Inhibition of Kv4.3/KChIP2.2 Channels by Bupivacaine and Its Modulation by the Pore Mutation Kv4.3V401I

Anna Solth, M.Sc.,* Cornelia C. Siebrands, M.Sc.,† Patrick Friederich, M.D.‡

Background: The transient outward current Ito is an important repolarizing K current in human ventricular myocardium mediated by Kv4.3 and KChIP2.2 subunits. Inhibition of Ito by amino-amide local anesthetics may be involved in severe cardiotoxic side effects. This study elucidates the molecular mechanisms of bupivacaine interaction with complexes formed by Kv4.3 and KChIP2.2 as well as the modulatory effect of KChIP2.2. For this purpose, the pharmacologic effects of bupivacaine on Kv4.3×/KChIP2.2 channels and on the pore mutant Kv4.3V401I were investigated.

Methods: Kv4.3/KChIP2.2 cDNA was transiently expressed in Chinese hamster ovary cells. Site-directed mutagenesis and patch clamp experiments were performed to analyze the effects of bupivacaine on wild-type and mutant channels.

Results: Inhibition of Kv4.3×/KChIP2.2 channels by bupivacaine was concentration-dependent and reversible. The IC50s for inhibition of the charge conducted by Kv4.3×/KChIP2.2 channels by bupivacaine and levobupivacaine were 55±8 and 50±5 μM, respectively. The local anesthetic accelerated macroscopic current decline of Kv4.3×/KChIP2.2 and slowed recovery from inactivation without altering steady state inactivation. KChIP2.2 altered the response of Kv4.3× channels to bupivacaine and bupivacaine modulated KChIP2.2 effects on Kv4.3× channels. The pore mutation V401I slowed macroscopic current decline of Kv4.3 channels and recovery from inactivation, and it diminished modulation of gating by KChIP2.2. Bupivacaine inhibition of Kv4.3V401I resembled Kv4.3×, and was not changed by coexpression of KChIP2.2.

Conclusions: These results indicate that bupivacaine blocks Kv4.3/KChIP2.2 channels from the open state. They furthermore give structural evidence that amino-amide local anesthetics interfere with the effects of KChIP2.2 on Kv4.3 by an indirect mechanism.

THE transient outward current Ito is an important repolarizing K current in the human heart.1 2 The reduction of Ito contributes to prolongation of the cardiac action potential and ventricular arrhythmia occurring in ventricular failure3 4 and in myocardial infarction.5 Although the exact molecular composition of human Ito in vivo remains unclear,6 it is widely accepted that Kv4.3 constitutes the principal α subunit in the human heart.6 7 8 Kv4.3 α subunits coassemble with accessory subunits of the KChIP2 family (Kv channel interacting protein).9 10 KChIP2 alters the level of surface expression as well as the gating properties of Kv4 channels.11 The level of KChIP2 gene expression decreases from the epicardium to the endocardium and correlates with the transmural gradient of Ito in canine,7 8 murine,12 and human myocardium.7 8 Knockout of the KChIP2 gene in mice leads to a complete absence of Ito, a prolongation of action potential duration, and an increased susceptibility to development of polymorphic ventricular arrhythmia of the torsades de pointes type.12 The precise mechanism of the interaction of KChIP with Kv4 channels has not been elucidated. However, it is clear that the N-terminal part of the Kv4.3 α subunit is crucial in binding and interacting with KChIP2.11 The amino acids valine 399 and valine 401 in the inner pore region of Kv4.3 channels are also considered important for the modulatory effects of KChIP2.13

Inhibition of K channels by bupivacaine has been regarded as the molecular mechanism responsible for QTc interval prolongation of the electrocardiogram observed during toxic plasma concentrations of this amino-amide local anesthetic.14 16 The pharmacologic effects of bupivacaine on cloned cardiac repolarizing K channel α subunits have been studied.17 24 Also, the pharmacologic action of bupivacaine on some cardiac ion channel complexes formed from α and β subunits has been investigated.18 22 24 The results of these studies demonstrate that amino-amide local anesthetics are not selective Na channel blockers but also block potassium channels in the same concentration range. In addition, these studies propose that amino-amide local anesthetics act as open channel blockers of Kv1.5 and Kv4.3 channels17 20 24 and that local anesthetics interfere with the effects of KChIP on Kv4.3 channels by an unknown mechanism.24

