Impaired stretch modulation in potentially lethal cardiac sodium channel mutants

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The presence of two slowly inactivating mutants of the cardiac sodium channel (hNa1.5), R1623Q and R1626P, associate with sporadic Long-QT3 (LQT3) syndrome, and may contribute to ventricular tachyarrhythmias and/or lethal ventricular disturbances. Cardiac mecanoelectric feedback is considered a factor in such sporadic arrhythmias. Since stretch and shear forces modulate hNa1.5 gating, detailed electrophysiological study of LQT3 mutants channel α subunit(s) might provide insights. We compared recombinant R1623Q and WT currents in control vs. stretched membrane of cell-attached patches of Xenopus oocytes. Macroscopic current was monitored before, during, and after stretch induced by pipette suction. In either mutant Na+ channel, peak current at small depolarizations could be more than doubled by stretch. As in WT, R1623Q showed reversible and stretch intensity dependent acceleration of current onset and decay at all voltages, with kinetic coupling between these two processes retained during stretch. These two Na1.5 channel α subunits differed in the absolute extent of kinetic acceleration for a given stretch intensity; over a range of intensities, R1623Q inactivation speed increased significantly less than did WT. The LQT3 mutant R1626P also retained its kinetic coupling during stretch. Whereas WT stretch-difference currents (I(V) during stretch minus I(V) without stretch) were mostly inhibitory (equivalent to outward current), they were substantially (R1623Q) or entirely (R1626P) excitatory for the LQT3 mutants. If stretch-modulated Na1.5 current (i.e., brief excitation followed by accelerated current decay) routinely contributes to cardiac mecanoelectric feedback, then during hemodynamic load variations, the abnormal stretch-modulated components of R1623Q and R1626P current could be pro-arrhythmic.

Introduction

Cardiac action potentials (AP) depend on time and voltage gated channels (VGCs), among which sodium channels (Na1.5) trigger and then modulate AP duration. VGC structure, as exemplified by Kv channels, is such that voltage sensors extensively contact the lipid bilayer. Therefore, the energetics of the lateral protein/lipid-matrix interface help determine transition rates (e.g., from a closed-resting to a closed-activated state) in VGCs. Assuming similar bilayer/sensor interactions prevail among all VGCs, perturbation of these interactions might underlie stretch modulation of VGCs (reviewed in refs. 2–4). Stretch increases current amplitude in certain VGCs without altering onset kinetics (e.g., Kv3 and Ca2 channels). For others, Kv1.5, Na1.5, Na1.6 and HCN2, transitions which regulate activation are stretch-sensitive so stretch changes both current onset speed and magnitude.

Mechano-dependent modifications in Na current kinetics were first reported on squid axons subjected to hyperbaric pressures. In these experiments, normalized (within-prep) Na current signals were time rescaled. The results showed that activation and inactivation reversibly co-decelerate in response to large reversible hyperbaric pressure changes. In previous work, we showed that recombinant hNa1.5 in Xenopus oocyte patches respond to membrane stretch with a reversible left-shift of the conductance vs. membrane voltage (g(V)) relation, and that current onset and decay both accelerate with stretch. This indicates that functional coupling between channel activation and fast inactivation is not lost during stretch. Coupling in Na1.5 signifies that depolarization-induced opening of the activation gate strongly facilitates the subsequent closure of the inactivation gate, thereby fostering the coordinated acceleration of activation and inactivation kinetics at increasingly depolarized voltages.

Because of this coupling, S4 mutations typically affect both activation and inactivation. Subtle Na1.5 mutations that generate kinetic abnormalities can result in life-threatening cardiac arrhythmias including type 3 Long-QT syndrome (LQT3). Most LQT3 mutations affect Na1.5 inactivation via residues located near the cytoplasmic inactivation gate. Na+ channels with mutated or missing gate-region residues have WT-like voltage-dependent inactivation but show abnormal re-opening events. By contrast, the R1623Q and the R1626P mutations, identified in patients with LQT3 syndrome, are located at the extracellular end of D4S4 (transmembrane segment four (S4) in domain 4 (D4)). Comparisons of current families reveal that both current onset and decay are slower in R1623Q and R1626P than...
Both mutant channels might also have a greater tendency to inactivate from closed states, based on left-shifted (~7 mV) steady-state availability. With inactivation slowed in R1623Q and R1626P, closing-then-reopening events (evident in single channel records of R1623Q) occur prior to eventual inactivation. The arginine-to-glutamine and arginine-to-proline mutations reduce or abolish the apparent voltage sensitivity of inactivation. Similar kinetic changes have been identified for the skeletal muscle sodium channel in which an equivalent D4S4 mutation (hNa1.4-R1448C in place of hNa1.5-R1623Q) causes congenital paramyotonia.

In Na$_A$ channels, voltage-dependent D4S4 motion has at least two components. The possibility of a D4S4 motion limiting opening remains controversial, but it is clear that a charge-moving D4S4 motion exposes the inactivation particle binding site. Na$_A$ channel current decay (often termed “inactivation”) then depends on the inherently voltage independent (but fast) binding of the inactivation particle. Normally (i.e., WT), this binding accelerates steeply where the g(V) relation (often termed “activation”) changes rapidly with voltage. Hence, for WT, “coupling” is also taken to refer to these overlapping voltage dependences. For R1623Q the rate of inactivation (1/τ$_{\text{max}}$) is less voltage sensitive than for WT in that range, while in R1626P inactivation rates are essentially voltage insensitive.

