEM. Control (n = 6); 1 μmol/L bupivacaine (n = 6); 10 μmol/L bupivacaine (n = 5); 50 μmol/L bupivacaine (n = 3); 100 μmol/L bupivacaine (n = 4). C: Plot of normalized fraction of peak I_{Na} against different concentrations of bupivacaine tested at 0.5, 1, and 2 Hz. ***P <0.001 for normalized I_{Na} tested at 1 Hz (open circles) compared with that at 2 Hz (open triangles); Student's t-test (n = 5–6). D: Time constants for the entry into the steady-state blockade of I_{Na} by bupivacaine tested at 0.5, 1, and 2 Hz. *P <0.05, ***P <0.001 *vs* control; one-way ANOVA (n = 5–6).

decreased in a nonlinear frequency-dependent manner, prominently for 0.5 and 2 Hz but less efficiently for 1 Hz (Fig. 2D).

Bupivacaine Shifts the Voltage-dependent Relationship of Activation and Inactivation

To give a full assessment of the pharmacological profile of bupivacaine, the voltage-dependent activation and steady-state inactivation of expressed $Na_v1.5$ channels were tested. Since 200 μmol/L bupivacaine significantly depressed the channel activity, amplitudes recorded at this concentration did not reflect its real pharmacological effect. Among the four concentrations tested (except 1 μmol/L), the voltage-dependent activation curves were substantially shifted to more depolarized potentials in a dose-dependent manner (7.71 mV for 10 μmol/L, 9.21 mV for 50 μmol/L, and 12.01 mV for 100 μmol/L) (Fig. 3B, Table 1).

Compared with the voltage-dependent activation, the steady-state inactivation was less sensitive to bupivacaine. All the inactivation curves were shifted to more hyperpolarized potentials, except for the 10 μmol/L bupivacaine treatment (Fig. 3B). Bupivacaine at 50 μmol/L hyperpolarized the steady-state inactivation curve by 8.25 mV (P <0.001, n = 6), about double that of 1 μmol/L (3.47 mV; P <0.001, n = 6) and 100 μmol/L (4.31 mV; P <0.001, n = 6). In contrast, 10 μmol/L bupivacaine slightly depolarized the steady-state inactivation by 1.26 mV (P <0.01, n = 6) (Table 1). Bupivacaine also significantly altered the slope factor of the inactivation curves at all concentrations except 1 μmol/L (Table 1).

Bupivacaine Modifies the Gating Kinetics of Inactivation

Since LAs have a higher affinity for channels in the inactivated state, we considered that the blockade of I_{Na} by bupivacaine may be due to changes in the fast and slow inactivation components of $Na_v1.5$. To test this hypothesis, the voltage-dependent relationships of these two components were explored.

The voltage-dependence of fast and slow inactivation was investigated using protocols with prepulses from −100 mV to +60 mV for different durations (10 ms for fast and 2 000 ms for slow inactivation) (Fig. S1A, B, inset).

Overall, the slow inactivation was more vulnerable to modulation by bupivacaine, with $V_{1/2}$ shifted to a more hyperpolarized potential than that of fast inactivation at all tested concentrations (1, 10, 50, and 100 μmol/L). In slow inactivation, 50 μmol/L bupivacaine induced the most pronounced hyperpolarization shift (20.22 mV) (P <0.001, n = 6) and modest shifts at 10 and 20 μmol/L (3.14 and 11.98 mV respectively; both P <0.001, n = 6), Bupivacaine at 1 μmol/L barely caused any shift in the slow inactivation curve (P >0.05, n = 6). Finally, the fraction of channels resistant to slow inactivation was decreased by bupivacaine in a dose-dependent manner (Fig. S2).

The effect of bupivacaine on the fast inactivation of $Na_v1.5$ was not as prominent as that on slow inactivation. At 100 μmol/L, bupivacaine even markedly depolarized the voltage-dependency by 5.46 mV (P <0.01, n = 6), accompanied by a decrease in the steepness of the inactivation curve (Δk = 5.08 mV), in contrast to the other groups (Table 2, Fig. S1).