Ito is suggested to constitute a potential molecular target involved in the cardiotoxic action of bupivacaine.25 26 However, the effects of bupivacaine on complexes formed by human Kv4 and KChIP subunits have not yet been studied. Furthermore, it is unknown whether local anesthetics directly or indirectly interfere with the action of KChIP subunits by binding to the ion channel pore of Kv4.3 channels. Kv4.3 and KChIP2.2 constitute the most abundant isoforms of Kv4 and KChIP subunits present in human heart.11 27 Therefore, this study was designed to investigate in detail bupivacaine effects on complexes formed by Kv4.3 and KChIP2.2 subunits cloned from human heart. Investigating the effects of bupivacaine on a pore mutation of Kv4.3 channels predicted to alter interaction with KChIP2.213


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could also demonstrate whether bupivacaine and KChIP interfere by a direct or indirect mechanism.

Materials and Methods

Cell Culture
Chinese hamster ovary cells were cultured in 50-ml flasks (NUNC, Roskilde, Denmark) at 37°C in Minimum Essential Medium Alpha medium (GIBCO; Invitrogen, Carlsbad, CA) with 10% fetal calf serum (Biochrom, Berlin, Germany), 100 U/ml penicillin, and 100 mg/ml streptomycin (GIBCO) in a humidified atmosphere (5% CO2). Cells were subcultured in 35-mm-diameter monodishes at least 1 day before transfection.

Molecular Biology and Transfection of Cells
The mutant Kv4.3V401I was created by site-directed mutagenesis. All constructs were cloned in the pcDNA3 expression vector. Chinese hamster ovary cells were transiently transfected with 0.1 µg Kv4.3wt (GenBank No. NM 004980) or Kv4.3V401I plasmid DNA, 1.0 µg KChIP2.2 plasmid DNA (GenBank No. NM 173191), 0.3 µg EFGP cDNA, and 3 µl lipofectamine reagent (GIBCO) per dish according to the manufacturer’s protocol. Cells were cotransfected with an EGFP pcDNA3 construct to verify successful transfection. Only green fluorescing cells were used for patch clamp experiments. Patch clamp experiments were performed at room temperature 1–2 days after transfection.

Electrophysiology of Potassium Channels
Whole cell currents were measured with the patch clamp method20 using an EPC-9 amplifier (HEKA Elektronik, Lambrecht, Germany) and Pulse software version 8.11 (HEKA Elektronik). Patch electrodes with a pipette resistance between 2.0 and 4.0 MΩ were pulled from borosilicate glass capillary tubes (World Precision Instruments, Saratoga, FL) and filled with the following solution: 160 mM KCl, 0.5 mM MgCl2, 10 mM HEPES, 2 mM Na-ATP (all from Sigma, Deisenhofen, Germany); pH 7.2 (adjusted with KOH). The external solution applied on the cells was of the following composition: 135 mM NaCl, 5 mM KCl, 2 mM CaCl2, 2 mM MgCl2, 5 mM HEPES, 10 mM sucrose, 0.1 mg/ml Phenol red (all from Sigma); pH 7.4 (adjusted with NaOH). The capacity of the cells was 29.8 ± 16.1 pF (n = 20). Series resistance was 2.5–5 MΩ and actively compensated for by at least 80%. A p/4 leak subtraction protocol was used in the study. Bupivacaine (Sigma), levobupivacaine (AstraZeneca, Södertälje, Sweden), and dextrobupivacaine (AstraZeneca) were dissolved in the extracellular solution. The drugs were superfused on the cells by a hydrostatically driven perfusion system.

The holding potential of the cell membrane was −80 mV, and Kv4.3 channels were activated by different protocols. For channel stimulation by the rectangle pulse protocol, the membrane was hyperpolarized for 200 ms to −100 mV and subsequently depolarized to +40 mV for 1,000 ms. Current activation was analyzed by depolarization of the membrane potential for 1,000 ms from −40 to +60 mV in 10-mV steps. Steady state inactivation was measured by depolarizing the membrane to +40 mV for 25 ms from prepulses of a duration of 4,000 ms increasing from −75 and −15 mV in steps of 5 mV. For measuring the recovery from inactivation, the initial depolarization of the membrane potential to +40 mV for 1,500 ms was followed by depolarizing the membrane potential to −80 mV for different duration of time increasing from 10 to 5,120 ms by doubling the time spent at −80 mV (Δt) after each individual depolarization. The repolarization was followed by a depolarizing step of 25 ms to +40 mV. Experiments were performed at room temperature. The recorded signal was filtered at 2 kHz and stored on a personal computer for later analysis with a sampling rate of up to 20 kHz.

