

# Membrane permeable local anesthetics modulate $\text{Na}_v1.5$ mechanosensitivity

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**Keywords:** voltage-gated, sodium channel, ion channel, stretch, mechanosensitive, pressure, lidocaine, benzocaine, QX-314

Voltage-gated sodium selective ion channel  $\text{Na}_v1.5$  is expressed in the heart and the gastrointestinal tract, which are mechanically active organs.  $\text{Na}_v1.5$  is mechanosensitive at stimuli that gate other mechanosensitive ion channels. Local anesthetic and antiarrhythmic drugs act upon  $\text{Na}_v1.5$  to modulate activity by multiple mechanisms. This study examined whether  $\text{Na}_v1.5$  mechanosensitivity is modulated by local anesthetics.  $\text{Na}_v1.5$  channels were expressed in HEK-293 cells, and mechanosensitivity was tested in cell-attached and excised inside-out configurations. Using a novel protocol with paired voltage ladders and short pressure pulses, negative patch pressure (-30 mmHg) in both configurations produced a hyperpolarizing shift in the half-point of the voltage-dependence of activation ( $V_{1/2a}$ ) and inactivation ( $V_{1/2i}$ ) by about -10 mV. Lidocaine (50  $\mu\text{M}$ ) inhibited pressure-induced shift of  $V_{1/2a}$  but not  $V_{1/2i}$ . Lidocaine inhibited the tonic increase in pressure-induced peak current in a use-dependence protocol, but it did not otherwise affect use-dependent block. The local anesthetic benzocaine, which does not show use-dependent block, also effectively blocked a pressure-induced shift in  $V_{1/2a}$ . Lidocaine inhibited mechanosensitivity in  $\text{Na}_v1.5$  at the local anesthetic binding site mutated (F1760A). However, a membrane impermeable lidocaine analog QX-314 did not affect mechanosensitivity of F1760A  $\text{Na}_v1.5$  when applied from either side of the membrane. These data suggest that the mechanism of lidocaine inhibition of the pressure-induced shift in the half-point of voltage-dependence of activation is separate from the mechanisms of use-dependent block. Modulation of  $\text{Na}_v1.5$  mechanosensitivity by the membrane permeable local anesthetics may require hydrophobic access and may involve membrane-protein interactions.

## Introduction

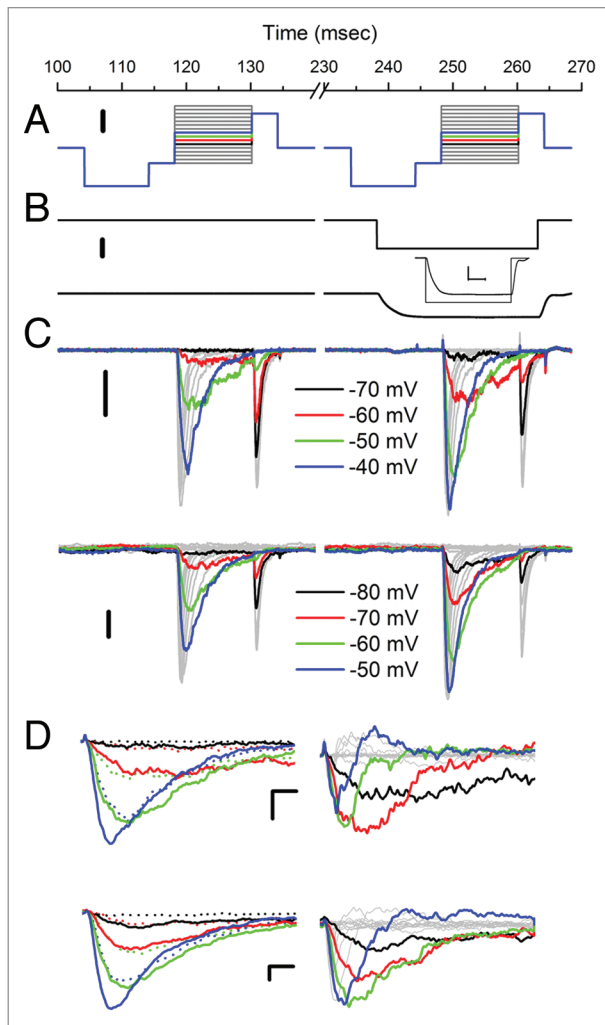
Voltage-gated sodium selective ion channel  $\text{Na}_v1.5$  is mechanosensitive.  $\text{Na}_v1.5$  is found in two mechanically active organs—the heart<sup>1</sup> and the gastrointestinal (GI) tract.<sup>2</sup> In cardiac myocytes  $\text{Na}_v1.5$  is responsible for the depolarizing upstroke of the action potential.<sup>1</sup> Mutations in  $\text{Na}_v1.5$  are clearly pathogenic in the heart, as exemplified by disorders such as long QT type 3 (LQT3).<sup>3</sup> In the human GI tract  $\text{Na}_v1.5$  is found in smooth muscle cells and interstitial cells of Cajal.<sup>4,5</sup> Sodium currents in the gastrointestinal tract contribute to setting the resting potential and affect the upstroke and frequency of the slow waves,<sup>6</sup> which are coordinating electrical events in the GI tract required for cyclical contractions. Mutations in  $\text{Na}_v1.5$  also result in GI pathologies.<sup>7,8</sup> In addition to being voltage-sensitive,  $\text{Na}_v1.5$  is also mechanically sensitive.<sup>6,9,10</sup> Recent evidence suggests that some disease-associated  $\text{Na}_v1.5$  mutations, both in the heart<sup>11</sup> and in the gut,<sup>8</sup> have abnormal mechanical sensitivity. Therefore, the mechanism of  $\text{Na}_v1.5$  mechanosensitivity is of scientific and clinical interest.

$\text{Na}_v1.5$  mechanosensitivity is established in both cell-attached patches and whole-cell.  $\text{Na}_v1.5$  is sensitive to mechanical stimulation in the whole-cell mode to shear-stress<sup>6</sup> and in cell-attached patches to pressure.<sup>9,10</sup> In both whole cell and

cell-attached modes, mechanical stimulation of  $\text{Na}_v1.5$  results in an increase in peak inward current<sup>8</sup> and acceleration of the voltage-dependent activation and inactivation kinetics.<sup>9,10</sup> In cell-attached membrane patches in HEK cells, negative pressure additionally hyperpolarizes the half-point of voltage-dependence of activation and inactivation by the same linear constant,  $\sim 0.7$  mV/mmHg.<sup>10</sup> The mechanisms of  $\text{Na}_v1.5$  mechanosensitivity are unclear. Whole-cell studies demonstrate that shear-sensitivity requires an intact f-actin cytoskeleton as f-actin disassembly or disruption of syntrophin, which likely links  $\text{Na}_v1.5$  channels with actin result in loss of shear-sensitivity.<sup>12,13</sup> Previous studies with other  $\text{Na}_v$  channels ( $\text{Na}_v1.4$  and  $\text{Na}_v1.6$ ) in patches have also demonstrated a requirement of the cytoskeleton for mechanosensitivity.<sup>14-16</sup> Patch excision resulted in an irreversible hyperpolarizing shift in the voltage-dependence and kinetic properties.<sup>14,16</sup> It is unclear from these studies whether the  $\text{Na}_v1.5$   $\alpha$ -subunit is mechanosensitive as a stand-alone unit.