Since in WT, voltage-induced D4S4 movements have been thought to kinetically couple inactivation to activation, we asked whether coupled activation/inactivation relations during stretch are altered when D4S4 is mutated in a way that should reduce (R1623Q) or abolish (R1626P) the apparent voltage dependence of inactivation. Our work revealed a difference between WT and both of the selected LQT3 related mutants: the ability of stretch to accelerate INa decay. In blinded tests, R1623Q decay was significantly less stretch-sensitive than WT decay. Reduced stretch-sensitivity rendered the mutants less able to rapidly inactivate excitatory Na$^+$ currents during stretch.

Our findings suggest that a voltage-dependent stabilization of the inactivated state by D4S4 could be crucial when membranes experience elevated tension. Both LQT3 mutations showed robust stretch-excitation but impaired stretch-inhibition. Whether inherent stretch-sensitivity in Na$_A$,4 (or other VGCs) has physiological ramifications is uncertain. For stretch-activated cation (SAC) channels a perennial issue has been where do cardiac myocyte membranes deform enough to modulate channel activity and the same applies for VGCs. During stretch-induced cardiac arrhythmias, Na$_A$ channel blockers are powerfully anti-arrhythmic (reviewed in refs. 21 and 22) but this falls short as evidence for in situ stretch-modulated INa. No available drugs impair only stretch-modulated components of VGC currents. Thus mutant VGCs with cardiomechanical phenotypes might help uncover in situ mechano-behavior.

Stress intensities that modulate Na$_A$ and other VGCs are the same as for SAC channels (reviewed in ref. 3). SAC channels (whose molecular identity is not settled) are evident in atrial but not ventricular myocytes, stretch-modulatable VGCs (K$_{\text{r1}}$, K$_{\text{r3}}$, K$_{\text{r7}}$, Ca$_{\text{r1}}$, HCN2, Na$_A$,1.6 and Na$_A$,1.5) occur in all cardiomyocytes. That SAC channels and not VGCs would contribute to mechanoelectric feedback seems implausible (reviewed in ref. 27).

Interestingly, Na$_A$,1.5 current in smooth muscle and gut mechanosensory cells (interstitial cells of Cajal) is shear-sensitive as is a neuronal-type endothelial cell Na$_A$ channel. Pacinian corpuscle mechanoreceptor current has a tetrodotoxin-sensitive component and DRG neurons robustly express a Na$_A$,1.5 splice variant. Fine Na$_A$,1.5-expressing neural processes arising from intra-cardiac autonomic neurons deeply penetrate the ventricular wall. Accordingly, ventricular or atrial wall stresses might deform these processes, modulating I$_{\text{Na}}$.

Whenever Na$_A$,1.5 channel currents are modulated by cardiac mechanoelectric feedback, the more weakly stretch sensitive responses of mutant Na$_A$,1.5 compared to WT would be expected to add insult to injury by failing to adequately hasten turn-off of already overly-prolonged I$_{\text{Na}}$.

**Results**

**Stretch modulation of sodium channels in ventricular myocyte.** The likelihood that recombinant Na$_A$,1.5 channel responses to applied stretch have patho/physiological relevance is greater if native cardiac Na$_A$ channels are mechanoresponsive. We therefore studied Na$_A$ channels in freshly isolated adult rat ventricular myocytes (see Suppl. material), a preparation where Na$_A$ channels are predominantly Na$_A$,1.5 (13 pm$^2$, ~40x more than other Na$_A$ isoforms). The myocyte was clamped 75 mV hyperpolarized to -155 to -165 mV. As reported by Sachs and colleagues and others, we never observed SAC channel events. Multiple Na$_A$ channel events were always present during depolarizing steps, but even without stretch, contractions disrupted most patch recordings. Figure 1 shows the one complete before/after stretch dataset; the traces of Figure 1A are normalized to peak then the time axis of stretch current is extended until the traces overlap the before/after traces (Fig. 1B). The ability to perform this rescaling of the time axis on peak normalized currents signifies that rate-limiting step(s) for activation and inactivation were accelerated by the same factor during stretch, as in recombinant Na$_A$,1.5 and Na$_A$,1.6. The stretch difference current (C) suggests that during a depolarizing excursion (e.g., action potential) of a ventricular myocyte in situ, stretch would be only briefly excitatory (excess early I$_{\text{Na}}$), with the major consequence of stretch being inhibitory (reduced I$_{\text{Na}}$).

**Recombinant hNa$_A$,1.5 and effect of lanthanum on the R1623Q conductance-voltage relation.** Since La$^{3+}$ was included in the recording pipette to inhibit the endogenous SAC channels in the oocyte, control experiments were carried out to assess the effects of La$^{3+}$ on the R1623Q g(V) relation. As expected, La$^{3+}$ progressively right-shifted the g(V) curves, with V$_{0.5}$ moving from -43 mV at 250 mM La$^{3+}$ to -14 mV at 1 mM La$^{3+}$ (data not shown). Empirically, we found that 350 μM [LaCl$_3$] inhibited SAC channel activity during stretch in most of the experiments. At this concentration, g(V) relations of WT and R1623Q Na$_A$,1.5 were not different (V$_{0.5}$ for WT -21 ± 1 mV, n = 5, for R1623Q, 12 ± 1 mV, n = 9).

**Figure 1**

- A: Normalized peak current before and after stretch for W1623Q. The traces are offset to show the time axis of current during stretch. The stretch amplitude is ∼3 × RMP, and the time of stretch is indicated by the black bar. The traces are normalized to peak current and the time axis of stretch current is extended until the traces overlap the before/after traces. 
- B: Normalized peak current before and after stretch for W1623Q. The traces are offset to show the time axis of current during stretch. The stretch amplitude is ∼3 × RMP, and the time of stretch is indicated by the black bar. The traces are normalized to peak current and the time axis of stretch current is extended until the traces overlap the before/after traces.
-22 ± 1 mV, n = 9, F test p > 0.2; errors are confidence limits), in good agreement with ref. 9 (data not shown).