Data Analysis
The current–time relation observed with the different protocols for potassium channel activation was used to determine the maximal outward current (I max) as well as to quantify the charge (Q) crossing the membrane during the duration of the depolarization induced by the rectangle protocol. The charge crossing the membrane is equivalent to the time integrals of current traces and was determined using Pulse software version 8.11 (HEKA Elektronik). Inhibition was quantified by the ratio of time integrals of current traces in the presence of the drug to the mean of the time integrals before application of the drug and after washout of the drug (inhibition Q = 1 − Q drug/(Q control + Q washout)/2). The concentration–response curves were fitted with the Hill equation c/e max = 1/(1 + (IC 50/c)Hill coefficient, and IC 50 = concentration of half-maximal effect). Regression analysis was performed using Kaleidagraph (Synergy Software, Reading, PA). The whole cell conductance G max was calculated using the following formula: G max = I max/(V m − E k), where I max is the maximum current during each test potential, V m is the membrane potential, and E k is the Nernst potential for potassium (−87.54 mV under our experimental conditions). The means of the whole cell conductance were mathematically described by a Boltzmann function G = G max/[1 + exp((V m − V 0.5)/k)], where G max is the maximal whole cell conductance, V 0.5 is the voltage of half-maximal activation, V m is the membrane potential, and k is the slope factor, using Kaleidagraph software. Time-dependent inactivation was mathematically described using a biexponential function y = C + A 1 exp(−t/τ 1) + A 2 exp(−t/τ 2), where τ 1 and τ 2 are the system time constants, A 1 and A 2 are the amplitudes.
of each component of the exponential, and C is the baseline value. Recovery from inactivation was determined by relating the maximal size of the outward current during the test pulse to the size of maximal outward current during the prepulse. The time-dependent increase of this ratio was mathematically described with a monoexponential or biexponential function as described above. The time course of the development of block was analyzed using the following equation: \( y = y_{control} - I_{drug}/I_{control} \), where \( I_{control} \) is the current at any given time under control conditions and \( I_{drug} \) is the current at the same time under drug conditions. Data points are presented as mean ± SD unless stated otherwise. Statistical significance was tested using analysis of variance and the Tukey-Kramer multiple comparisons test (Graph Pad; Prism, San Diego, CA) or two-sided paired and unpaired Student t tests as appropriate (Excel; Microsoft, Redmond, WA). A value of \( P \leq 0.05 \) was regarded as significant. The number n is the number of experiments.

Results

\( \text{Kv4.3}_{\text{wt}}/\text{KChIP2.2} \) currents activated with a midpoint of 0 ± 4 mV (n = 10) and were fully activated at a potential of +40 mV. Therefore, a rectangle protocol with a depolarization to +40 mV was chosen to test the influence of bupivacaine and levobupivacaine on the channel complexes. The rectangle protocol evoked a rapidly activating and inactivating A-type current (fig. 1A). Bupivacaine (30 \( \mu \)M) had two effects on the \( \text{Kv4.3}_{\text{wt}}/\text{KChIP2.2} \) current. It reduced the current, and it accelerated macroscopic current decline in a such way that a crossing phenomenon occurred upon overlay of control and drug traces. Both effects were reversible upon washout (fig. 1A). The current decline of \( \text{Kv4.3}_{\text{wt}}/\text{KChIP2.2} \) currents was described using two time constants. Bupivacaine, 30 \( \mu \)M, reduced the fast time constant \( \tau_1 \) from 35 ± 14 ms (n = 9) to 10 ± 3 ms (n = 5; \( P < 0.01 \)), and it increased the slow time constant \( \tau_2 \) from 96 ± 44 ms (n = 9) to 147 ± 37 ms (n = 5; \( P < 0.05 \)). The ratio of the amplitudes of the time constants, amplitude 1 and amplitude 2, was changed by bupivacaine from 62 ± 14% for amplitude 1 and 38 ± 14% for amplitude 2 under control and washout conditions to 80 ± 5% for amplitude 1 and 20 ± 5% for amplitude 2 when 30 \( \mu \)M bupivacaine was applied (n = 5; \( P < 0.05 \)). This resulted in the crossing phenomenon. Consequently, bupivacaine action on the channel complex was inhibitory before the crossing but stimulatory after the crossing of currents.

