

# Hyperpolarized shifts in the voltage dependence of fast inactivation of Na<sub>v</sub>1.4 and Na<sub>v</sub>1.5 in a rat model of critical illness myopathy

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**Critical illness myopathy is a disorder in which skeletal muscle becomes electrically inexcitable. We previously demonstrated that a shift in the voltage dependence of fast inactivation of sodium currents contributes to inexcitability of affected fibres in an animal model of critical illness myopathy in which denervated rat skeletal muscle is treated with corticosteroids (steroid-denervated; SD). In the current study we examined whether expression of Na<sub>v</sub>1.5 contributes to the altered voltage dependence of sodium channel inactivation in SD muscle. We used TTX and  $\mu$ -conotoxin GIIIB to selectively block Na<sub>v</sub>1.4 in SD muscle and found that the level of Na<sub>v</sub>1.5 did not correlate closely with the shift in fast inactivation. Surprisingly, we found that the voltage dependence of inactivation of Na<sub>v</sub>1.4 was similar to that of Na<sub>v</sub>1.5 in skeletal muscle *in vivo*. In severely affected fibres, inactivation of both Na<sub>v</sub>1.4 and Na<sub>v</sub>1.5 was shifted towards hyperpolarized potentials. We examined the role of denervation and steroid treatment in the shift of the voltage dependence of inactivation and found that both denervation and steroid treatment contribute to the shift in inactivation. Our results suggest that modulation of the voltage dependence of inactivation of both Na<sub>v</sub>1.4 and Na<sub>v</sub>1.5 *in vivo* contributes to loss of electrical excitability in SD muscle.**

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Critical illness myopathy is a disorder of skeletal muscle in which acute weakness in patients is caused by failure of muscle fibres to generate action potentials (Rich *et al.* 1996, 1997; Bird & Rich, 2002). We have examined the cause of loss of muscle excitability in an animal model of critical illness myopathy in which denervated rat muscle is treated with corticosteroids *in vivo* (steroid-denervated; SD; Rich *et al.* 1998). We found that a hyperpolarized shift in the voltage dependence of sodium channel fast inactivation plays a central role in loss of muscle fibre excitability (Rich & Pinter, 2001, 2003). The cause of altered voltage dependence of sodium channel inactivation in SD muscle is unknown.

Innervated adult skeletal muscle expresses only the Na<sub>v</sub>1.4 sodium channel isoform (Yang *et al.* 1991; Rich *et al.* 1999). In SD muscle, however, RNA for a second sodium channel isoform (Na<sub>v</sub>1.5) is expressed at high levels, suggesting Na<sub>v</sub>1.5 protein may be abundant (Rich *et al.* 1999). *In vitro*, heterologously expressed Na<sub>v</sub>1.5 channels gate at more negative potentials than Na<sub>v</sub>1.4 channels so the presence of Na<sub>v</sub>1.5 could shift the voltage dependence of sodium current in SD muscle (Wang *et al.*

1996; Zhang *et al.* 1999). Thus, one explanation for the shift in the voltage dependence of sodium channel inactivation in SD muscle is the presence of high levels of Na<sub>v</sub>1.5. However, a study of sodium channel gating in denervated muscle found that although Na<sub>v</sub>1.5 contributed less than 30% of total sodium conductance, there was a 10 mV shift of sodium channel activation and inactivation towards more hyperpolarized potentials (Pappone, 1980). This raises the possibility that the voltage dependence of Na<sub>v</sub>1.4 gating is altered following denervation. Since the animal model of critical illness myopathy that we use involves denervation of skeletal muscle, we also considered a second possibility: that altered Na<sub>v</sub>1.4 gating contributes to the hyperpolarized shift in sodium channel inactivation in SD muscle.

To determine whether expression of Na<sub>v</sub>1.5 causes the shift in gating of sodium current in SD muscle we selectively blocked Na<sub>v</sub>1.4 with tetrodotoxin (TTX) and  $\mu$ -conotoxin GIIIB. We found that hyperpolarized shifts in the voltage dependence of sodium current gating did not correlate closely with the amount of Na<sub>v</sub>1.5 present. Instead, in more severely affected fibres, there was a

hyperpolarized shift in the voltage dependence of fast inactivation of both Na<sub>v</sub>1.4 and Na<sub>v</sub>1.5. Our data suggest that modulation of the voltage dependence of inactivation of the Na<sub>v</sub>1.4 and Na<sub>v</sub>1.5 sodium channel isoforms plays an important role in loss of muscle fibre excitability in the animal model of critical illness myopathy.