Effects of stretch on peak Na⁺ conductances: R1623Q vs. WT. As demonstrated previously, stretch reversibly accelerated $I_{Na}$ activation and fast inactivation (measured from the time course of $I_{Na}$ onset and decay, respectively) for WT Na⁺.1.5 Figure 2A shows a g(V) relation before, during and after application of pipette suction. For R1623Q, as for WT, stretch reversibly accelerated current onset and decay, as illustrated in Figure 2B for a stretch test done at three voltages. Stretch-induced effects on current onset kinetics were not compared for WT and R1623Q because the recording bandwidth was more likely to attenuate WT than R1623Q currents. Note that near the foot of the g(V), as shown at -60 mV, stretch clearly increased $I_{Na}$. After peak $I_{Na}$ is normalized (shown for the -50 mV step) then further expanded (left) to illustrate the kinetics (for -50 mV), the stretch-accelerated onset and decay are evident. In both R1623Q and WT, peak inward current near the foot of the g(V) more than doubled with stretch (Suppl. Fig. S1). However as peak values depend on both onset and decay rates, both of which changed with stretch, we did not pursue peak value comparisons further.

Figure 2C confirms that, as for WT, applied stretch can left-shift the R1623Q g(V) curve; significant stretch-induced $V_{0.5}$ left shifts of the same magnitude were not observed at both 350 µM and 1 mM La³⁺ (see legend for details). Thus, lanthanide-induced right-shift and lanthanum-dependent diminution of current did not demonstrably interfere with stretch modulation of the rate-limiting transition for activation in NaV1.5-R1623Q. This is consistent with previous findings for NaV1.5-WT exposed to gadolinium (a lanthanide), as well as for other voltage gated channels and applied lanthanides (Ki,1, HCN2).2,37

Coupling between activation and inactivation in R1623Q. In WT NaV1.5, activation and inactivation kinetics both accelerate with stretch, and this also was observed for native channels (Fig. 1B). This finding indicates that the strong coupling of fast inactivation to activation in NaV1.5 channels is not impaired by stretch. It was suggested that abnormal NaV1.5 kinetics upon mutation of arginine-1623 in D4S4 to glutamine might reflect altered movements of the domain 4 voltage sensor that compromise coupling between activation and inactivation processes (i.e., channel opening and binding of the inactivation particle).3 Since in R1623Q current onset and decay kinetics are slower than in WT and since we observed co-acceleration of these kinetics upon stretch, we addressed the question of kinetic coupling in R1623Q using double rescaling analyses (as described for Fig. 1B). Figure 3A shows R1623Q current traces elicited by pulses to different test voltages (-30, -20 and 0 mV). Note that traces in Figure 3Ai superimpose closely after current normalization and time axis rescaling (Fig. 3Aii). Thus, based on double rescaling analysis (an indicator of strong kinetic coupling between activation and inactivation) coupling was not impaired in R1623Q; evidently a channel that exhibits “uncoupling” of g(V) from $r_{inactivation}$ (V)$^9$ can retain WT-like kinetic coupling between current onset and current decay.

Next we assessed whether, in R1623Q, current onset and decay remained kinetically coupled during stretch. In Figure 3B and C (as in Fig. 1), amplitude-normalized traces were rescaled to determine if this could yield the expected overlap of control and stretch traces. R1623Q traces from near the foot and the maximum of the g(V) relation (i.e., at -45 mV and 0 mV) from a multipulse experiment (like Fig. 2B but different test voltages) show that for precisely the same stretch stimulus, peak $I_{Na}$ increased dramatically near the foot (Fig. 3Bi) whereas at the maximum (Fig. 3Bii) current amplitude did not change. Since $g_{max}$ was unchanged during stretch, it seems that stretch did not alter membrane area. Instead, increased $I_{Na}$ at, e.g., -45 mV, reflects a stretch-induced effect on channel gating, not on membrane area. It should be recalled that since these experiments are performed using cell-attached patches (not whole cell clamp) membrane area cannot be assessed by capacitance measurements.

Figure 3B and C demonstrate that, indeed, in R1623Q channels, stretch co-accelerated both current onset and decay. After amplitude normalization, extending the ‘stretch trace’ time base (Fig. 3Bii) demonstrated complete overlap with the no-stretch trace (overlap of the “during” and “before” trace is shown). At 0 mV, peak $I_{Na}$ required no normalization (see trace expansion) but both current rise (“activation”) and current decay (“inactivation”) were faster during stretch. Again, time base expansion of the stretch trace (Fig. 3Gi) produced complete overlap. The factor by which the timescale was increased is the “stretch acceleration factor” (SAF). This double rescaling was robust, applying over a broad range of stretch intensities and at different voltages. For one patch, Figure 3D shows traces at suction levels from 0 mmHg (black) up to -30 mmHg. Current traces are expanded in the panel below to show stretch-modulated onsets. Note that these data, taken at 0 mV, have not been amplitude normalized; again this emphasizes the point that once $g_{max}$ has been reached (by the depolarizing step), pipette suction did not increase peak current. From twelve R1623Q patches, comparisons at -40 and
Figure 2. Recombinant WT and R1623Q cardiac Na⁺ channels. (A) Normalized g(V) curves for WT channels before/during/after stretch (-30 mmHg used to stretch the membrane, 250 μM La³⁺ in the pipette, -9 ± 3 mV left shift. In another patch using 1 mM La³⁺ (data not shown) -30 mmHg caused a -12 ± 2 mV left shift. (B) R1623Q current during depolarizing steps. Throughout, black, red and grey signify before/during/after stretch (due to -16 mmHg in this instance). I_{Na} increased with stretch. As here (and in A for WT) reversibility was not always complete upon release of stretch. Currents at -50 mV were normalized to peak then (below) early current expanded (asterisks give a reference) to demonstrate that, as in WT, stretch reversibly increased rates of current onset and decay. (C) Stretch-induced left shift of R1623Q g(V). -20 mmHg was used to stretch the membrane patches. With 350 μM La³⁺ in the patch pipettes (V0.5 = -22 ± 1 mV (n = 9) for control vs. -35 ± 1 mV (n = 5) for stretch), Δ = -12 ± 2 mV, and with 1 mM La³⁺ (V0.5 = -12 ± 1 mV (n = 4) for control vs. -25 ± 1 mV (n = 4) for stretch), Δ = -11 ± 2 mV (errors are confidence limits of fits; between patches F test, p < 0.0001).