**Mechanism and pharmacology of  $\text{Na}_v1.5$  mechanosensitivity is unclear.** The mechanism of voltage-gated ion channel mechanosensitivity independent of the cytoskeleton is unclear. Previous studies suggest that amphiphiles alter mechanosensitivity of voltage-gated sodium channels,<sup>17</sup> but their specificity is unclear.<sup>18</sup> Further, no pharmacologic tools exist that specifically address mechanosensitivity of voltage-gated ion channels, and

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Submitted: 05/07/12; Revised: 06/14/12; Accepted: 06/20/12  
<http://dx.doi.org/10.4161/chan.21202>



**Figure 1.**  $\text{Na}_v1.5$  is reversibly pressure-sensitive in cell-attached and inside-out patches. **(A)** Voltage-ladder protocol includes a pre-pulse to  $-200$  mV for 10 msec to accelerate recovery from inactivation. Patches are stepped from  $-140$  to  $+10$  mV in 10 mV increments to test activation and then a step to  $-10$  mV to test inactivation. Scale bar is 50 mV. **(B)** Pressure step to  $-35$  mmHg was applied during the second voltage ladder. Scale bar is 20 mmHg. Lower trace shows pressure at the patch in response to a  $-30$  mmHg step. Inset shows that a  $-35$  mmHg applied pressure results in a  $-30$  mmHg pressure at the patch after a 5 msec risetime. **(C)** Voltage-dependent currents in a cell-attached (upper) and inside-out patches (lower) at rest (0 mmHg, first ladder) and during pressure stimulation ( $-30$  mmHg, second ladder). Color highlighted steps demonstrate typical differences (larger currents, faster kinetics, greater inactivation) between 0 mmHg and  $-30$  mmHg at multiple voltages spanning the steep voltage-dependent activation. Scale bar is 20 pA for upper trace and 50 pA for lower trace. **(D)** On the left voltage-dependent currents highlighted in **(C)** overlaid for 0 mmHg (dotted traces) and  $-30$  mmHg (solid traces) for cell-attached (top) and inside-out (bottom) patches. These traces show larger currents and faster kinetics with pressure. On the right are difference currents  $I_{\text{diff}} = I_{-30\text{mmHg}} - I_{0\text{mmHg}}$  for cell-attached (top) and inside-out (bottom) patches. Pressure-induced changes in the voltage-dependent currents for cell-attached and inside-out patches are very similar. Scale bars 20 pA and 1 msec for cell-attached (upper) traces and 50 pA and 1 msec for inside-out (lower) traces.

there is little knowledge on the impact of voltage-gated ion channel drugs on their mechanosensitivity. Our recent work showed that lidocaine and a piperazine derivative ranolazine, a molecule similar to lidocaine and with similar mechanism of  $\text{Na}_v1.5$  block,<sup>19</sup> both inhibit  $\text{Na}_v1.5$  mechanosensitivity.<sup>20</sup> We showed in cardiac myocytes and HEK cells heterologously expressing  $\text{Na}_v1.5$  that ranolazine and lidocaine inhibited mechanosensitivity to shear stress in whole cells and patch pressure in cell-attached patches. Initial evaluation suggested that block of mechanosensitivity by ranolazine was similar to lidocaine, and if the molecules were charged they were ineffective blockers of  $\text{Na}_v1.5$  mechanosensitivity when applied from the outside. This work is limited by the availability of the extracellular face of the membrane to pharmacologic manipulation. Our aim here was to thoroughly test the extracellular and intracellular facing membrane responses to modulation by local anesthetics.

**Reversible  $\text{Na}_v1.5$  mechanosensitivity in excised patches has not been shown.** Direct examination of ion channel mechanosensitivity requires fast (millisecond), reversible and reproducible responses that are independent of other factors.<sup>21</sup> The duration of mechanical stimulation is important in directly assessing mechanosensitivity. Previous work on  $\text{Na}_v1.5$  mechanosensitivity used pressure pulses spanning at the shortest hundreds of milliseconds to seconds,<sup>9,10</sup> which results in significant cell membrane restructuring<sup>22</sup> and allows the possibility of other mechanosensitive factors contributing to response. Shorter duration pulses are therefore needed. Further, excised patches allow testing of mechanosensitivity in an environment where cytoskeleton and other cytoplasmic factors are less important. However, of the voltage-gated ion channels studied to date only the *Shaker*  $\text{K}_v$  channel has been shown to be reversibly mechanosensitive in excised patches.<sup>23</sup>

In this work we demonstrate a robust method for studying reversible mechanosensitivity of  $\text{Na}_v1.5$  in excised patches. We test  $\text{Na}_v1.5$  mechanosensitivity of inside-out patches and compare results to the established behavior in cell-attached patches. We then demonstrate lidocaine modulation of  $\text{Na}_v1.5$  mechanosensitivity in excised patches and that the mechanism of lidocaine inhibition of the pressure-induced shift in the voltage-dependence of activation is separate from the mechanisms of use-dependent block.

## Results

### Reversible pressure-dependent response in membrane patches.

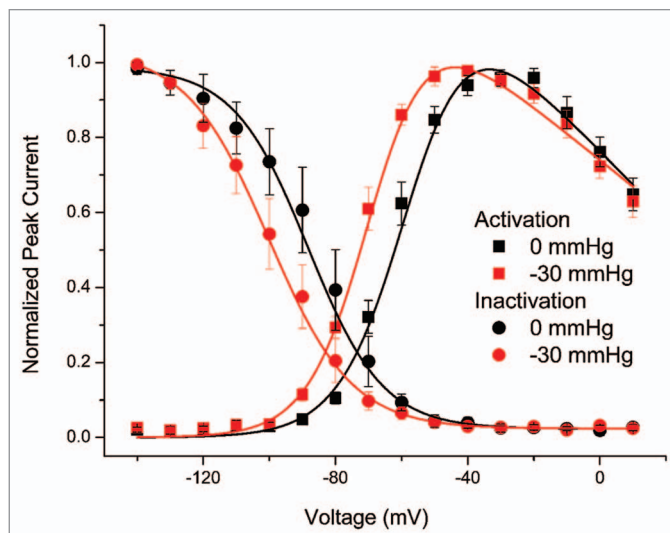
In this study we aimed to minimize membrane restructuring by using the shortest possible pressure pulses. In preliminary experiments for this study we determined that 25 msec pressure steps resulted in a sufficient stimulation and fully reversible behavior. We used a protocol with paired voltage ladders to test voltage-dependence of activation (steps from  $-140$  mV to  $+10$  mV) and voltage-dependence of inactivation (step to  $-10$  mV) (Fig. 1A). By using a 10 msec pre-pulse to  $-200$  mV we were able to accelerate recovery from inactivation which allowed us to obtain a 5-fold average of 16 voltage steps for both control and test without using inter-pulse delays. We tested this protocol extensively for fidelity.

In both cell-attached and inside-out patch configurations when no pressure was applied, the voltage-dependent currents were identical for the two ladders, meaning that complete recovery from fast-inactivation had taken place between steps (Fig. S1).