Figure 1B shows the effect of different concentrations of bupivacaine on the \( \text{Kv4.3}_{\text{wt}}/\text{KChIP2.2} \) channel complex. The reduction of current and the change of current shape were concentration dependent. The inhibition of \( \text{Kv4.3}_{\text{wt}}/\text{KChIP2.2} \) channels was quantified as reduction of the charge transfer only until the crossing of currents was reached (Q_{cross}). These two parameters of inhibition were plotted versus the concentration and fitted using Hill equations (n = 3–10 for each concentration). (D) The local anesthetic accelerated the fast time constant \( \tau_1 \) of \( \text{Kv4.3}_{\text{wt}}/\text{KChIP2.2} \) inactivation. This effect was quantified as inhibition of \( \tau_1 \) (see Materials and Methods), and the concentration–response curve of inhibition of \( \tau_1 \) was fitted with a Hill equation (n = 3–10 for each concentration).
Table 1. Parameters of Hill Functions

<table>
<thead>
<tr>
<th></th>
<th>Inhibition Q Bupivacaine</th>
<th>Inhibition Q Levobupivacaine</th>
<th>Inhibition Q cross Bupivacaine</th>
<th>Inhibition Q cross Levobupivacaine</th>
<th>Acceleration τ1 Bupivacaine</th>
<th>Acceleration τ1 Levobupivacaine</th>
</tr>
</thead>
<tbody>
<tr>
<td>e\textsubscript{max} (%)</td>
<td>101 ± 5</td>
<td>93 ± 3</td>
<td>99 ± 7</td>
<td>93 ± 2</td>
<td>100 ± 9</td>
<td>93 ± 1</td>
</tr>
<tr>
<td>Hill coefficient</td>
<td>1.02 ± 0.12</td>
<td>1.31 ± 0.14</td>
<td>0.88 ± 0.18</td>
<td>1.01 ± 0.08</td>
<td>0.68 ± 0.24</td>
<td>1.09 ± 0.06</td>
</tr>
<tr>
<td>IC\textsubscript{50} (\mu M)</td>
<td>55.0 ± 8.2</td>
<td>50.4 ± 5.1</td>
<td>29.7 ± 6.9</td>
<td>25.8 ± 2.0</td>
<td>17.2 ± 5.8</td>
<td>16.6 ± 0.7</td>
</tr>
<tr>
<td>n</td>
<td>20</td>
<td>26</td>
<td>24</td>
<td>28</td>
<td>26</td>
<td>29</td>
</tr>
</tbody>
</table>

Parameters of the Hill function describing concentration-dependent inhibition of charge (Q), charge until crossing (Q\textsubscript{cross}), and fast time constant of current delay (\(\tau_1\)) of Kv4.3/KChIP2.2 channels by bupivacaine and levobupivacaine. Data are presented as mean ± SEM.

IC\textsubscript{50} values for inhibition of Q, and Q\textsubscript{cross} by bupivacaine were 55 ± 8 \(\mu\)M (\(n = 20\)) and 30 ± 7 \(\mu\)M (\(n = 24\)), respectively (table 1). The acceleration of macroscopic current decline was concentration-dependent as well, and the time until the crossing occurred increased with concentration (fig. 1B). The Hill function describing the concentration–response data for reduction of \(\tau_1\) yielded an IC\textsubscript{50} value of 17 ± 6 \(\mu\)M (\(n = 26\)) (fig. 1D and table 1). Although \(\tau_2\) was increased by 30 \(\mu\)M bupivacaine, it was impossible to be analyzed under higher drug concentrations (300 \(\mu\)M and 1 mM) because of the small size of the residual current. Levobupivacaine had similar effects on Kv4.3\textsubscript{wt}/KChIP2.2. The IC\textsubscript{50} values for inhibition of Q, Q\textsubscript{cross}, and reduction of \(\tau_1\) by levobupivacaine were 50 ± 5 \(\mu\)M (\(n = 26\)), 26 ± 2 \(\mu\)M (\(n = 28\)), and 17 ± 1 \(\mu\)M (\(n = 29\)), respectively (table 1). There was no significant difference in any parameter of Kv4.3\textsubscript{wt}/KChIP2.2 inhibition by racemic bupivacaine and by levobupivacaine (\(P > 0.05\)).