Figure 3. Stretch accelerates R1623Q current onset and decay rates to the same extent. (Ai) typical set of R1623Q records at 3 voltages and (Aii) after the set of records was double rescaled, as described for Figure 1B (scales: 10 pA, 5 ms). (B and C) show R1623Q current during depolarizing steps. Throughout, black, red and grey signify before/during/after stretch (due to -16 mmHg in this instance). I_{Na} increased with stretch. As here (and in A for WT) reversibility was not always complete upon release of stretch. Currents at -50 mV were normalized to peak then (below) early current expanded (asterisks give a reference) to demonstrate that, as in WT, stretch reversibly increased rates of current onset and decay. (C) Stretch-induced left shift of R1623Q g(V). -20 mmHg was used to stretch the membrane patches. With 350 μM La³⁺ in the patch pipettes (V0.5 = -22 ± 1 mV (n = 9) for control vs. -35 ± 1 mV (n = 5) for stretch), Δ = -12 ± 2 mV, and with 1 mM La³⁺ (V0.5 = -12 ± 1 mV (n = 4) for control vs. -25 ± 1 mV (n = 4) for stretch), Δ = -11 ± 2 mV (errors are confidence limits of fits; between patches F test, p < 0.0001).

(E) From the rescaling procedure illustrated in (B and C) stretch acceleration factor (SAF) vs. stretch stimulus intensity plots from data at -40 and 0 mV (n = 4 and n = 5 respectively).
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Figure 4. Inactivation time constants for \( I_{Na} \). (A) Current inactivation kinetics of a “before” (black; 0 mmHg) and a stretch-modulated (red; -30 mmHg) R1623Q current during a step to -30 mV from -120 mV. Best-fit single exponential relations (continuous light grey lines) from which \( \tau_{inactivation} \) was obtained. (B) Voltage dependence of \( \tau_{inactivation} \) for WT and R1623Q. Dotted lines are 95% confidence limits. WT inactivation was faster and more steeply voltage dependent than R1623Q: e-fold/36 ± 4 mV for WT vs. e-fold/57 ± 2 mV in R1623Q (F test p < 0.0005) (the number of averaged recordings per point is given in (C), controls; standard errors are shown). (C) WT \( \tau_{inactivation} \) changed with stretch without altering voltage dependence: the e-fold change factors were 36 ± 4 mV without and 33 ± 6 with stretch (F test p > 0.2). In R1623Q (Ci), \( \tau_{inactivation} \) changed less strongly with stretch than in WT but voltage dependence steepened: the e-fold change factor dropped from 57 ± 2 mV in control to 38 ± 3 during stretch (F test p < 0.0005). (Cii) best fits for WT and R1623Q (“RQ”) with/without stretch are replotted for direct comparison. Throughout, 350 µM La³⁺ and -30 mmHg were used. Control data are \( \tau_{inactivation} \) values averaged from independent fits to traces before and after stretch.

0 mV with suction levels from -5 to -30 mmHg are plotted in Figure 3E. Stretch acceleration increased with increasing suction intensity. These results show that for voltages near both the foot (minimum) and the upper asymptote (maximum) of the g(V) relation, co-accelerated activation/inactivation was a stretch intensity-dependent phenomenon.

Stretch and voltage dependence of the inactivation time constants: R1623Q vs. WT. As expected, both current onset and decay were slower in R1623Q than in WT. In experiments with sufficiently good signal to noise that bandwidth demonstrably did not limit detection of current activation, time to 90% current peak was measured at -30 mV before stretch on WT and R1623Q. Time to 90% peak for WT was 1.65 ± 0.09 ms (n = 7) and for R1623Q 2.02 ± 0.06 ms (n = 6) (one-tailed t-test, p = 0.02). Figure 4 compares \( \tau_{inactivation} \) in oocyte patches with/without stretch. Experiments and analyses were done blinded as to whether an oocyte expressed WT or R1623Q channels. Statistics are given in the legend. For both R1623Q (Fig. 4A) and WT channels (not shown) decays were fitted to a single exponential. Figure 4B plots \( \tau_{inactivation} \) (V) for WT and R1623Q. As expected, at all voltages studied R1623Q inactivated slower than WT, and \( \tau_{inactivation} \) (V) was significantly shallower in R1623Q than in WT. Inactivation was 1.3-fold slower than WT at -40 mV and 2.6-fold slower at 0 mV, amounting to e-fold/36 mV in WT vs. e-fold/57 mV in R1623Q for \( \tau_{inactivation} \) (V). WT current (Fig. 4Ci) accelerated more strongly with stretch than R1623Q current (Fig. 4Cii). In WT, voltage dependence (slope of \( \tau_{inactivation} \) (V)) was unaffected by stretch. For R1623Q, stretch acceleration was weaker, but \( \tau_{inactivation} \) (V) steepened significantly (from e-fold/57 mV without stretch to e-fold/38 mV with stretch). Thus, while stretch due to -30 mmHg accelerated inactivation across all voltages in both channels, Figure 4Ciii shows that this moderately intense stimulus still left R1623Q decay slower than for WT in non-stretched membrane. Stretch acceleration factors obtained by double-rescaling versus fitting a single exponential to current decay (\( \tau_{inactivation} \)) were in good agreement, differing by <10% in 78% of cases for R1623Q (total of 49). Since it was possible to successfully apply the double rescaling analysis to R1623Q currents, we can conclude that the observed \( \tau_{inactivation} \) trends with stretch also apply for current onset periods. However, because WT rise times are more bandwidth-limited than R1623Q rise times, formal comparisons of WT vs. R1623Q stretch acceleration factors were based only on \( \tau_{inactivation} \).