To test mechanosensitivity we used -30 mmHg patch pressure, and each voltage step was obtained first at 0 mmHg (rest) and then -30 mmHg stimulus (pressure) (Fig. 1A and B). In our system -35 mmHg of applied pressure translated to -30 mmHg pressure in the patch (Fig. 1B) with a rise-time of ~5 msec (Fig. 1B, inset). Thus, voltage was stepped 10 msec after the start of the pressure pulse. A reverse protocol in which the ladder at -30 mmHg preceded the one at 0 mmHg yielded consistent results with those we present below (Fig. S2).

Negative pressure altered the voltage-dependent currents as illustrated by records obtained from a representative patch in Figure 1C (upper cell-attached, lower inside-out). Qualitatively, no significant differences were noted between the currents in the cell-attached and inside-out configurations. In both sets negative pressure resulted in activation (first voltage step) at more negative voltages (black traces), larger peak currents (blue traces) and faster kinetics (red, green and blue traces) (Fig. 1C and D). Following activation, channel availability (second voltage step) was diminished more significantly with pressure (black, red and green traces) suggesting that the increase in peak current is not due to an increase in channel numbers. In the left panels of Figure 1D (top is cell-attached, bottom is inside-out) we overlay voltage-dependent activation currents for select voltage steps to demonstrate the increases in peak currents and kinetics of activation and inactivation for -30 mmHg (solid) vs. 0 mmHg (dot). In the right panels we show the resulting difference current, which is excess  $\text{Na}^+$  current when the patch is stretched ( $I_{\text{diff}} = I_{30\text{mmHg}} - I_{0\text{mmHg}}$ ). Again, no qualitative difference between cell-attached and inside-out patches was noted for difference currents. In both preparations there was an increase (downward) in difference current for all time points at more hyperpolarized activating voltages (black, red and green traces). For the more depolarized voltages (blue traces) there was a biphasic response—the large negative early peaks in the difference current demonstrated the accelerated kinetics of activation, while faster inactivation resulted in upward deflection in at later time points.

**Pressure hyperpolarizes  $\text{Na}_v1.5$  half-point of voltage-dependence of activation and inactivation in inside-out patches.** Previous work in cell-attached patches showed pressure-induced changes in  $\text{Na}_v1.5$  voltage-sensitivity.<sup>10,11</sup> In excised inside-out patches at rest (0 mmHg) voltage-dependent peak currents were fit to a two-state Boltzmann function with half-point of activation  $V_{1/2a}$  of  $-58.7 \pm 5.4$  mV, slope  $dV_a$  of  $8.8 \pm 1.6$  mV and  $G_{\text{max}}$  of  $1.7 \pm 1.8$  nS (range 0.27 to 5.85) ( $n = 9$ ) (Fig. 2, black squares). When these patches were stimulated by a -30 mmHg pulse  $V_{1/2a}$  was  $-68.8 \pm 6.2$  mV,  $dV_a$  was  $8.4 \pm 1.7$  mV and  $G_{\text{max}}$  was  $1.6 \pm 1.8$  nS (range 0.3 to 5.8 nS) ( $n = 7$ ) (Fig. 2, red squares). There was a significant difference between -30 mmHg and 0 mmHg in half-points  $\Delta V_{1/2a} = -10.0 \pm 1.5$  mV ( $p < 0.01$  by paired two-tailed t-test), while the changes in  $dV_a$  and  $G_{\text{max}}$  were not significant ( $n = 7$ ).



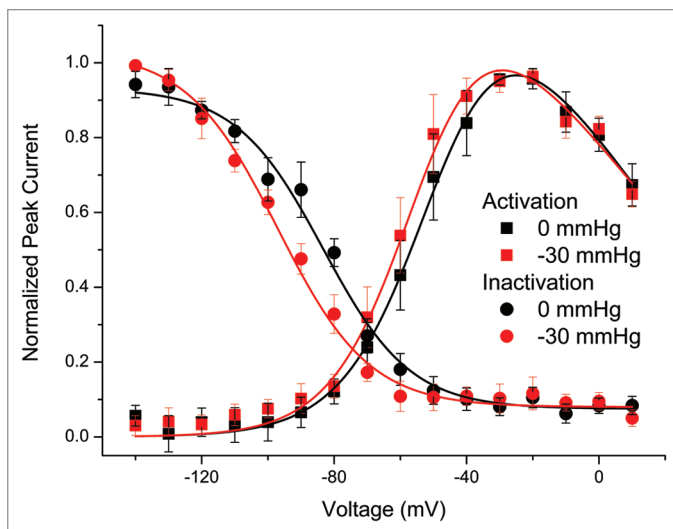
**Figure 2.** Negative pressure hyperpolarizes  $\text{Na}_v1.5$  voltage-dependence of activation ( $V_{1/2a}$ ) and inactivation ( $V_{1/2i}$ ). Shown here are voltage-dependent current vs. voltage (V) of activation (squares) and inactivation (circles) from inside-out patches at resting pressure (0 mmHg, black) and with applied pressure (-30 mmHg, red). Data were fit to two-state Boltzmann functions (solid lines) and shows a hyperpolarizing shift in the half-points of voltage dependence of activation ( $\Delta V_{1/2a} = -10$  mV) and inactivation ( $\Delta V_{1/2i} = -12$  mV) ( $n = 5$ ,  $p < 0.01$ ).

In our protocol each 12 msec activation ladder voltage step was followed by a step to -10 mV for 4 msec to test voltage-dependence of fast inactivation (Fig. 1A). Voltage-dependent peak currents were then fit with two-state Boltzmann function. In inside-out patches at rest (0 mmHg) the half-point of voltage-dependence of inactivation ( $V_{1/2i}$ ) was  $-88.5 \pm 12.0$  mV and slope ( $dV_i$ ) was  $10.5 \pm 2.5$  ( $n = 5$ ) (Fig. 2, black circles). Pressure (-30 mmHg) in these patches resulted in  $V_{1/2i}$  of  $-100.3 \pm 10.3$  mV and  $dV_i$  of  $11.5 \pm 1.1$  mV ( $n = 5$ ) (Fig. 2, red circles). Therefore, pressure hyperpolarized the half-point of inactivation, with  $\Delta V_{1/2i} = -11.7 \pm 2.8$  mV ( $p < 0.01$  by paired t-test) but there was no significant change in  $dV_i$ .

These data show that in inside-out patches -30 mmHg pressure steps hyperpolarized the  $\text{Na}_v1.5$  half-points of voltage-dependence of activation ( $V_{1/2a}$ ) and inactivation ( $V_{1/2i}$ ) by -10 and -12 mV, respectively (Fig. 2).