To further analyze the possible stereospecific effect of bupivacaine, paired tests were performed with the pure enantiomers \((−)\)-levobupivacaine and \((+)-dextrobupivacaine at a concentration of 30 \(\mu\)M. This concentration was chosen because it was near the IC\textsubscript{50} value for the inhibition of Q and hence at the steepest part of the concentration–response curve. A gentle difference in potency between the enantiomers should best be detectable at this part of the concentration–response curve. Levobupivacaine and dextrobupivacaine inhibited Q at 30 \(\mu\)M with nearly identical potencies (levobupivacaine: 51.4 ± 1%, dextrobupivacaine: 50.5 ± 1%; \(n = 11\); \(P > 0.05\)). Therefore, only racemic bupivacaine was used in the following experiments.

To establish the effect of bupivacaine on Kv4.3\textsubscript{wt}/KChIP2.2 channel gating, the effects of 100 \(\mu\)M bupivacaine on voltage-dependent activation, steady state inactivation, and recovery from inactivation were investigated (figs. 2 and 3). Bupivacaine affected voltage-dependent activation as well as recovery from inactivation without changing steady state inactivation. Figure 2 shows original current traces elicited by the activation protocol (fig. 2A), the inactivation protocol (fig. 2B), and the recovery protocol (fig. 2C). Application of 100 \(\mu\)M bupivacaine reduced the current during every protocol. This effect was always reversible upon washout. Bupivacaine (100 \(\mu\)M) modulated the voltage dependence of activation by shifting the midpoint of current activation in the depolarizing direction by 10 ± 4 mV (\(n = 5\); \(P < 0.05\)). The slope of the activation curve was increased by 2 ± 1 mV (\(n = 5\); \(P < 0.05\); fig. 3A and table 2). The

![Fig. 2. Original current traces demonstrating bupivacaine effects on different Kv4.3\textsubscript{wt}/KChIP2.2 gating parameters. The influence of bupivacaine on activation (A), inactivation (B), and recovery from inactivation (C) was studied using the appropriate pulse protocols depicted as an inset next to the traces. Shown are currents under control conditions, after application of 100 \(\mu\)M bupivacaine, and after the washout of the substance. All currents were reduced by bupivacaine.](http://anesthesiology.pubs.asahq.org/pdfaccess.ashx?url=/data/journals/jasa/931080/ on 10/14/2018)
Voltage dependence of inactivation was not altered by 100 μM bupivacaine (fig. 3B). However, the local anesthetic slowed the recovery from inactivation (fig. 3C). Under control and washout conditions, the time dependence of the recovery from inactivation was adequately fitted with one time constant (τ_rec 57 ± 7 ms; n = 6). Application of 100 μM bupivacaine altered Kv4.3/KChIP2.2 gating in such a way that two time constants were necessary to adequately describe the time dependence of recovery (τ_rec1 = 42 ± 6 ms, τ_rec2 = 2,339 ± 728 ms; n = 6). Both time constants differed significantly from the time constant under control and washout conditions (P < 0.05; table 2).

Table 2. Effect of Bupivacaine on Channel Gating

<table>
<thead>
<tr>
<th></th>
<th>Control/Washout</th>
<th>100 μM Bupivacaine</th>
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<tbody>
<tr>
<td><strong>Activation</strong></td>
<td></td>
<td></td>
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<tr>
<td>V_0.5 mV</td>
<td>-0.1 ± 3.8</td>
<td>9.6 ± 2.0</td>
</tr>
<tr>
<td>Slope factor, mV</td>
<td>12.4 ± 2.6</td>
<td>14.6 ± 1.6</td>
</tr>
<tr>
<td>G_max %</td>
<td>96.9 ± 1.9</td>
<td>100.4 ± 0.9</td>
</tr>
<tr>
<td>n</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td><strong>Voltage-dependent inactivation</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>V_0.5 mV</td>
<td>-44.4 ± 2.7</td>
<td>-44.1 ± 3.0</td>
</tr>
<tr>
<td>Slope factor, mV</td>
<td>-3.4 ± 0.3</td>
<td>-3.7 ± 0.3</td>
</tr>
<tr>
<td>G_max %</td>
<td>101.0 ± 1.2</td>
<td>100.0 ± 0.8</td>
</tr>
<tr>
<td>n</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td><strong>Recovery from inactivation</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>τ_rec1, ms</td>
<td>57.2 ± 7.0</td>
<td>41.7 ± 5.6</td>
</tr>
<tr>
<td>τ_rec2, ms</td>
<td>2,338.5 ± 728.1</td>
<td></td>
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<tr>
<td>n</td>
<td>6</td>
<td>6</td>
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</tbody>
</table>