## Methods

### Tissue preparation and perfusion

Denervation, treatment of rats and viewing of muscle fibres were done as previously described (Rich *et al.* 1998; Rich & Pinter, 2001). Briefly, rat muscle was denervated by removing a 10-mm segment of the left sciatic nerve in anaesthetized (ketamine, 5 mg kg<sup>-1</sup>, and zylaxine, 15 mg kg<sup>-1</sup>, administered intraperitoneally) adult female Wistar rats (250–350 g body weight). Dexamethasone (5 mg kg<sup>-1</sup>) was injected daily intraperitoneally beginning on the day of denervation and continuing for 7–11 days. Rats were killed by carbon dioxide inhalation, the extensor digitorum longus (EDL) was dissected tendon to tendon and muscle fibres were labelled with 10 μM 4-(4-diethylaminostyryl)-N-methylpyridinium iodide (4-Di-2-ASP) and visualized using an upright epifluorescence microscope. For all experiments the recording chamber was continuously perfused with solution containing (mM): NaCl, 118; KCl, 3.5; CaCl<sub>2</sub>, 1.5; MgSO<sub>4</sub>, 0.7; NaHCO<sub>3</sub>, 26.2; NaH<sub>2</sub>PO<sub>4</sub>, 1.7; glucose, 10.8 (pH 7.3–7.4, 20–22°C) equilibrated with 95% O<sub>2</sub> and 5% CO<sub>2</sub>. All animal protocols were performed in accordance with Emory University IACUC guidelines.

### Loose patch voltage clamp

Loose patch voltage recording and analysis was performed as previously described (Rich & Pinter, 2001, 2003). Briefly, patch electrodes were made from soft glass (catalogue no. 22-358739, Fisher Scientific) using a horizontal pipette puller (Flaming/Brown type) and were heat polished. Patch pipettes were filled with the normal external solution (see above) containing 1 ng ml<sup>-1</sup> sulforhodamine. The seal factor ( $R_s/(R_s + R_p)$ , where  $R_s$  is the shunt resistance and  $R_p$  is the patch pipette resistance) was kept greater than 0.4. The leak current was compensated manually by adjusting the compensation current (Stuhmer *et al.* 1983). Shunt resistance was measured on-line immediately before the application of each voltage step and used to adjust the step amplitude.

Fibres were not impaled to measure the resting potential prior to seal formation. Instead the resting potential was assumed (based on an average of 5–10 fibres) during voltage protocols and then measured after patch recordings were complete. The difference between the assumed and measured resting potential was then used to correct

step voltages used during data acquisition. By impaling the muscle fibre after patch recordings were complete, potential problems related to issues of muscle damage and depolarization due to impalement were avoided.

### Tetrodotoxin and μ-conotoxin GIIIB application

Tetrodotoxin in citrate (TTX) was purchased from Alomone Laboratories (Jerusalem). Prior to application of TTX we measured the voltage dependence of fast inactivation in several fibres in each muscle. We then applied 300 nM TTX to the bath and used suction to fill the pipette with the same TTX solution. We then reformed seals on the same muscle fibres and measured sodium currents. Muscle fibres were re-identified using pictures we had taken during the initial recording of sodium currents (Fig. 2). To help identify fibres we stained endplates by applying 5 μg ml<sup>-1</sup> rhodamine-conjugated bungarotoxin (Molecular Probes, Eugene OR, USA) for 30 s. Sodium currents were recorded near (within 100–200 μm), but never over muscle fibre endplates. By assuming a  $K_d$  of 1.9 μM for Na<sub>v</sub>1.5 (White *et al.* 1991) and 5 nM for Na<sub>v</sub>1.4 (Trimmer *et al.* 1989; for review see Goldin, 2001) one can estimate that 300 nM TTX blocked 97% of Na<sub>v</sub>1.4 and 23% of Na<sub>v</sub>1.5 (Lupa *et al.* 1995). We then solved for the percentage of Na<sub>v</sub>1.4 and Na<sub>v</sub>1.5 using the following equations:

$$\text{Na}_{v1.4} + \text{Na}_{v1.5} = \text{Na}_{\text{tot}}$$

$$(0.03)\text{Na}_{v1.4} + (0.77)\text{Na}_{v1.5} = \text{Na}_{\text{TTX}}$$

where  $\text{Na}_{\text{tot}}$  is the amplitude of the sodium current before application of TTX and  $\text{Na}_{\text{TTX}}$  is the sodium current after application of 300 nM TTX.