**Lidocaine decreases pressure-sensitivity of  $\text{Na}_v1.5$  half-point of voltage-dependence of activation ( $V_{1/2a}$ ) but not voltage-dependence of fast inactivation ( $V_{1/2i}$ ).** At rest and with 50  $\mu\text{M}$  lidocaine in the bath, average  $V_{1/2a}$  was  $-56.7 \pm 7.8$  mV,  $dV_a$  was  $-10.3 \pm 2.1$  mV and  $G_{\text{max}}$  was  $0.87 \pm 0.75$  nS (range 0.10 to 1.92 nS) ( $n = 7$ ) (Fig. 3, black squares). With -30 mmHg pressure,  $V_{1/2a}$  was  $-60.7 \pm 5.1$  mV,  $dV_a$  was  $-10.6 \pm 1.9$  mV,  $G_{\text{max}}$  was  $0.87 \pm 0.75$  nS (range 0.075 to 1.92 nS) ( $n = 7$ ) (Fig. 3, red circles). The resulting difference between 0 and -30 mmHg was  $\Delta V_{1/2a}$  of  $-3.9 \pm 4.3$  mV ( $p < 0.05$  by paired t-test) without significant differences noted for  $\Delta dV_a$  and  $\Delta G_{\text{max}}$  ( $n = 7$ ). However, compared with controls lidocaine resulted in a 6.1 mV smaller  $\Delta V_{1/2a}$  ( $p < 0.05$  for control vs. lidocaine by two-sample t-test).





**Figure 3.** Lidocaine inhibits pressure-induced hyperpolarization of  $\text{Na}_v1.5$  voltage-dependence of activation ( $V_{1/2a}$ ) and but not inactivation ( $V_{1/2i}$ ). Shown here are voltage-dependent current vs. voltage (IV) of activation (squares) and inactivation (circles) from five inside-out patches at resting pressure (0 mmHg, black) and with applied pressure (-30 mmHg, red). Data was fit to two-state Boltzmann functions (solid lines) and shows a  $-4$  mV shift in the half-point of voltage dependence of activation ( $\Delta V_{1/2a}$ ) ( $n = 7$ ,  $p < 0.05$ ), and a  $-10$  mV shift in the half-point of voltage dependence of inactivation ( $n = 7$ ,  $p < 0.05$ ).

We also tested the effects of lidocaine on the pressure-induced shift in voltage-dependence of  $\text{Na}_v1.5$  fast inactivation. Lidocaine is known to hyperpolarize the voltage-dependence of fast inactivation.<sup>24</sup> Indeed, at rest using our protocol with  $50 \mu\text{M}$  lidocaine there was a shift in the voltage-dependence of inactivation (Fig. S3). In inside-out patches in the presence of  $50 \mu\text{M}$  lidocaine in the bath at 0 mmHg the average voltage-dependence of half-point of inactivation ( $V_{1/2i}$ ) was  $-90.5 \pm 3.8$  mV, and slope  $dV_i$  was  $14.1 \pm 3.8$  mV ( $n = 5$ ,  $p > 0.05$  compared with no lidocaine controls by two-sample t-test). With -30 mmHg stimulation,  $V_{1/2i}$  shifted to  $-101.0 \pm 4.4$  mV and  $dV_i$  to  $13.0 \pm 2.3$  mV ( $n = 5$ ). The shift of  $V_{1/2i}$  with stretch with lidocaine was significant for 0 mmHg vs. -30 mmHg  $\Delta V_{1/2i}$  of  $-10.5 \pm 6.0$  mV ( $p < 0.05$  by paired t-test) and similar  $\Delta V_{1/2i}$  to controls of  $-11.7 \pm 2.8$  mV ( $p > 0.05$  between lidocaine and control by two-sample t-test).

The data above show that lidocaine inhibits the pressure-induced hyperpolarizing shift of  $\text{Na}_v1.5$  half-point of voltage-dependence of activation ( $V_{1/2a}$ ) but does not inhibit the shift in the half-point of voltage-dependence of fast-inactivation ( $V_{1/2i}$ ) (Fig. 3). The effect of lidocaine on the mechanosensitivity of  $V_{1/2i}$  is likely complicated by lidocaine's hyperpolarizing effect on  $V_{1/2i}$  at rest.<sup>24</sup> Therefore, in the subsequent experiments we used voltage-dependence of activation to assess the effects of local anesthetics on  $\text{Na}_v1.5$  mechanosensitivity.

**Lidocaine inhibits the pressure-induced increase in peak  $\text{Na}_v1.5$  current but pressure does not alter use-dependent block by lidocaine.** In the absence of mechanical stimulation, lidocaine block of  $\text{Na}_v1.5$  channels is known to involve multiple mechanisms.<sup>25</sup> The mechanism with the highest affinity is

a use-dependent block by lidocaine.<sup>26</sup> We examined the interaction between stretch and use-dependent block by lidocaine in inside-out patches. In controls, a 10 Hz use-dependence protocol resulted in a  $10.8 \pm 3.3\%$  loss of current between the 1st and 20th steps ( $n = 3$ ,  $p < 0.05$ , Fig. 4). At -30 mmHg, this protocol resulted in a mean increase in peak current by  $13.5 \pm 2.6\%$  at each step and a mean loss of current of  $9.8 \pm 9.7\%$  between 1st and 20th steps ( $n = 3$ ,  $p < 0.05$ , Fig. 4). When lidocaine ( $50 \mu\text{M}$ ) was added to the bath, at rest (0 mmHg) the same protocol produced a significant use-dependent decrease in mean current of  $51.4 \pm 6.0\%$  between 1st and 20th steps ( $n = 3$ ,  $p < 0.05$ , Figure 4). In the presence of lidocaine, pressure failed to increase peak currents at each step as in controls but did not otherwise alter use-dependent block by lidocaine. The average difference between peaks for all steps at 0 mmHg and -30 mmHg pressure was  $0.3 \pm 3\%$  ( $p > 0.05$  by paired t-test). These results suggest that the mechanisms of use-dependence and mechanosensitivity block of  $\text{Na}_v1.5$  by lidocaine may not overlap. Next, we explore whether lidocaine use-dependence and mechanosensitivity block mechanisms are related.

**Benzocaine inhibits pressure-induced shift in  $\text{Na}_v1.5$  half point of voltage-dependence of activation ( $V_{1/2a}$ ).** If the mechanism of  $\text{Na}_v1.5$  mechanosensitivity block by lidocaine is separate from the mechanism of use-dependent block, then other local anesthetics that are known to have no use dependent block may be effective blockers of  $\text{Na}_v1.5$  mechanosensitivity. We tested this hypothesis with benzocaine which is a neutral local anesthetic known to lack the ability of use-dependent block.<sup>27</sup> Benzocaine also inhibited mechanosensitivity. In inside-out patches,  $50 \mu\text{M}$  benzocaine added to the bath solution resulted in a pressure-induced shift in the voltage-sensitivity of activation  $\Delta V_{1/2a}$  of only  $-4.8 \pm 3.3$  mV ( $n = 4$ ,  $p > 0.05$  for 0 mmHg compared with -30 mmHg but  $p < 0.05$  for  $\Delta V_{1/2a}$  for 0 mmHg vs. -30 mmHg for control vs. benzocaine). These data show that local anesthetics other than lidocaine modulate  $\text{Na}_v1.5$  mechanosensitivity and suggest that use-dependent block mechanism is distinct from blockade of mechanosensitivity.