Bupivacaine (100 μM) induced effects on the voltage dependence of activation, voltage dependence of inactivation, and recovery from inactivation of Kv4.3/KChIP2.2. Data are presented as mean ± SD. V_0.5 = voltage of half-maximal activation; G_max = maximal conductance; n = number of experiments; τ_rec = time constant of recovery.

Voltage dependence of Kv4.3/KChIP2.2 block was characterized first. Figures 3A-D show the voltage dependence of Kv4.3 wt/KChIP2.2 activation to more depolarized potentials and increased the slope factor, which resulted in a steeper rise of the conductance–voltage curve under bupivacaine than under control and washout conditions. (B) The voltage dependence of channel inactivation was not altered by bupivacaine (100 μM). (C) The recovery from inactivation was slowed by bupivacaine (100 μM). Under control and washout conditions, one time constant was sufficient to describe the conductance–time relation of recovery from inactivation adequately, but two time constants were necessary to describe this curve when bupivacaine was applied (see also table 2). (D) Inhibition of Q increased with voltage in the range from -10 to +40 mV. (E) The fast time constant of inactivation was voltage dependent under control and washout as well as under drug conditions. (F) The distribution of the amplitudes was voltage dependent under control and washout conditions. At 0 mV, the amplitude of the fast time constant of inactivation (amplitude 1) was much larger than amplitude 2. Toward more positive potentials, the curves for both amplitudes were converging. This voltage dependence was reversed by 100 μM bupivacaine, with amplitude disparity becoming more marked with voltage.

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Table 3. Comparison of Current Decay between Kv4.3wt and Kv4.3V401I

<table>
<thead>
<tr>
<th></th>
<th>Kv4.3wt</th>
<th>Kv4.3wt/KChIP2.2</th>
<th>Kv4.3V401I</th>
<th>Kv4.3V401I/KChIP2.2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$\tau_1$, ms</td>
<td>20±2</td>
<td>35±14</td>
<td>33±6</td>
<td>33±9</td>
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<tr>
<td>$\tau_2$, ms</td>
<td>130±25</td>
<td>96±44</td>
<td>293±48</td>
<td>196±49</td>
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<tr>
<td>Amplitude 1, %</td>
<td>84±6</td>
<td>61±13</td>
<td>83±4</td>
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<tr>
<td>Amplitude 2, %</td>
<td>16±6</td>
<td>39±13</td>
<td>17±4</td>
<td>15±5</td>
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<tr>
<td>n</td>
<td>10</td>
<td>9</td>
<td>7</td>
<td>9</td>
</tr>
<tr>
<td>30 $\mu$M bupivacaine</td>
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</tr>
<tr>
<td>$\tau_1$, ms</td>
<td>9±2</td>
<td>10±3</td>
<td>22±6</td>
<td>9±2</td>
</tr>
<tr>
<td>$\tau_2$, ms</td>
<td>90±14</td>
<td>147±37</td>
<td>238±53</td>
<td>139±47</td>
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<tr>
<td>Amplitude 1, %</td>
<td>79±6</td>
<td>80±5</td>
<td>75±6</td>
<td>83±3</td>
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<tr>
<td>Amplitude 2, %</td>
<td>21±6</td>
<td>20±5</td>
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<tr>
<td>n</td>
<td>10</td>
<td>5</td>
<td>7</td>
<td>9</td>
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</table>

Time constants ($\tau$) and amplitudes for biexponential fit of current decay of Kv4.3 channels under control conditions and under bupivacaine (30 $\mu$M). Data are presented as mean ± SD.