Effect of stretch intensity on \( \tau_{inactivation} \): R1623Q vs. WT. \( \tau_{inactivation} \) for WT and R1623Q without and with stretch was quantified at stretch levels up to -20 mmHg in patches clamped to -30 mV and 0 mV (Fig. 5A). These procedures were blinded. The results are summarized as a ratio (control/stretch). At both voltages, the extent to which the ratio increased with increasing stretch intensity was significantly greater for WT than for R1623Q channels. Again, therefore, R1623Q channel currents were more weakly accelerated by stretch than for WT. Supplementary Figure S2 summarizes this at 3 voltages for WT vs. R1623Q responses to -16 mmHg (which generated moderate intensity stretch since -50 mmHg usually ruptured patches during 40 s applications; oocyte patches tolerate -100 mmHg for a few seconds).

Figure 5Bi illustrates, for R1623Q, typical control and -16 mmHg (stretch) currents from an experiment in which a series of
voltage clamp steps was applied. Figure 5Bi contrasts the stretch difference current for Figure 5Bi data to a comparable (i.e., also -16 mmHg) WT channel difference current. Whether cardiac myocytes experience stretch of comparable intensity is unknown. Nevertheless, the curved arrows emphasize that if NaV1.5-rich membranes were to experience stretch during voltage excursions (i.e., action potentials), stretch-induced excitatory current (downward component of Na+ difference current) would persist substantially longer in R1623Q than in WT myocytes.

Response of R1626P channels to stretch. NaV1.5-R1626P is another LQT3-related NaV1.5 D4S4 mutant reported from a human patient. R1626P in HEK cells exhibits an essentially voltage-independent current inactivation time course over the range -35 mV to 5 mV (half-time $\tau_{\text{inact}} \sim 3.2 \text{ ms}$). Thus, during action potentials in situ this mutant would yield abnormally prolonged $I_{\text{Na}}$. The flat $\tau_{\text{inact}} (V)$ suggests that substituting arginine with proline, a small neutral amino acid that kinks helical secondary structures, impairs voltage sensing by D4. From the originally envisaged model for D4S4 involvement in activation-inactivation coupling one can infer (a) that a NaV1.5 mutant with voltage-insensitive fast inactivation should have stretch-insensitive fast inactivation and (b) that current onset should accelerate with stretch without affecting the rate of current inactivation. We tested these predictions on R1626P channels in oocyte patches.

Figure 6A shows an R1626P current family. In the oocyte patches, as in HEK cells, R1626P current inactivated within milliseconds at all voltages. This process was voltage independent in the voltage range where activation changes steeply (Fig. 6B). In each of four patches tested for reversible

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**Figure 5.** Relative effectiveness of stretch on WT vs. R1623Q. (A) With increasing stretch-intensity, $\tau_{\text{inact}}$ changed more in WT than in R1623Q as seen from $\tau_{\text{inact}}$ ratios (without/with stretch for suctions up to -20 mmHg) for currents at -30 mV and at 0 mV (n = 3 at -30 mV and n = 6 at 0 mV for both WT and R1623Q). (Bii) R1623Q current with/without stretch (before/after averaged). (Bii) the stretch difference current obtained from those records overlapped with a comparable stretch difference current for WT channels. The two were peak normalized for -30 mV (arrow). Curved arrows show when WT stretch-induced excitatory current declined to zero to make the point that for R1623Q, stretch-induced excitatory current persisted longer than for WT.