**Lidocaine inhibition of  $\text{Na}_v1.5$  mechanosensitivity does not require the F1760 binding site.** We next examined whether the inhibition of  $\text{Na}_v1.5$  mechanosensitivity by lidocaine requires the established local anesthetic binding site. While multiple residues are known to affect local anesthetic binding within the pore of  $\text{Na}_v1.5$ , F1760 is of most importance,<sup>28,29</sup> and previously shown to be vital for use-dependent block.<sup>30</sup> We introduced a point mutation in this residue (F1760A) and determined whether the inhibition of  $\text{Na}_v1.5$  mechanosensitivity was affected. In excised inside-out patches F1760A  $\text{Na}_v1.5$  showed voltage dependent activation properties similar to the wild-type  $\text{Na}_v1.5$  channels ( $V_{1/2a} = -54.0 \pm 2.9$  mV,  $dV_a = -8.5 \pm 1.7$  mV,  $G_{\text{max}} = 1.5 \pm 1.7$  nS (range 0.65 to 4.0 nS),  $n = 4$ ). At -30 mmHg,  $V_{1/2a}$  was  $-64.6 \pm 3.3$ ,  $dV_a$  was  $-9.8 \pm 1.9$  mV,  $G_{\text{max}}$  was  $1.5 \pm 1.5$  nS (range 0.42 – 3.78 nS) ( $n = 4$ ) (Fig. 5A). Thus, negative pressure (-30 mmHg) produced a significant shift in the half-point of the voltage-dependence of activation ( $\Delta V_{1/2a} = -10.6 \pm 3.6$  mV,  $p < 0.05$  by paired t-test) (Fig. 5, gray area in all panels) with no changes to other parameters ( $n = 4$ ). The hyperpolarizing shift in  $V_{1/2a}$  was not different from

the wild-type channels ( $\Delta V_{1/2a} = -10.1 \pm 1.5$  mV) ( $p > 0.05$  by two-sample t-test).

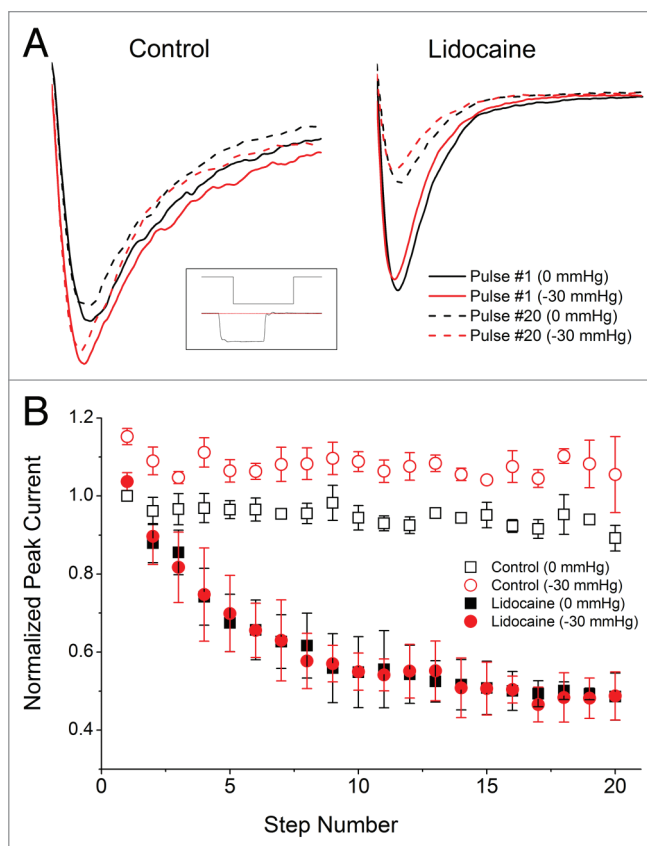
We next tested whether lidocaine blocked mechanosensitivity in F1760A  $\text{Na}_v1.5$ . In inside-out patches the addition of 50  $\mu\text{M}$  lidocaine to the bath solution of inside-out patches with F1760A  $\text{Na}_v1.5$  resulted in no changes to peak current or voltage sensitivity at 0 mmHg ( $G_{\text{max}} = 1.5 \pm 1.5$  nS,  $V_{1/2a} = -53.4 \pm 4.2$  mV, both  $p > 0.05$  compared with wt  $\text{Na}_v1.5$ ) (Fig. 5B). In the presence of lidocaine, -30 mmHg stretch resulted in a shift of  $V_{1/2a}$  to  $-54.8 \pm 2.4$  mV or a  $\Delta V_{1/2a}$  of  $-1.4 \pm 2.0$  mV for change from 0 mmHg to -30 mmHg ( $n = 4$ ,  $p > 0.05$  for 0 mmHg vs. -30 mmHg and  $p < 0.05$  for control vs. 50  $\mu\text{M}$  lidocaine) (Fig. 5B, gray area is  $\Delta V_{1/2a}$  for controls). The other parameters ( $dV_a$ ,  $G_{\text{max}}$ ) did not change significantly for 0 mmHg vs. -30 mmHg, consistent with controls.

These results show that in inside-out patches F1760A  $\text{Na}_v1.5$  are mechanosensitive to a similar extent as the wildtype  $\text{Na}_v1.5$  channels and that lidocaine inhibits mechanosensitivity of F1760A  $\text{Na}_v1.5$ . This suggests that the mechanism of  $\text{Na}_v1.5$  mechanosensitivity block by lidocaine does not involve the principal component of the local anesthetic binding site.

**QX-314 does not alter pressure sensitivity of F1760A  $\text{Na}_v1.5$ .** QX-314 is a permanently charged quaternary ammonium analog of lidocaine. It does not partition into the hydrophobic core of the lipid membrane. QX-314 efficiently and use-dependently blocks  $\text{Na}_v1.5$  from the intracellular side with slow off-rate and from the extracellular side as a slow block that requires permeation through the pore.<sup>31</sup> We tested QX-314 on either side of the membrane to assess aqueous accessibility of binding sites for mechanosensitivity block and the need for membrane partitioning. In order to eliminate the use-dependent and slow off-rate block of  $\text{Na}_v1.5$  by QX-314 we used F1760A  $\text{Na}_v1.5$ .

First, 50  $\mu\text{M}$  QX-314 was added to the bath to assess the intracellular side of the inside-out patches. At rest and with 50  $\mu\text{M}$  QX-314 in the bath, average  $V_{1/2a}$  was  $-55.5 \pm 1.5$  mV,  $dV_a$  was  $8.7 \pm 1.4$  mV,  $G_{\text{max}}$  was  $2.0 \pm 3.1$  nS (range 0.4 to 6.6 nS) ( $n = 4$ ) (Fig. 5C). With the application of -30 mmHg pressure to these patches, average  $V_{1/2a}$  was  $-64.3 \pm 3.8$  mV,  $dV_a$  was  $9.0 \pm 1.0$  mV,  $G_{\text{max}}$  was  $2.2 \pm 3.5$  nS (range 0.4 – 7.5 nS) ( $n = 4$ ). The result was a significant difference between 0 and -30 mmHg pressure for  $\Delta V_{1/2a}$  of  $-8.8 \pm 2.6$  mV ( $n = 4$ ,  $p < 0.05$  by paired t-test) with no differences in  $dV_a$  and  $G_{\text{max}}$ . Further, pressure sensitivity of  $\Delta V_{1/2a}$  was not different between F1760A  $\text{Na}_v1.5$  in the presence and absence of intracellular QX-314 ( $n = 4$ ,  $p > 0.05$  by two-sample t-test) (Fig. 5C, gray area is  $\Delta V_{1/2a}$  for controls).