The time-constants of decay for the fast and slow components were obtained by fitting the current decay of the activation traces to the double exponential equation. The time constant was several milliseconds in the fast component but dozens of milliseconds in the slow component (Fig. 4A). Bupivacaine preferentially acted on the slow component, in that 50 μmol/L bupivacaine increased the time constants at most of the potentials tested (−20 mV, τ_{slow} = 11.01 ± 1.08 ms, n = 5; –10 mV, τ_{slow} = 21.05 ± 4.61 ms, n = 5; 0 mV, τ_{slow} = 44.26 ± 2.71 ms, P <0.001, n = 5; +10 mV, τ_{slow} = 87.65 ± 8.32 ms, P <0.001,

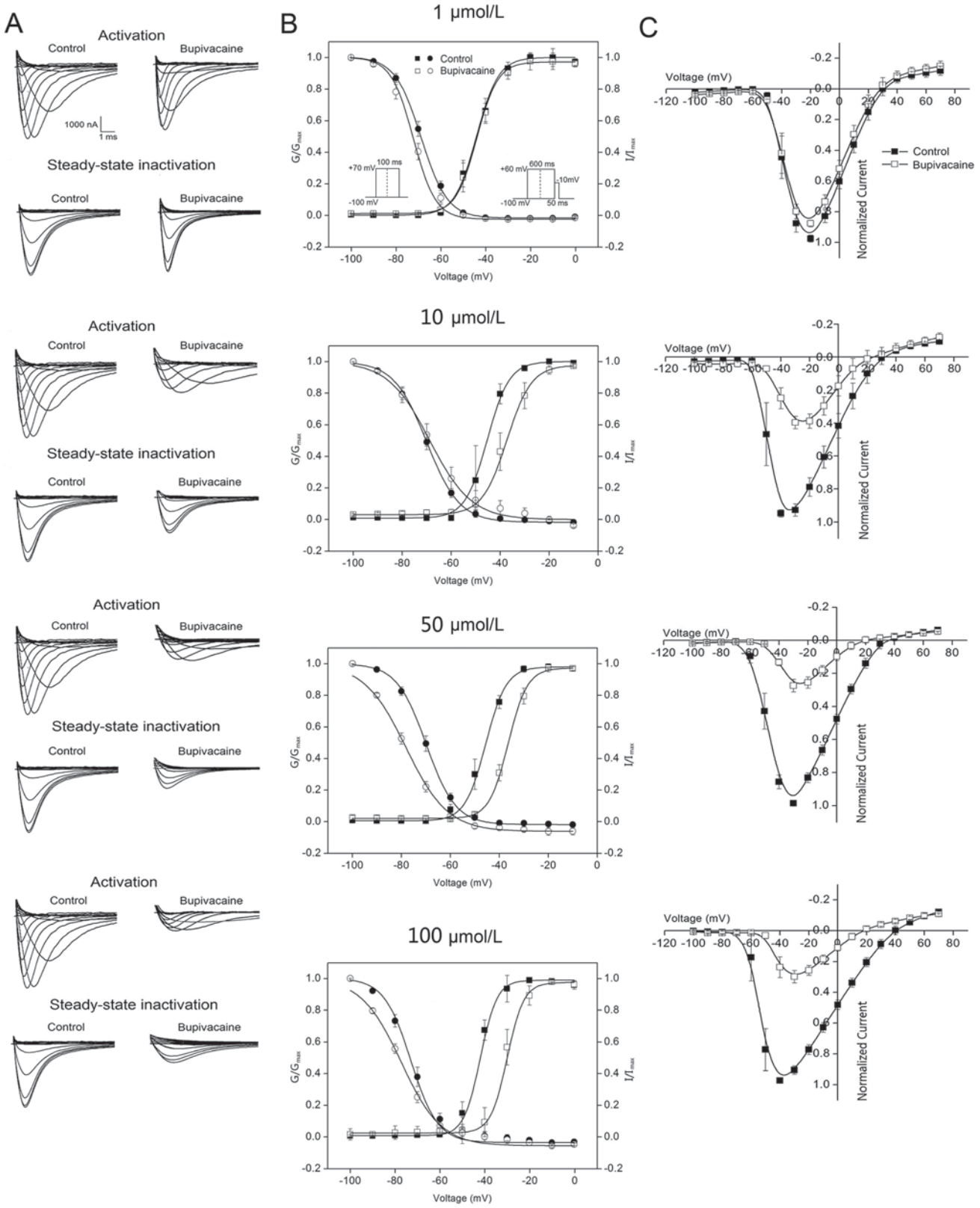
A
Activation
Control
Bupivacaine
1000 nA
1 ms
Steady-state inactivation
Control
Bupivacaine
B
1 μmol/L
Control
Bupivacaine
+70 mV
100 ms
-100 mV
+60 mV
600 ms
10mV
-100 mV
50 ms
G/G_max
I/I_max
Voltage (mV)
10 μmol/L
50 μmol/L
100 μmol/L
C
Voltage (mV)
Control
Bupivacaine
Normalized Current