n = number of experiments.
the β subunit as well as with inhibition of the pore mutant Kv4.3V401I expressed with and without KChIP2.2 (figs. 5A–C). Bupivacaine (30 μM) inhibited Kv4.3wt, Kv4.3V401I, and Kv4.3V401I/KChIP2.2 channels (figs. 5A–C). However, in contrast to Kv4.3wt/KChIP2.2, bupivacaine did not induce a crossing phenomenon in Kv4.3wt, Kv4.3V401I, or Kv4.3V401I/KChIP2.2 channels (compare figs. 5A–C with fig. 1A). As a consequence, inhibition (Q) of Kv4.3wt/KChIP2.2 was significantly less than inhibition (Q) of Kv4.3wt (36 ± 6% for Kv4.3wt/KChIP2.2, n = 5, vs. 54 ± 5% for Kv4.3wt, n = 10; P < 0.01; fig. 5D). The reduction of bupivacaine sensitivity of Kv4.3wt/KChIP2.2 was not observed with the pore mutant Kv4.3V401I. Inhibition (Q) of Kv4.3V401I amounted to 53 ± 9% (n = 6) in the absence of KChIP2.2, and it was 50 ± 7% (n = 7) in the presence of KChIP2.2 (P > 0.05; fig. 5D). Inhibition of Kv4.3V401I and Kv4.3wt channels did not significantly differ (P > 0.05; fig. 5D). KChIP2.2 slowed the development of block by bupivacaine of Kv4.3wt channels but not of Kv4.3V401I channels. The time constant for the development of Kv4.3wt block was 3 ± 1 ms (n = 6), 6 ± 1 ms for Kv4.3wt/KChIP2.2 (n = 5), 4 ± 1 ms for Kv4.3V401I (n = 6), and 4 ± 1 ms for Kv4.3V401I/KChIP2.2 (n = 8). Block of both Kv4.3V401I and Kv4.3V401I/KChIP2.2 channels developed significantly slower than block of Kv4.3wt/KChIP2.2 channels (P < 0.01).

**Discussion**

This study established the effects of the local anesthetic bupivacaine on Kv4.3wt/KChIP2.2 and Kv4.3V401I/KChIP2.2 channel complexes expressed in Chinese hamster ovary cells. The biophysical properties of the wild-type ion channel complex were in accord with biophysical properties of Kv4/KChIP2 channel complexes reported previously. The local anesthetics bupivacaine and levobupivacaine reduced Kv4.3wt and Kv4.3V401I/KChIP2.2 currents in a concentration-dependent and reversible manner. Both drugs exhibited the same effects on Kv4.3wt/KChIP2.2 and did not differ with regard to inhibitory potency. Bupivacaine caused a depolarizing shift of voltage-dependent current activation. The local anesthetic decreased the time constants of macroscopic current decline, slowed recovery from channel inactivation, and induced a crossing phenomenon of currents.

Acceleration of macroscopic current decline is a characteristic feature of open channel block. This mechanism has already been described previously for the inhibition of several Kv α subunits, including Kv4.3 by bupivacaine. Inhibition of Kv1.5 channels by bupivacaine is mediated by threonine 477 (current number according to GenBank No. NM 002234: T477) within the tetraethylammonium binding domain in the pore region of Kv1.5 channels and also by threonine 505, lysine 508, and valine 512 in the S6 segment of Kv1.5 channels (current numbers according to GenBank No. NM 002234: T505, K510, V514). These amino acids are also important for the stereoselective inhibition of Kv1.5 channels by bupivacaine. Mutation of T505 to valine effectively reduces stereoselectivity. The corresponding position in Kv4.3 channels is valine 394. Therefore, the results of our study are in accord with the previous mutagenesis studies on Kv1.5, as well as with results obtained with native Igα. However, in contrast to bupivacaine, ropivacaine interacted with Kv4.3/KChIP2.2 channels in a stereoselective manner. The lipophilicity of these local anesthetics may therefore be inversely related to their ability of stereospecific molecular inter-
action with Kv4.3/KChIP2.2 channels. Further study is needed to finally resolve this issue.