Second, 50  $\mu\text{M}$  QX-314 was added to the patch pipette to assess the extracellular side of the inside-out patches. At rest and with 50  $\mu\text{M}$  QX-314 in the pipette, average  $V_{1/2a}$  was  $-52.8 \pm 5.8$  mV,  $dV_a$  was  $-8.1 \pm 1.3$  mV,  $G_{\text{max}}$  was  $1.3 \pm 1.6$  nS (range 0.4 to 3.7 nS) (Fig. 5D). With the application of -30 mmHg pressure to these patches, average  $V_{1/2a}$  was  $-61.1 \pm 6.1$  mV,  $dV_a$  was  $9.1 \pm 1.6$  mV,  $G_{\text{max}}$  was  $2.2 \pm 3.5$  nS (range 0.4 – 7.5 nS) ( $n = 4$ ). The resulting significant difference between 0 and -30 mmHg pressure was  $\Delta V_{1/2a}$  of  $-8.4 \pm 1.5$  mV ( $n = 4$ ,  $p < 0.05$  by paired t-test), but no significant differences were found for  $dV_a$  and  $G_{\text{max}}$  ( $n = 4$ ,  $p > 0.05$ , Figure 5D, gray area is  $\Delta V_{1/2a}$  for controls).

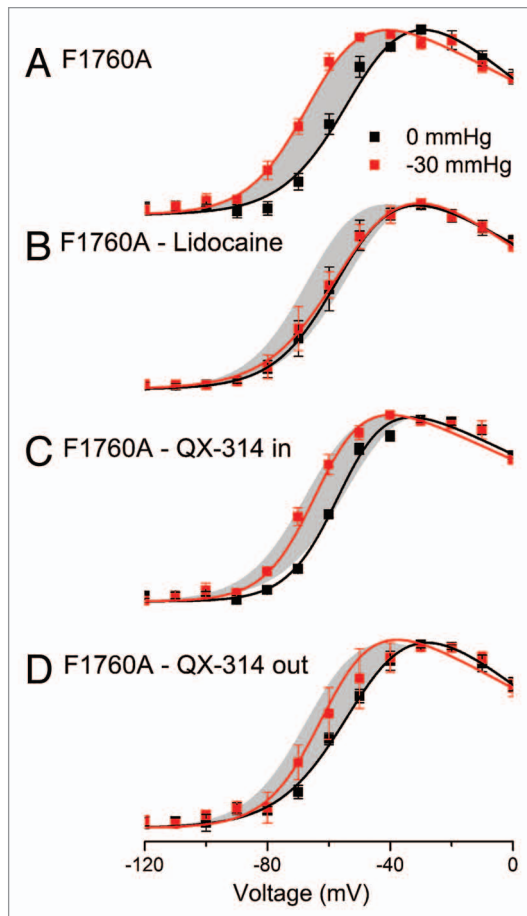


**Figure 4.** Lidocaine eliminates pressure-induced increase in  $\text{Na}_v1.5$  peak current. Inset shows a voltage step (upper trace) of the 10 Hz use-dependence protocol with (lower black trace) and without (lower red trace) -30 mmHg pressure. (A) On the left, in a typical control inside-out patch at resting tension (0 mmHg) there was no significant difference between 1st (solid black) and 20th (dash black) steps. In the same patch, -30 mmHg pressure produced a tonic increase in peak current both 1st (solid red) and 20th (dash red) steps but no significant difference between steps. On the right, in a typical inside-out patch at rest lidocaine (50  $\mu\text{M}$ ) resulted in a significant use-dependent block as shown by a large difference between 1st (solid black) and 20th (dash black) steps compared with at rest controls. (B) Peak currents normalized to 1st step at 0 mmHg for all 20 steps in the 10 Hz use dependence protocol. Peak currents for all 20 steps are increased with pressure in the controls by 14% ( $n = 3$ ,  $p < 0.05$ ). In the presence of lidocaine there is a substantial use dependent block (51%,  $n = 3$ ,  $p < 0.05$ ) but no tonic increase in peak current with pressure at each step (0.3%,  $n = 3$ ,  $p > 0.05$ ).

These data show that QX-314 applied separately from both intracellular and extracellular sites of the channel fails to block  $\text{Na}_v1.5$  mechanosensitivity, suggesting that mechanosensitivity block does not involve a site with solely aqueous access.

## Discussion

**$\text{Na}_v1.5$  is robustly mechanosensitive in excised patches.** Inside-out patches have an advantage over cell attached patches in that they allow precise control of the transmembrane voltage, partial elimination of cytoskeleton and other cytoplasmic factors and



**Figure 5.** Lidocaine effect on  $\text{Na}_v1.5$  mechanosensitivity does not involve F1760 but requires a charge neutral form. Voltage-dependence of activation was tested at rest (0 mmHg, black) and with pressure (-30 mmHg, red curves) in the absence and presence of lidocaine and QX-314. **(A)** Voltage-dependent activation of F1760A at rest (0 mmHg, black) and in the presence of pressure (-30 mmHg, red), showing a -11 mV shift in half-point of activation with stretch ( $\Delta V_{1/2a}$ ) ( $n = 4$ ,  $p < 0.05$ ). **(B)** With the addition of lidocaine (50  $\mu\text{M}$ ) inhibits the shift of the voltage-dependence of activation curve ( $\Delta V_{1/2a} = 1.4$  mV) ( $n =$ ,  $p > 0.05$ ) (gray area is the shift by F1760A control). **(C)** With QX-314 (50  $\mu\text{M}$ ) applied to the inside the shift in the half-point of voltage-sensitivity with pressure is -9 mV ( $n = 4$ ,  $p < 0.05$ ). **(D)** With QX-314 (50  $\mu\text{M}$ ) applied to the outside the shift in the half-point of voltage-sensitivity with pressure is -8 mV ( $n = 4$ ,  $p < 0.05$ ).

drug application to the cytoplasmic side. All of the previously published studies on  $\text{Na}_v1.5$  mechanosensitivity in patches used cell-attached configuration.<sup>9-11,20</sup> Previous work in pressurized patches showed a fast capacitance rise (milliseconds), followed by a prolonged rise to steady-state (tens to hundreds of milliseconds).<sup>22</sup> We assessed the fast and reversible response using a short 25 msec stretch stimulus and paired voltage ladder protocol, so patches were only stimulated by pressure for ~20% of the protocol duration (Fig. 1A and B). Given strong voltage-dependence of  $\text{Na}_v1.5$  we were able to test  $\text{Na}_v1.5$  voltage-dependent pressure sensitivity within 10 msec of pressure pulse initiation. In addition, these short pulses allowed testing without addition of

lanthanides which block endogenous mechanosensitive channels,<sup>32</sup> but are also known to affect  $\text{Na}_v1.5$  function.<sup>33</sup> Reversible mechanosensitive response at the millisecond scale in excised patches further reinforces stand-alone mechanosensitivity of the  $\text{Na}_v1.5$   $\alpha$ -subunit.