Fig. 3. Voltage-dependent activation and steady-state inactivation of $Na_v1.5$ before and after application of 1, 10, 50, and 100 μmol/L bupivacaine. A: Na^+ currents were elicited by depolarizing pulses from a holding potential of −100 mV to +70 mV in 10-mV increments. The voltage-dependence of steady-state inactivation was determined using a two-step protocol in which a conditioning pulse to potentials ranging from –100 mV to +60 mV was followed by a test pulse to –10 mV to measure the peak current amplitude (protocols in B, insets). B: Conductance values were calculated by dividing the peak current amplitude by the driving force at each potential and normalizing to the maximum conductance. For steady-state inactivation, the peak current amplitude during the test pulse was normalized to the maximum current amplitude and plotted as a function of the conditioning pulse potential. Values are averages, and error bars indicate SEMs. The data were fitted to a two-state Boltzmann equation, and the parameters of the fits are shown in Table 1. Sample sizes of each group are shown in Table 1. C: Normalized current-voltage (*I-V*) relationship of $Na_v1.5$ before and after bupivacaine adiministration. Mean ± SEMs.

Table 1. Parameters for activation and steady-state inactivation of $Na_v1.5$ in blank and bupivacaine treatment conditions.

Concentration	Treatment	Activation			Steady-state inactivation		
		n	$V_{1/2}$ (mV)	*k*	*n*	$V_{1/2}$ (mV)	*k*
1 μmol/L	Control	6	–44.03±0.51	4.89±0.29	6	–68.74±0.34	5.84±0.20
	Bupivacaine	6	–43.84±0.49	4.62±0.91	6	–72.21±0.36***	5.91±0.29
10 μmol/L	Control	6	–44.96±0.61	4.49±0.09	6	–70.02±0.34	6.70±0.36
	Bupivacaine	6	–37.25±0.60*	5.03±0.28	6	–68.76±0.29**	8.51±0.30***
50 μmol/L	Control	5	–45.59±0.82	4.21±0.46	6	–69.90±0.35	6.01±0.32
	Bupivacaine	5	–36.38±0.41*	3.91±0.23	6	–78.15±0.34***	8.52±0.35***
100 μmol/L	Control	6	–41.93±1.98	3.69±1.21	6	–72.94±0.37	6.00±0.40
	Bupivacaine	6	–29.92±1.08***	3.71±0.93	6	–77.25±0.29***	8.91±0.39***

*P <0.05, **P <0.01 and ***P <0.001, one way ANOVA; *n* indicates the number of samples tested and *k* is the slope factor. Values are mean ± SEM.

Table 2. Parameters for fast and slow inactivation of $Na_v1.5$ in blank and bupivacaine treatment conditions

Treatment	Concentration	Fast inactivation			Concentration	Slow inactivation		
		n	$V_{1/2}$ (mV)	*k*		*n*	$V_{1/2}$ (mV)	*k*
Control	1 μmol/L	6	–46.68±0.55	6.83±0.47	1 μmol/L	6	–58.46±0.53	14.97±0.31
Drug		6	–48.23±0.79	7.12±0.43		6	–58.88±0.78	14.07±0.57
Control	10 μmol/L	6	–45.36±0.53	6.86±0.21	10 μmol/L	6	–61.77±0.65	13.19±0.61
Drug		6	–47.14±0.77	8.35±0.48*		6	–64.91±0.21***	9.89±0.32***
Control	50 μmol/L	6	–49.89±0.73	6.20±0.33	20 μmol/L	6	–61.64±0.67	15.64±0.42
Drug		6	–51.19±1.21*	7.93±0.79		6	–73.62±0.43***	12.14±0.68***
Control	100 μmol/L	6	–51.75±0.64	6.63±0.27	50 μmol/L	6	–61.65±0.73	14.65±0.31
Drug		6	–46.29±0.74**	11.71±0.57***		6	–81.87±0.49***	11.02±0.46***