The crossing of currents under drug condition with the currents under drug free condition may be explained by a reversal of the effects of KChIP2.2 on Kv4.3 wt channel gating. A possible interaction of bupivacaine with KChIP2.2 may also explain why the block of Kv4.3 wt/KChIP2.2 developed significantly slower than block of Kv4.3 wt channels. Because KChIP2.2 changes the inhibition of Kv4.3 wt channels by the open pore blocker bupivacaine, it could be further hypothesized that KChIP2.2 also binds to the pore region. However, this seems less likely for several reasons.

It is well established that the N-terminus is crucial for KChIP interaction with Kv4 channels. By impairing the movement of the N-termini to the internal mouth of the pore, KChIPs reduce N-type inactivation from the open state and increase closed state inactivation, resulting from conformational changes at the internal vestibule of the pore (V-type inactivation). This effect of KChIP can be antagonized by intracellular application of an N-terminal peptide. As these changes in channel gating occur without a direct interaction of KChIP with the ion channel pore, sterical interaction of the pore blocker bupivacaine and KChIP2.2 is unlikely to occur. This view is supported by the results obtained with the Kv4.3 pore mutant V401I. The mutation V401I slows macroscopic current decline of Kv4.3/KChIP2.2 channel complexes as well as recovery from inactivation. As the current density of both Kv4.3 wt and Kv4.3 V401I channels is increased by coexpression with KChIP2.2, binding of KChIP2.2 to Kv4.3 V401I channels does not seem impaired. In addition, the voltage dependence of activation and inactivation of Kv4.3 V401I is very similar to that of Kv4.3 wt. Hence, the gating properties of the channel and of the channel complex with KChIP2.2 are only marginally influenced by this mutation, making it particularly suitable for pharmacologic studies. In contrast to Kv4.3 wt channels, inhibition of Kv4.3 V401I channels by bupivacaine is not reduced by coexpression with KChIP2.2, and bupivacaine does not induce a crossing of inhibited currents with control currents. Furthermore, the onset of block of Kv4.3 V401I is not altered by coexpression with KChIP2.2. The lack of a crossing phenomenon may be explained by different inactivation time constants of Kv4.3 wt/KChIP2.2 and Kv4.3 V401I/KChIP2.2. \( \tau_1 \) of current decline is significantly decreased by the local anesthetic in all channels investigated, whereas \( \tau_2 \) is significantly increased by bupivacaine only in Kv4.3 wt/KChIP2.2 complexes. When comparing the absolute values of the time constants of current decline and their amplitudes (table 3), it becomes obvious that despite different inactivation time constants under control conditions, both \( \tau_1 \) and \( \tau_2 \) of Kv4.3 wt/KChIP2.2 and Kv4.3 V401I/KChIP2.2 current decline behave in exactly the same way under the influence of bupivacaine. The reduction of Kv4.3 inhibition caused by coexpression with KChIP2.2, therefore, is a consequence of the relatively small \( \tau_2 \) value of Kv4.3 wt/KChIP2.2 under control conditions. Because KChIP2.2 binds to both Kv4.3 wt as well as Kv4.3 V401I, the interaction of bupivacaine with the ion channel pore is unlikely to be altered by direct interaction of the local anesthetic with KChIP2.2.

The results of this study add further evidence to the model suggested previously to describe the interference of effects of amino-amide local anesthetics and KChIP subunits while acting on Kv4.3 channels. Kv4.3 channels inactivate from a preopen closed state by a mechanism involving conformational changes at the internal vestibule of the pore, as well as from the open state by a mechanism involving the N-termini. The interaction of KChIP with the N-termini impairs inactivation from the open state by preventing the channel’s N-termini to interact with the internal mouth of the pore. Consequently, inactivation from the preopen closed state predominates in complexes formed by Kv4 and KChIP subunits. By interacting with the channel pore, bupivacaine introduces a blocked open state of Kv4.3/KChIP2.2 channels reminiscent of N-type inactivation. Bupivacaine thus reverses KChIP2.2 effects on Kv4.3 channels by mimicking the interaction of the N-termini of the \( \alpha \) subunit with its ion channel pore.

In summary, the amino-amide local anesthetic bupivacaine inhibited complexes formed by Kv4.3/KChIP2.2 in a concentration-dependent and reversible manner. The results of our study are consistent with the idea that bupivacaine blocks Kv4.3/KChIP2.2 channels from the open state. By influencing channel gating, KChIP2.2 indirectly altered the response of Kv4.3 channels to bupivacaine.

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References


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