Compared with the typical voltage-dependent behavior in whole-cells, both inside-out and cell-attached patches showed larger variability in the half-points of voltage-dependence ( $V_{1/2}$ ) and shallower slopes (dV). This may be due to non-zero resting tension<sup>34</sup> and channel location in the rim of the seal.<sup>35</sup> Nevertheless, our current protocol allowed adequate averaging in space and time and patches provide a robust platform for studying mechanosensitivity.

**Membrane-permeable local anesthetics modulate  $\text{Na}_v1.5$  mechanosensitivity by a mechanism different than use-dependent block.**  $\text{Na}_v1.5$  channels are mechanosensitive in excised inside-out patches, and the response type and amplitude of pressure sensitivity are similar to previously published data in cell-attached patches.<sup>10,11,20</sup> Specifically, in response to -30 mmHg pressure applied to an inside-out patch we saw a hyperpolarizing shift in  $V_{1/2a}$  and  $V_{1/2i}$  of about -10 mV without changes in  $dV_a$ ,  $dV_i$  or  $G_{\text{max}}$  (Fig. 2). Lidocaine (50  $\mu\text{M}$ ) altered  $\text{Na}_v1.5$  mechanosensitivity as demonstrated by a decrease in shift of  $V_{1/2a}$  with -30 mmHg pressure by 67% ( $\Delta V_{1/2a}$  from -12 mV for control to -4 mV for lidocaine), similar to previous finding in cell-attached patches.<sup>20</sup> On the other hand, lidocaine did not alter the pressure-induced shift in  $V_{1/2i}$  (Fig. 3). Since lidocaine stabilizes inactivation at rest (Fig. S3), resulting in a hyperpolarizing shift of  $V_{1/2i}$ ,<sup>24</sup> the lack of pressure-induced effect on  $V_{1/2i}$  in the presence of lidocaine likely relates to the drug's effects at rest.

Block by lidocaine of  $\text{Na}_v1.5$  currents in the absence of pressure is known to be use-dependent and we next tested whether there were any changes to the use-dependence of lidocaine block of  $\text{Na}_v1.5$ . Using a 10 Hz use-dependence protocol with and without -30 mmHg we show in control  $\text{Na}_v1.5$  (0  $\mu\text{M}$  lidocaine) an increase in peak current at each step without use-dependent loss of current (Fig. 4A and C). Lidocaine blocked the pressure-induced increase in the peak current, but otherwise use-dependence did not change with application of pressure. We provide two pieces of evidence that suggest that mechanisms of inhibition of  $\text{Na}_v1.5$  mechanosensitivity and use-dependent block by lidocaine are separate mechanisms. First, we used another local anesthetic, benzocaine, which unlike lidocaine is permanently neutral and does not show use-dependent block of  $\text{Na}_v1.5$  likely due to escape via the hydrophobic pathway.<sup>27,36</sup> Yet, benzocaine efficiently blocked  $\text{Na}_v1.5$  mechanosensitivity as shown by a decrease in shift of  $V_{1/2a}$  by 59%. The magnitude of the  $\text{Na}_v1.5$  mechanosensitivity block by benzocaine was similar to lidocaine. Second, we mutated a critical residue for use-dependent block (F1760A).<sup>30</sup> F1760A  $\text{Na}_v1.5$  were mechanosensitive to the same extent as the wild-type  $\text{Na}_v1.5$  (Fig. 5A). Lidocaine continued to block mechanosensitivity F1760A  $\text{Na}_v1.5$  at the level similar to the control wild-type channels (Fig. 5B). These data suggest that the mechanisms of lidocaine block of  $\text{Na}_v1.5$  mechanosensitivity and use-dependence are separate.



**Na<sub>v</sub>1.5 mechanosensitivity block by local anesthetics may require membrane partitioning.** Both lidocaine and benzocaine are membrane permeable and there are sufficient data that suggest that the mechanism of voltage-gated ion channel mechanosensitivity may be mediated by bilayer-protein interactions.<sup>17,18,37,38</sup> We examined whether membrane partitioning by local anesthetics is critical for block of Na<sub>v</sub>1.5 mechanosensitivity. For this we used a membrane-impermeable permanently charged lidocaine homolog QX-314. QX-314 is structurally very similar to lidocaine, with the only difference being lidocaine's secondary amine group being replaced by a charged quaternary ammonium. Thus, QX-314 and lidocaine access the same aqueous sites, but only lidocaine is able to access hydrophobic pathway.<sup>31</sup> Unlike lidocaine and benzocaine, the application of QX-314 to either side of the membrane did not inhibit mechanosensitivity of F1760A Na<sub>v</sub>1.5 (Fig. 5C and D). First, these data suggest that not all local anesthetics modify mechanosensitivity, as lidocaine and benzocaine were effective inhibitors and QX-314 was not. Second, differential effects of Na<sub>v</sub>1.5 mechanosensitivity by QX-314 and lidocaine/benzocaine suggest that the mechanism of Na<sub>v</sub>1.5 mechanosensitivity involves the lipid bilayer and potentially the lipid-protein interface.

**The potential mechanisms of Na<sub>v</sub>1.5 mechanosensitivity modulation by membrane permeable local anesthetics likely involve protein-bilayer interface.** Membrane partitioning of local anesthetics may play a role in their inhibition of Na<sub>v</sub>1.5 mechanosensitivity. Several possibilities exist with respect to the mechanism, broadly divided into non-specific and specific effects. First, local anesthetics may non-specifically alter bulk mechanical or electrical membrane properties. Second, bilayer stretch may alter interactions between the membrane permeable local anesthetics and the bilayer. Third, local anesthetics may non-specifically alter lipid-protein interactions in vital lipid exposed channel domains (e.g., voltage-sensors, S4–5 linkers, pore). Fourth, specific binding of local anesthetics membrane accessible protein site(s) may inhibit Na<sub>v</sub>1.5 mechanosensitivity.

Bulk membrane mechanical (e.g., elasticity<sup>17</sup> and thickness<sup>39</sup>) and electrical (e.g., electrostatic potential<sup>40</sup> and capacitance<sup>41</sup>) properties are known to affect voltage-gated channel function. Previous work showed that increasing lipid thickness hyperpolarized Na<sub>v</sub> voltage-dependence of activation and inactivation,<sup>39</sup> and disruption of the bilayer order by cholesterol depletion or amphiphiles hyperpolarized Na<sub>v</sub>1.4 voltage-dependence of inactivation.<sup>17</sup> Further, local anesthetics partitioning into bilayers alter membrane structure<sup>42</sup> and thus the mechanical<sup>43</sup> and electrical<sup>44</sup> functional properties. Interestingly, both charged and uncharged forms of local anesthetics interact with lipid bilayers, albeit the neutral forms show deeper penetration and larger effects.<sup>43</sup> Yet, it is unlikely that the effects we have observed are due to alteration of bulk membrane mechanical properties at rest. First, at the 50 μM doses of local anesthetics used in this study the drug density is much less than 1% of the lipid, and unlikely to have a significant effect on bulk membrane properties.<sup>45</sup> Second, the addition of lidocaine and benzocaine to Na<sub>v</sub>1.5 and QX-314 to F1760A Na<sub>v</sub>1.5 did not change the voltage-dependence properties ( $V_{1/2a}$  and  $dV_a$ ) at rest ( $p > 0.05$  for all compared with no drug Na<sub>v</sub>1.5 control).