*P <0.05, **P <0.01, ***P <0.001; *n* indicates the number of samples tested. Values are mean ± SEM.

n = 3), while the time constants of the fast component were resistant to bupivacaine at all concentrations. These findings were consistent with the voltage-dependent relationship of inactivation described above. Accordingly, with the delayed time constants of the slow component induced by bupivacaine, the fractions of the fast component were slightly increased. Among all the potentials considered, the increased proportion of fast component

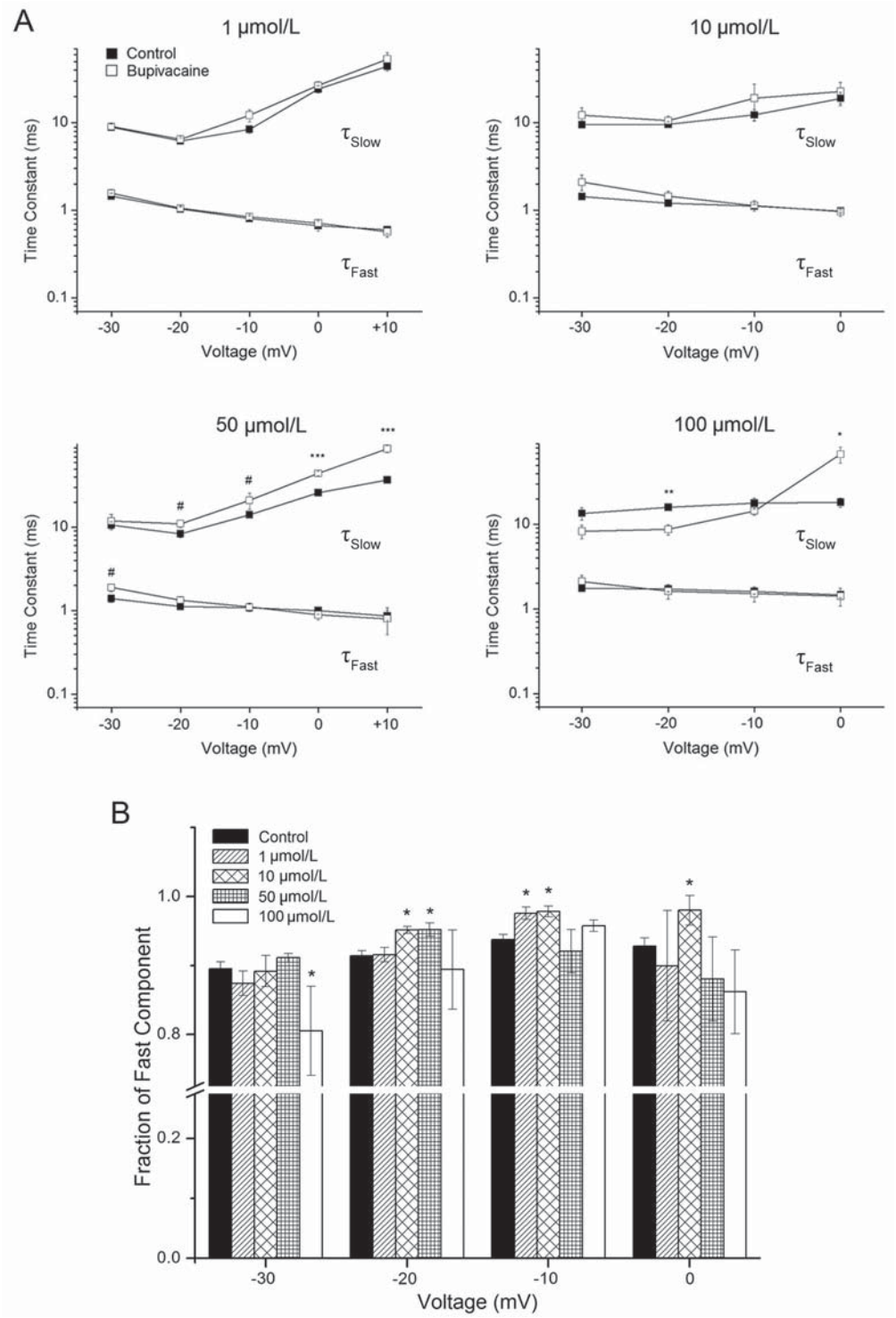

Fig. 4. Modulation of the inactivation kinetics of $Na_v1.5$ before and after 1, 10, 50, and 100 μmol/L bupivacaine. *n* = 5–6. A: Time-courses of decay of Na^+ currents at −30, −20, −10, and +10 mV were fitted to a double-exponential equation. The currents were separated into two inactivation components (τ_{fast} and τ_{slow}) based on the inactivation rate. The effect of bupivacaine was calculated at 1, 10, 50, and 100 μmol/L (open boxes). B: Modulation of the fast component of inactivation of $Na_v1.5$ by bupivacaine. **P* <0.05, ***P* <0.01, ****P* <0.001 *vs* control, Student's *t*-test.