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**Figure 6.** Stretch-induced effects on R1626P current. (A) A family of R1626P currents recorded without stretch (single sweeps from $V_{\text{hold}} = -100 \text{ mV}$, steps from -50 to 40 mV in 10 mV intervals); traces at -30 mV and +10 mV are darkened, the trace at 0 mV is marked. (B) $\tau_{\text{inact}} (V)$ obtained for 4 patches from single exponential fits to the inactivation phase of current from families like that in (A). Inset shows data from a different patch. Traces at the plotted values with fitted exponentials (green) and their respective $\tau_{\text{inact}} (V)$ values are listed. (C) average current before, during, after stretch (-20 mmHg suction used, 40 runs averaged for steps from -100 mV to -30 mV at 1 Hz). (Cii) double rescaling of the during-stretch current to the no-stretch (before/after averaged) current onset, yielding completely overlapping time courses for onset and decay. (Ciii) stretch difference currents (averaged before/after currents subtracted from during stretch currents; the multistep protocol included steps to -30 mV and 0 mV). 500 $\mu$M La$^{3+}$ was used throughout.
responses to stretch, R1626P channels reacted as in Figure 6Ci. Stretch increased both current onset and decay rates, though the absolute stretch effect was weaker than in WT. In response to -20 mmHg and clamp steps to -30 mV, the R1626P \( \tau_{\text{inactivation}} \) ratio for control/stretch (same parameter as for R1623Q, Fig. 5A) was significantly lower than for WT: 1.23 ± 0.02 (n = 3) for R1626P, compared to 1.79 ± 0.07 (n = 4) for WT (one-tailed t-test, p = 0.05). As in WT and R1623Q, peak current normalization alone did not produce overlapping traces but, in four of four R1626P patches, the double rescaling demonstrated complete overlap of current onset and decay time courses (Fig. 6Cii). As with WT and R1623Q, the stretch-induced fold-increase in current decay precisely matched the stretch-induced fold-increase in current onset rate. Thus, while R1626P kinetics (like R1623Q) responded more weakly to stretch than did WT, the prediction that coupling would fail in this D4 mutant was not met (as it was not for R1623Q).Stretch difference currents for R1626P (Fig. 6Ciii) reveal that for this mutant, stretch was exclusively excitatory (stretch is inhibitory when it yields a positive \( I_{Na} \) difference current). With a D4S4 dependent voltage-sensitive contribution to current decay missing, the stretch-activated component was also missing, which suggests that the primary (i.e., rate-limiting) effect of stretch on current decay in Na\(_{1.5}\) channels does not require stretch-acceleration of a D4S4-dependent process.

**Discussion**

Overview of the Na\(_{1.5}\) mutants studied: R1623Q and R1626P. In Long-QT syndrome, prolonged action potentials may contribute to ventricular arrhythmias that can result in syncope and sudden death. Deficient Na\(_{1.5}\) inactivation that yields excess \( I_{Na} \) is a primary cause of LQT3.\(^{13,38} \) In LQT3 mutations that affect the inactivation gate or its putative docking sites, inactivation rates are similar to WT. Post-inactivation burst openings explain the observed late macroscopic current component. However, some LQT3 mutants affect the voltage-dependence of inactivation kinetics in Na\(_{1.5}\). This depends on D4S4 motion.\(^{9,13} \) In the sporadically lethal D4-sensor neutralization (R-Q) mutant, R1623Q,\(^{11,39} \) what differs from WT, as confirmed here for oocyte patches (La\(^{3+}\) present), is that inactivation is slow and \( \tau_{\text{inactivation}} \) shows less steep voltage-dependence.\(^9 \) The LQT3 mutant R1626P,\(^{14} \) three amino acids beyond R1623 in D4S4, seems to be a more extreme case,\(^{16} \) in that a presumed proline kink in D4S4 evidently “paralyzes” the D4 sensor. In R1626P, inactivation is present but it is slow and \( \tau_{\text{inactivation}} \) is flat.\(^{14} \) In R1623Q, a reduced tendency to inactivate when open plus an elevated tendency to inactivate when closed allows the channel to deactivate then reactivate before inactivating (Fig. 7).\(^{15} \) The net outcome: a slower rise and decay of \( I_{Na} \) in WT and a more shallow \( \tau_{\text{inactivation}} \).\(^{9,15} \) During action potential clamp of R1623Q-expressing HEK cells, \( I_{Na} \) decay is relatively slow and on repolarization the \( I_{Na} \) increase exceeds that for WT, especially at low frequencies.\(^{11,39} \)

**Responses of Na\(_{1.5}\) R1623Q to stretch.** Moderately strong stretch elicited hyperpolarizing shifts of both the R1623Q and WT g(V) relations. At small depolarizations, stretch-induced peak \( I_{Na} \) increases were equal in R1623Q and WT Na\(_{1.5}\) records. During late diastole, therefore, either WT or mutant channels could contribute to the premature ventricular APs elicited by abrupt ventricular stretch stimuli.\(^{40} \) Lanthanum shifted Na\(_{1.5}\)-R1623Q gating but did not impair its stretch modulation, consistent with findings for Na\(_{1.5}\)-WT and other VGCs (K\(_{1.1}\), HCN2) with lanthanides present.\(^2,37 \) The conclusion of Li and Baumgarten, from their study of ventricular myocyte \( I_{Na} \),\(^{27} \) that lanthanide inhibition of \( I_{Na} \) is likely to contribute to suppression of stretch-induced arrhythmias acquires added significance knowing (a) that \( I_{Na} \) itself is stretch-modulated and (b) that SAC channels (the presumed target of the lanthanide at the time) are not present in adult ventricular myocytes. Cardiac myocytes (bearing either WT or the mutant Na\(_{1.5}\) channels) stretched to a degree that strained the sarcolemma, should, extrapolating from our data, exhibit stretch-augmented peak \( I_{Na} \).\(^* \) For lanthanide-sensitive stretch-induced ventricular arrhythmias, stretch-modulated VGC current, including stretch-modulated \( I_{Na} \), provides a plausible explanation (reviewed in ref. 4). When cardiac AP models begin incorporating stretch-induced changes in VGC responses, modeling effects of stretch on WT Na\(_{1.5}\) via rate changes yielding ~1.2-fold faster current rise and decay times (as expected for small stretch intensities) should be a meaningful starting point for comparison against experimental outcomes.

Inactivation kinetics in WT and R1623Q differed in that stretch did not alter the voltage-sensitivity of \( \tau_{\text{inactivation}} \) for WT but steepened this parameter for R1623Q. Even with the moderately strong stretch used in our experiments, however,
inactivation speed in R1623Q never matched that of WT without stretch. Nevertheless, at all tested voltages and stretch intensities, R1623Q, (like WT) exhibited stretch induced co-acceleration of activation and inactivation.