Another possibility is that membrane stretch alters the interaction properties of the bilayer with the local anesthetics. It is known that the partition coefficient of the local anesthetics depends on the state of the bilayer, preferring to partition into the liquid-crystal over solid-gel membranes.<sup>45</sup> This suggests that lidocaine partitioning likely increases with stretch, such that the drug's effects on Na<sub>v</sub>1.5 may appear stretch dependent. In the use-dependence protocol lidocaine inhibited pressure-dependent increase in peak current, but otherwise did not show additional use-dependent block as may be expected if lidocaine availability increased with stretch. Nevertheless, this possibility exists and requires further exploration.

It is clear that the lipid bilayer serves as an important pathway for the function of local anesthetics,<sup>24</sup> and the neutral forms of these drugs pack readily within the lipids. Voltage-gated ion channel function depends on lipids<sup>46</sup> and the recent crystal structure of Na<sub>v</sub>Ab shows lipids as important structural elements, intruding through side entries into the pore of the channel.<sup>47</sup> The findings of this study do not allow for specific localization of the target of the membrane permeable anesthetics with respect to the mechanism of Na<sub>v</sub>1.5 mechanosensitivity modulation. However, potential targets are hydrophobic protein domains that may either rely on lipids for appropriate function<sup>46</sup> or identified binding sites for local anesthetics.<sup>48</sup>

In conclusion, in this study we demonstrate fast and reversible Na<sub>v</sub>1.5 mechanosensitivity in excised inside-out patches using a protocol with short pressure steps. Patch pressure hyperpolarizes Na<sub>v</sub>1.5 the half-points of voltage-dependence of activation and inactivation by about -10 mV. Lidocaine inhibits the pressure-induced shift in the voltage-dependence of activation but not inactivation. The uncoupling by lidocaine of the pressure-induced left-shifts in the half-points of voltage dependence of activation and inactivation may have therapeutic potential by decreasing pressure-induced excitability.<sup>49</sup> The mechanism of modulation of Na<sub>v</sub>1.5 mechanosensitivity by local anesthetics appears to require membrane partitioning and likely protein-lipid interaction.

## Methods

**Cell culture.** Human embryonic kidney 293 (HEK) cells were cultured and transfected as previously described.<sup>10</sup> Cells were grown in T25 flasks in Minimum Essential Medium, 10% horse serum, non-essential amino acids, sodium pyruvate and penicillin-streptomycin (Invitrogen). Cells were grown to about 75% confluency. One day prior to the experiments, flasks were transfected with SCN5A encoding hH1c3 or hH1c1 F1760A (kind gift of Dr Jonathan Makielski), with green fluorescent protein (GFP) pEGFP-C1 (Clontech), using Lipofectamine 2000 reagent and OPTI-MEM I reduced serum medium (Invitrogen). On the morning of experiments, cells were trypsinized, plated onto poly-L-lysine glass coverslips and used after about 5 h.

**Recording solutions and pharmacology.** For cell-attached patches, bath solution (extracellular) contained (in mM): 149 KCl, 2.45 CaCl<sub>2</sub>, 5.0 HEPES, 5.5 mM glucose. For excised inside-out patches, bath solution (intracellular) contained (in mM): 120 CsCl, 40 CsF, 1 NaCl, 2 MgCl<sub>2</sub>, 0.68 CaCl<sub>2</sub>, 10 HEPES,

2 EGTA. Free  $\text{Ca}^{2+}$  was calculated to be 100 nM (<http://www.stanford.edu/~cpatton/CaEGTA-TS.htm>). The patch pipette solution (extracellular) contained (in mM): 160 NaCl, 1 CsCl, 1  $\text{MgCl}_2$ , 2  $\text{CaCl}_2$  and 10 HEPES. For all solutions osmolality was  $300 \pm 10$  mmol/kg and pH 7.2. Local anesthetic solutions (lidocaine, benzocaine and QX-314) were prepared daily from 100 mM ethanol stocks.

**Electrophysiology.** Electrodes were pulled using Sutter Instruments P-97 puller and coated with heat cured Dow Corning R6101 compound from Garner 8250 glass. Electrodes were fire polished specifically for patch mechano-activation as previously published.<sup>10</sup> Axon 200A amplifier, Digidata 1322A, and Clampex 9 software were used for voltage-clamp and data acquisition. For voltage-ladder protocols, a 1.7 Hz stimulation frequency was used with no inter-pulse delay. Patches were held at -100 mV, and preceding each test step were pre-pulses of 10 msec to -204 mV and 4 msec to -140 mV which accelerated recovery from inactivation. Following the pre-pulse steps were 16 test voltage steps in 10 mV increments from -140 mV to 10 mV for 12 msec per step to test activation and finally a step to -10 mV for 4 msec to test availability. At each tested voltage a pair of pulses were obtained for control (0 mmHg) and pressure (-30 mmHg), which were separated by 230 msec. Currents were corrected with a P/6 protocol (hyperpolarizing steps from HP -120 mV) and a 5x average was obtained for each voltage step.

**Mechanical activation of voltage-clamped currents.** A specialized rapid pressure clamp was used to apply pressure (courtesy of Dr. Fred Sachs laboratory).<sup>50</sup> Negative pressure was monitored during patch formation and pressure delivery for all protocols.<sup>32</sup> Due to the mechanical remodeling of patches with prolonged

stretch pulses<sup>32</sup> we used a rapid pressure clamp (rise time to -30 mmHg in 5 msec). Stretch steps were limited to 25 msec obtained during activation/inactivation steps only. Seal history is important for such experiments,<sup>32</sup> therefore we recorded the pressures required for patch formation. Patches were typically formed at < 5 mmHg. Patches with seals that were formed at > 10 mmHg were discarded.

**Data analysis.** Voltage dependence of activation for each experiment was fit individually and averages provided in results. The figures show averages and fits of raw data. For voltage-dependence of activation we used a two-state Boltzmann model ( $I = [(V - V_{rev})G_{max} / (1 + e^{(V - V_{1/2a})/dVa})]$ ), where peak current ( $I$ ) is a function of voltage ( $V$ ),  $V_{1/2a}$  was half-point,  $dVa$  was the slope,  $V_{rev}$  was reversal potential, and  $G_{max}$  was maximal conductance. We fit voltage dependence of inactivation using a two-state Boltzmann model ( $I = (A_1 - A_2) / [1 + e^{(V - V_{1/2i})/dVi}] + A_2$ ), where  $A_1$  and  $A_2$  were constants,  $V_{1/2i}$  was half-point,  $dVi$  was the slope. Voltage dependence and dose-response curves were fit in pClamp9 and Origin 8.61.

#### Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

#### Acknowledgments

The authors would like to thank Drs David Linden and Arpad Mike for their critical review of the manuscript. This work was supported by NIDDK 52766 and P30DK84567.

#### Supplemental Material

Supplemental materials may be found here: [www.landesbioscience.com/journals/channels/article/21202](http://www.landesbioscience.com/journals/channels/article/21202)

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