induced by 10 μmol/L bupivacaine was evident, which was increased by 3.8% at −20 mV (P <0.05, n = 5), 4.1% at −10 mV (P <0.05, n = 4), and 5.2% at 0 mV (P <0.05, n = 3) (Fig. 4B). In addition, this increase was also found at −20 mV (3.9%, P <0.05, n = 5) for 50 μmol/L, and at −10 mV (3.8%, P <0.05, n = 6) for 1 μmol/L (Fig. 4B). Notably, a small but notable decrease for 100 μmol/L at −30 mV (9.0%, P <0.05, n = 4) was seen, which may have been caused by the disrupted gating property of channels at high concentrations of bupivacaine.

Bupivacaine Modulates the Development of Slow Inactivation and the Recovery from Inactivation

Since slow inactivation is an important factor governing

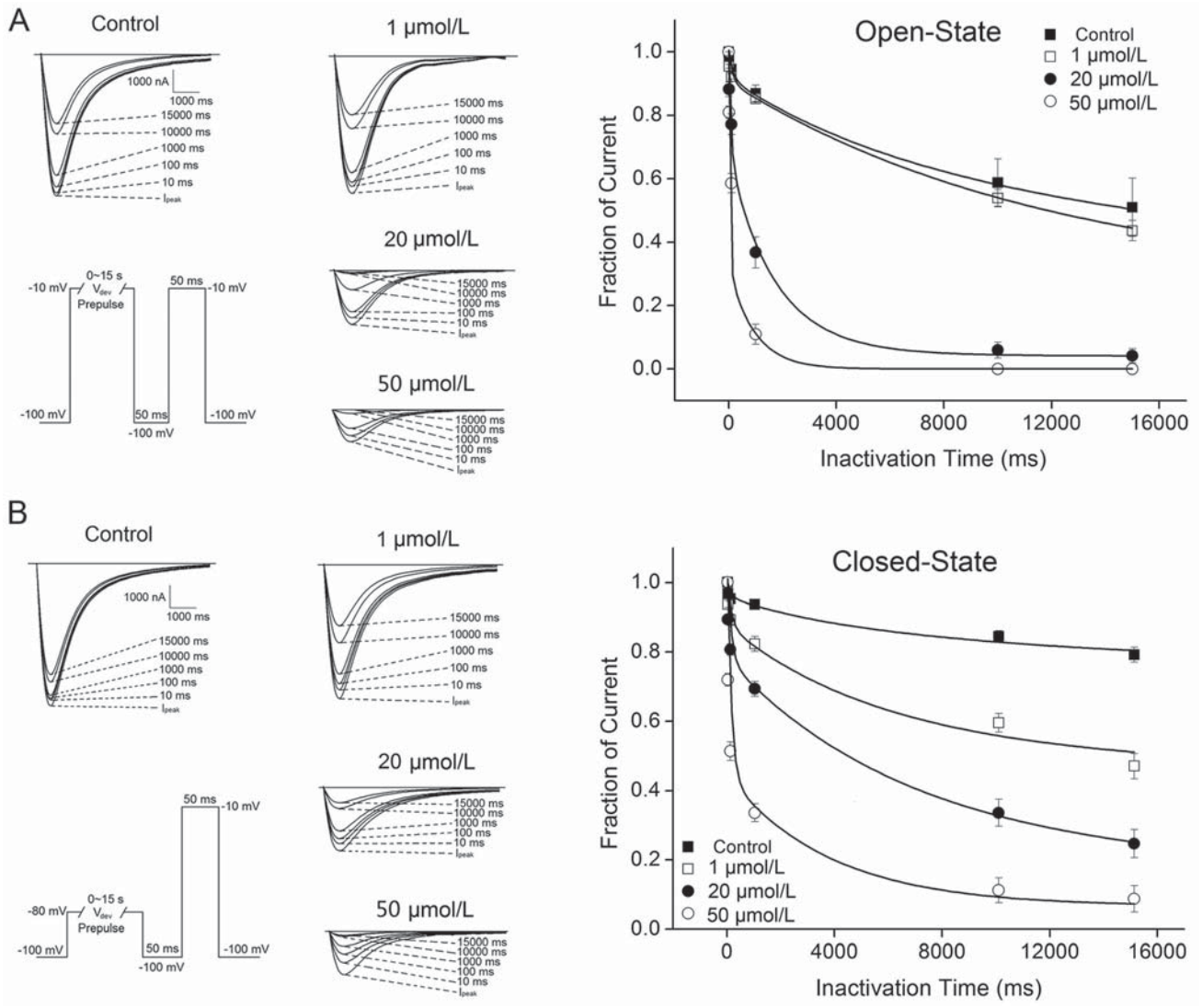

Fig. 5. Development of slow inactivation of $Na_v1.5$ is accelerated by different concentrations of bupivacaine. A: Superimposed current traces of $Na_v1.5$ in control or with different concentrations of bupivacaine showing the rate of development of open-state inactivation. B: Superimposed current traces of $Na_v1.5$ showing the rate of development of closed-state inactivation. Right panels in A and B: time courses of development of inactivation for the peak $Na_v1.5$ currents. Insets: oocytes were prepulsed to V_{dev} for increasing durations, then stepped to −10 mV to determine the fraction of current inactivated during the prepulse. The duration of the inactivation prepulse for each trace is indicated. Averaged data are presented at a V_{dev} of −10 mV (A, n = 6) or −80 mV (B, n = 6) to compare the extent of inactivation. Normalized currents are plotted as a function of V_{dev} duration.