In WT, kinetic coupling between activation and inactivation seems near-optimal, with thermally-driven closing of the cytoplasmic-side inactivation gate following quickly once the activation gates concerted “open”. The resulting open-plugged condition is thought to be further stabilized by voltage-dependent D4S4 movements. Membrane stretch would be felt by all four voltage sensors. This, in principle, should accelerate both the coupled activation/inactivation events and possibly, enhance voltage-dependent stabilization of the open-plugged state. Although stretch steepened the voltage dependence of current decay for R1623Q, it did not do so for WT, in keeping with the view that WT unplugging probabilities are extremely low. In R1623Q (as in WT), depolarization-and-stretch-accelerated activation would tend to quickly result in an open state but the more sluggish mutant D4S4 might allow time for a deactivation (followed eventually by reactivation) before the stabilizing D4S4 movement could occur. Both without (see state diagrams, Fig. 7) and with stretch, R1623Q would tend to open-deactivate-reopen (etc.) more than in WT, thereby coupling the slower current onset to a slower current decay. Thus, kinetic coupling was observed without and with stretch in WT and in the mutants. This view of how D4S4 contributes to NaV channel inactivation kinetics and to activation/inactivation coupling fits with the picture of D4 voltage sensor operation inferred from ionic current kinetics of homotetrameric VGC chimeras fabricated by placing NaV channel D4 voltage sensors into a K,2 channel background. That approach implied that inherent speeds of NaV D4 voltage sensors in their structured lipid surround are what establish voltage dependent inactivation kinetics and not, for instance, mechanical linkages between the D4 sensor and inactivation gate machinery.

**Stretch modulation in a NaV1.5 mutant with simple kinetics.** R1626P current decay kinetics are (as in mammalian cells) insensitive to voltage changes within the activation range. Coupling of activation to inactivation as originally envisaged by Chen et al. would have implied that coupled stretch-accelerated NaV1.5 inactivation requires a voltage-dependent D4S4 motion. If so, the (voltage-insensitive) current decay rate of R1626P might be expected to be unresponsive to stretch and it would be expected that current decay would not couple kinetically to current onset during stretch.

Contrary to predictions from that model, R1626P current decay was accelerated by stretch and kinetic coupling between activation and inactivation was retained during stretch. Together with our observations for R1623Q, this R1626P behavior seems understandable if NaV1.5 stretch modulation is dominated by effects on the D1-D3 voltage sensors. However, even with proline substituting for arginine at location 1626 in D4S4, the domain 4 voltage sensor might be partially operational. NaV channel gating current and site-directed fluorescence changes during stretch have not been studied, so speculation on stretch-modulated motion differences in WT vs. D4S4 mutants is not warranted. Whatever the mechanistic details, current decay remained fully coupled to stretch-accelerated current onset in WT and the two mutants, with the extent of stretch acceleration maximal in WT.

In WT, R1623Q and R1626P, binding of the inactivation “particle” is evidently fast (i.e., not limiting for current decay). As stretch speeds activation, it proportionally speeds the time course of current decay because activation is rate limiting for inactivation. In WT and to a lesser extent R1623Q, but evidently not in R1626P, slower voltage-dependent (and, presumably, stretch-sensitive) conformation changes involving D4S4 accelerate the time course of current decay with depolarization and, perhaps, by countering the frequency of open channel deactivation (see Fig. 7 legend), also accelerate macroscopic current onset. As for WT NaV channels, the current decay time course of R1626P channels depends on the (fast) voltage independent rate of open state “plugging” by the inactivation particle plus the particle’s dissociation rate, with voltage dependent D4S4 movement somehow reducing the rate of particle dissociation. Our data for R1626P verified that when stretch enhances the probability that a NaV1.5 channel opens it also enhances (by the same-fold) the channel’s probability of inactivating. Thus, stretch acceleration of the current decay time course was fully explained by kinetic coupling to the stretch accelerated activation of current. Since this process of kinetic coupling evidently requires no voltage sensitivity in D4S4, D1-D3 responses to stretch appear to dominate stretch modulation of the NaV1.5 ionic current time course.

The contrast between the two LQT3-associated mutants and WT is evident in stretch difference currents for recombinant as well as native (rat ventricular myocyte) NaV channels (Fig. 1D). In WT, stretch inhibition dominated stretch modulation, such that over much of the action potential voltage range, stretch accelerated activation of I Na (stretch excitation) would quickly give way to stretch inhibition of I Na (in other words the stretch difference current for I Na would quickly switch from negative to positive, equivalent to a switch from inward to outward membrane current). For R1623Q, the excitation/inhibition balance would shift strongly towards excitation while for R1626P it would be exclusively excitatory; in both cases the effect would be pro-arrhythmic.