$Na_v1.5$ availability in the activated state, we then determined whether bupivacaine affects the entry into slow inactivation (Fig. 5). Prepulses to −10 mV and −80 mV of variable durations were used to monitor the inactivation onset of $Na_v1.5$ in the open (Fig. 5A) and closed states (Fig. 5B). By fitting the decay currents to the double-exponential equitation, we compared the time constants for the development of slow inactivation at different concentrations of bupivacaine (Fig. 5, right panels). At −10 mV, bupivacaine at all concentrations accelerated both phase I (t_1) and phase II (t_2) of the decay in a dose-dependent manner (Table 3). But at −80 mV, bupivacaine at all concentrations delayed phase I, and the time constants of phase II were delayed at 20 and 50 μmol/L (Table 3). These results suggest that bupivacaine is capable of accelerating the development of open-state slow inactivation of $Na_v1.5$ but not that of closed-state slow inactivation.

The acceleration of entry into slow inactivation may be one of the reasons for the voltage-dependent block of $Na_v1.5$ currents by bupivacaine. However, the high-affinity binding of bupivacaine to the inactivated Na^+ channel might also affect the recovery time from inactivation, which would reduce the number of channels available to reopen, as seen in the actions of lidocaine[21]. Therefore, the recovery time and rate of kinetics were examined for $Na_v1.5$ at different concentrations of bupivacaine.

Recovery from inactivation was determined using a two-pulse protocol consisting of a 50-ms conditioning

Table 3. Time constants for the development of slow inactivation modulated by different concentrations of bupivacaine

Concentration(μmol/L)	Open-State			Closed-State		
	n	t_1 (ms)	t_2 (ms)	*n*	t_1 (ms)	t_2 (ms)
Control	6	151.57±0.49	17889.56±0.59	6	130.48±0.31	13469.90±0.52
1	6	132.93±0.33	12011.27±0.64	6	155.37±0.23	12945.21±0.91
20	6	83.75±0.41	2158.37±0.83	6	141.25±0.44	13511.22±1.03
50	6	35.66±0.21	958.46±0.77	6	147.11±0.56	14034.52±1.67

n indicates the number of samples tested. Values represent means ±S.E.M.

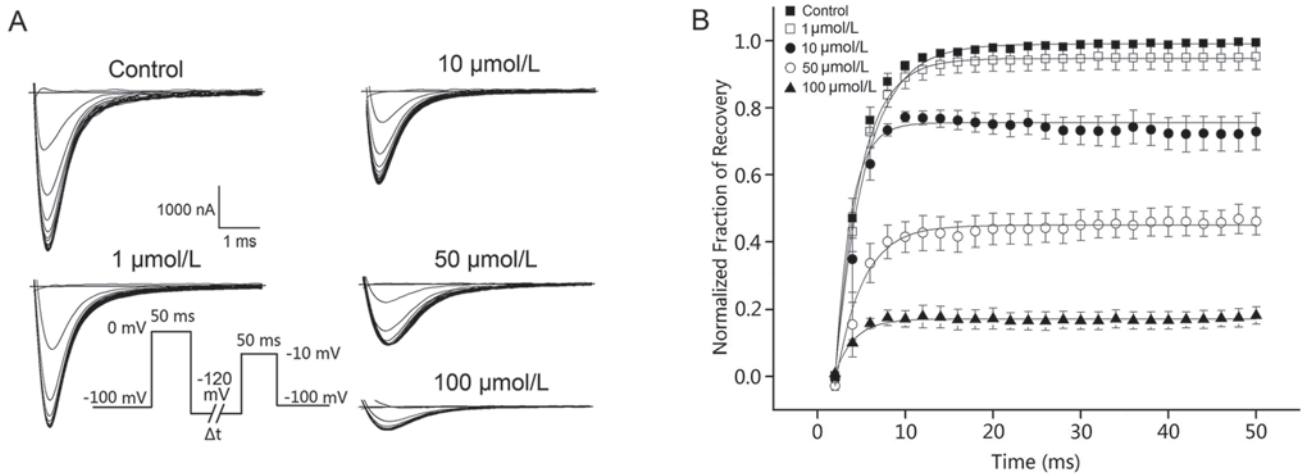

Fig. 6. Bupivacaine attenuates the recovery kinetics from inactivation. A: Time course of recovery at −120 mV as determined by a two-pulse protocol (below). Currents were recorded at a test pulse to −10 mV for 50 ms after a variable (0–100 ms) recovery time at −120 mV from a 50-ms conditioning prepulse at 0 mV. B: The recovery time course was then fitted to a single-exponential function to obtain the time constant of recovery. *n* = 3–6; mean ± SEM.

prepulse at 0 mV followed by a varied recovery time (0–100 ms) at −120 mV, after which a test pulse to −10 mV for 50 ms was applied (Fig. 6A, inset). The recovery kinetics of currents was measured at –10 mV and single exponential fits were used to estimate the recovery time constants and the proportion of recovered Na^+ channels. Under control conditions, 99.02 ± 0.10% of $Na_v1.5$ readily recovered after 50-ms depolarization at 0 mV, with a recovery time constant of 1.76 ± 0.09 ms. Bupivacaine reduced not only the number of channels recovered but also the rates of recovery from inactivation. The proportions of recovered Na^+ channels and time constants for the recovery (τ_{rec}) after treatment with bupivacaine were 94.70 ± 0.17% and 1.44 ± 0.03 ms for 1 μmol/L; 75.57 ± 0.53% and 0.89 ± 0.10 ms for 10 μmol/L; 44.92 ± 0.29% and 1.51 ± 0.10 ms for 50 μmol/L; and 17.17 ± 0.19% and 1.10 ± 0.06 ms for 100 μmol/L. These results suggest that bupivacaine is capable of attenuating the recovery potency of $Na_v1.5$ and slightly accelerating the time constant for partial recovery from the inactivated state (Fig. 6B).

DISCUSSION

Regarded as the most long-acting and efficient LA widely used in clinical management, bupivacaine is still associated with severe cardiac and CNS toxicity, which restricts its use as a safe and controllable LA[22]. The major mechanism for bupivacaine depression of cardiac conduction is considered to be the fast block of Na^+ channels during action potential transmission, which results in slow recovery from block during diastole[23]. Recently, levobupivacaine, a single enantiomer of bupivacaine, has been introduced as a new long-acting LA with potentially reduced toxicity compared with bupivacaine[24]. Even so, bupivacaine has not been replaced in the market, probably due to the lack of perceived safety benefits and/or the consideration of additional costs for the switch to levobupivacaine, which is ~57% more expensive than bupivacaine[22]. Therefore, a safer strategy for the current use of bupivacaine is urgently needed. To achieve this, an in-depth investigation of how bupivacaine interacts with Na^+ channels and the underlying mechanisms need to be illuminated.