**Mechanoelectric feedback, the LQT3 syndrome and mutations in D4S4.** Cardiac myocytes contact and thus generate stresses felt by adjacent myocytes. Myocytes can also experience exertion-dependent externally-generated mechanical forces (e.g., wall, valve and Purkinje fiber distension during elevated blood volume, altered shear stresses). Whenever such mechanical events alter cardiac electrophysiological responses, mechanoelectric feedback is said to have occurred. In humans, atrial and ventricular pacing experiments suggest that mechanoelectric feedback is intrinsic to a situation wherein ventricular tachycardia triggers atrial fibrillation; right atrial blood forced against closed atrio-ventricular valves distends the atrium causing shorter APs and shorter effective AP refractory periods in stretched atrioventricular valves. The channels responsible are unknown, but stretch-modulated VGC currents, including NaV1.5, are plausible candidates. With each beat, systole generates a new round of mechanical stress in cardiac myocytes just as NaV1.5 channels are inactivating.
Of the structures thought to sense cardiac stress and strain at least three are associated with Na\textsubscript{v}1.5 channels: intercalated discs, Z-lines (mechanically linked to Na\textsubscript{v}1.5-rich t-tubules), and caveolae. At the endocardial surface (where Na\textsubscript{v}1.5 channel expression is particularly high) fluid shear over the sarcolemma might generate Na\textsubscript{v}1.5-mediated mechanoelectric feedback responses (reviewed in ref. 26). Normally, stretch-enhanced inactivation of I\textsubscript{Na} would dampen out mechanically-induced changes in membrane voltage. Comparison of WT and R1623Q responses to increasing stretch intensities suggests that such dampening would be impaired in hearts carrying the R1623Q mutation. Consider, for example, cardiomyocytes firing APs and repolarizing while experiencing moderately intense stretch (equivalent to -16 mmHg applied to a patch); at 0 mV, in a WT heart, I\textsubscript{Na} would inactivate ≥2x as fast as in a heart with R1623Q (τ\textsubscript{inactivation (0)} at -16 mmHg for R1623Q vs. WT would be ~1.8 ms vs. ~0.7 ms). For R1623Q, the relative insensitivity of the inactivation process to stretch and to voltage would tend to enhance net inward depolarizing current.

Cardiac mechanoelectric feedback may contribute to normal electrophysiology homeostasis. Since VGC current is time dependent, VGC contributions to mechanoelectric feedback should be more evident the longer the time spent at voltages where stretch-modulated current is dramatic. With all VGCs being stretch-modulated, AP modeling will be required but, a priori, effects should be greater at lower heart rates. In pig heart, load-induced shortening of AP duration is most pronounced at low AP frequencies, with a weaker load-induced shortening of AP duration at high AP frequencies. Stretch difference currents for recombinant pacemaker channels (HCN2) driven to AP waveforms show pronounced stretch-inhibition at low frequencies and mild stretch-enhancement at high frequencies.

Adverse LQT events (arrhythmic) are often associated with low heart rate, the pacing range where mechanoelectric feedback is relatively important. Increased late or non-inactivating I\textsubscript{Na} is common among identified LQT3 mutants however, for some LQT3 mutants including the two studied here the dominant abnormality is a slow inactivation rate. Perhaps stretch-modulation linked to impaired voltage-sensitive Na\textsubscript{v}1.5 inactivation can exacerbate this syndrome. Stretch modulation was qualitatively different in WT (mostly inhibitory) vs. R1623Q or R1626P channels (largely or exclusively excitatory).

Careful inspection of stretch modulation in diverse cardiac arrhythmia mutants with plausible mechano-phenotypes might provide a much-needed entrée for testing whether and how VGCs contribute to cardiac mechanoelectric feedback. A mechanoelectric feedback-mediated arrhythmia is implied for a LQT1-associated K\textsubscript{s}7 mutant (R14C-KCNQ1) linked to familial atrial fibrillation. In that case abnormal sensitivity of the channel to osmotic swelling (abnormal stretch-modulation?) might link age-related hypertension to late onset atrial fibrillation.

**Methods**

**Oocyte preparation and electrophysiology.** Experiments were done as previously described (additional detail is given in the Suppl. Material) at a room temperature of 22°C. Briefly, *Xenopus laevis* oocytes were injected with cRNA for human heart WT Na\textsubscript{v}1.5 (plasmid: pSP64T-hH1) or its R1623Q mutant (pSP64T-hH1-R1623Q), kindly provided by A.L. George and Jeffrey Balser respectively, or R1626P (see Suppl. Material). Plasmids were linearized with XbaI and transcribed with SP6 RNA polymerase (SP6 Message Machine, Ambion, Austin TX). Oocytes were exposed to hyperosmotic solution and devitellinized prior to patch clamp experiments.

The high-K bath solution contained (in mM) 89 KCl, 0.4 CaCl\textsubscript{2}, 5 HEPES, 0.8 MgCl\textsubscript{2}, (pH 7.4 with KOH). In the recording pipette, KCl was substituted with 89 mM NaCl (pH adjusted with NaOH). LaCl\textsubscript{3} (250 μM, 350 μM, 500 μM or 1 mM as indicated) was added to the pipette. Borosilicate pipettes (~2–4 MΩ) were sylgard-coated and tips were fire-polished using a soda glass-covered platinum filament. An Axopatch 200B amplifier recorded and filtered (5 KHz) currents via pClamp6 software (Axon Instruments). Currents were corrected by P/N linear subtraction (N = 8; depolarizing steps from -120 mV).

Cell-attached patches were stretched via pipette suction which was monitored by digital manometer. Voltage clamp protocols were structured to yield before/during/after data sets with currents collected as running averages of multiple protocol runs (40 runs repeated at 1 s intervals; each run consisted of three 20 ms test pulses separated by 20 ms at holding potential of -120 mV). To obtain data during stretch, suction was applied continuously. P/N subtraction was performed with/without stretch as appropriate. To prevent bias, stretch ‘dose response’ maneuvers and analysis were performed blinded as to whether oocytes expressed WT or R1623Q channels.

**Statistics.** Mean values are plotted with standard errors. Comparisons between averaged datasets were made with two-tailed (except where one-tailed is specified) unpaired t-tests with p = 0.05 the significance threshold. Comparisons between best fitting curves of sigmoid datasets (Origin software, OriginLab Northampton MA) were made via the F-test with p < 0.05 indicating significant differences with 95% confidence.

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**Note**

Supplementary materials can be found at: www.landesbioscience.com/supplement/BanderaliCHAN4-1-Sup.pdf
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