In the present study, we examined the pharmacological profile of bupivacaine on $Na_v1.5$, with particular interest in how it interacts with channels expressed in background-free *Xenopus* oocytes.

It is well-established that bupivacaine has a higher affinity for inactivated Na^+ channels[21]. However, apart from the consistency of hyperpolarized inactivation found in this research, bupivacaine also shifted the voltage-dependent activation of $Na_v1.5$ to more depolarized values. The enhanced inactivation and impaired activation of $Na_v1.5$ caused by bupivacaine would reduce cell excitability since larger depolarizing stimuli would be required to activate the $Na_v1.5$ channel.

To date, the mechanisms underlying the blockade of VGSCs by class I LAs have been investigated to follow two independent stages. One is related to the voltage-dependent block, which involves voltage sensor inhibition in the open state. The other is defined as a lipophilic block resulting from interaction with the drug in the closed state[25]. The latter type of inactivation only occurs at very high concentrations and is therefore considered to be a low-affinity block, so this was not a concern in the current study. Consistent with the previous findings, bupivacaine greatly affected the inactivation of $Na_v1.5$ and decreased the number of Na^+ channels that recovered. Moreover, the development of slow inactivation and the voltage-dependent delay in slow inactivation time constants means that bupivacaine has an apparent bias for the open-state Na^+ channel (Fig. 5A). All the results support the idea that the blockade of $Na_v1.5$ by bupivacaine is due to a voltage-dependent block in the open state.

The binding sites of class I LAs on Na^+ channels are localized in DIII-S6 and DIV-S6. However, the key residues involved in bupivacaine binding have been less investigated, unlike that of lidocaine. Currently, it is generally considered that the voltage-dependent blockade may be attributed to the hydrophobic and aromatic residues within S6, such as L1280 and P1759[10]. Here, we found that between the fast and slow inactivation, bupivacaine preferably acted on the latter, for which the voltage-dependency and time constants were substantially changed. This finding is in agreement with the fact that the slow inactivation is thought to be accompanied by rearrangement of the channel pore in DIV[26]. On the other hand, the significant changes in steepness of voltage-dependency of fast inactivation induced by bupivacaine indicated an interaction between bupivacaine and the fast-inactivation lid associated with DIII-S6. Together, we

postulate that the binding of bupivacaine to the inactivated $Na_v1.5$ channel involves DIII-S6 and DIV-S4.

In addition, at extremely high concentrations, bupivacaine dramatically inhibited $Na_v1.5$ activity, leading to a permanent block that barely recovered. In contrast, at lower concentrations, bupivacaine induced a relatively small, reversible inhibition of $Na_v1.5$ currents. This phenomenon indicates that the CNS toxicity induced by bupivacaine may follow a two-stage process: at lower concentrations, inhibitory neurons are blocked first resulting in generalized convulsions, and at higher concentrations a global CNS depression occurs[2]. Hence, although treatment with bupivacaine may have the risk of clinical CNS syndromes, permanent damage can be avoided at appropriate dosages.

Of note, the bupivacaine-induced block of the inactivated state of the Na^+ channel displays stereoselectivity. Both enantiomers of bupivacaine bind with high affinity to the activated or open-state cardiac Na^+ channel, with binding kinetics faster for S(-)- than for R(+)-bupivacaine. The higher potency of R(+)-bupivacaine in blocking the inactivated state of the cardiac Na^+ channel may explain its higher toxicity because of the large contribution of the inactivated-state blockade during the plateau phase of the cardiac action potential. These results support the use of the S(-)-enantiomer to reduce cardiac toxicity[11]. However, as pure S(-)-enantiomer is far more expensive and bupivacaine is clinically used as a racemic mixture, this study provides clues, at least in part, for a safer strategy of the current use of bupivacaine.

In summary, our results revealed that the voltage-dependent block of $Na_v1.5$ by bupivacaine arises not only from a depolarized shift in voltage-dependent activation but also from hyperpolarized inactivation. In particular, bupivacaine has a preference for the open-state inactivated channels, the binding sites of which may rely on the hydrophobic residues within DIII-S6 and DIV-S6. In addition, overdose of bupivacaine could cause a drastic decrease in channel activity that may partially contribute to the clinical cardiac or CNS toxicity.

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SUPPLEMENTAL DATA

Supplemental data include two figures and can be found online at http://www.neurosci.cn/epData.asp?id=192.

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