μ-Conotoxin GIIIB was purchased from Sigma (St Louis, MO, USA) and was applied to muscle fibres in the same way as TTX. In control fibres ( $n = 10$ ) we found that the dose of μ-conotoxin GIIIB required to block close to 100% of the current carried by Na<sub>v</sub>1.4 was 600 nM.

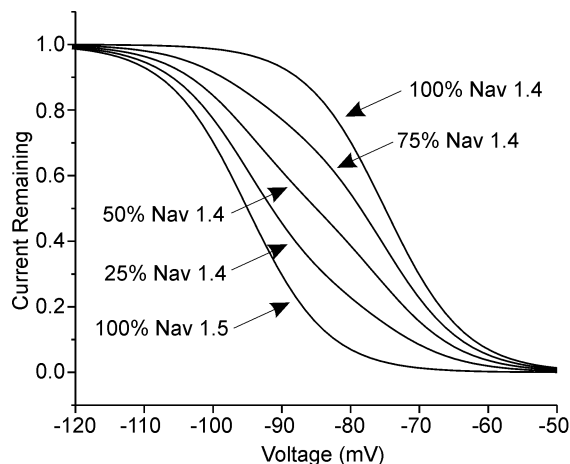
### Measurement of Inactivation of Na<sub>v</sub>1.4 and Na<sub>v</sub>1.5

Measures of fast inactivation were performed as previously described and all data was fitted to a Boltzmann function (Rich & Pinter, 2003). Briefly, measurement of fast inactivation was performed using a 50 ms pre-pulse to the test potential followed by a 10 ms pulse to -20 mV. For calculation of Na<sub>v</sub>1.4 inactivation, fibres were analysed in one of two ways. In SD fibres in which sodium current remaining after application of TTX or μ-conotoxin GIIIB was less than 10% of the amplitude prior to application of toxin, it was assumed that current was carried entirely by Na<sub>v</sub>1.4 since the error introduced by the presence of Na<sub>v</sub>1.5 was minimal. In fibres in which more than 10% of the sodium current remained after application of toxin, the voltage dependence of the Na<sub>v</sub>1.4 gating was

calculated as follows: sodium current remaining after application of toxin (TTX currents were corrected for amplitude using the equations given above) was subtracted from the sodium current prior to toxin application at each voltage step. The current amplitudes after subtraction of current carried by  $Na_v1.5$  were then fitted with Boltzmann distributions and analysed.

### Statistics and generation of modelled Boltzmann curves

All data other than linear fits were compared using Student's *t* test to determine statistical significance. Bonferroni correction was applied in all instances where multiple comparisons were made between treatment groups. All means are given  $\pm$  the s.e.m. Fitting of the data analysing correlations between parameters was performed using linear regression analysis. All fits (linear and Boltzmann) were performed using Origin software (OriginLab Corp., Northampton, MA, USA). Boltzmann curves for simulation of  $Na_v1.4$  and  $Na_v1.5$  gating were generated, scaled and added using Origin software. For example, to generate a curve made up of 75%  $Na_v1.4$ , the values from the  $Na_v1.4$  curve were multiplied by 0.75 and added to the 0.25 times the values from the  $Na_v1.5$  curve. Curves generated by mixing various percentages of  $Na_v1.4$  and  $Na_v1.5$  were then fitted with Boltzmann curves



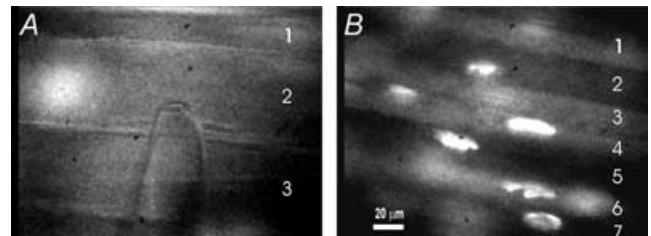
**Figure 1.** Simulation of the voltage dependence of sodium current inactivation in SD fibres with increasing percentages of  $Na_v1.5$

Values for the voltage dependence of inactivation were taken from Wang *et al.* (1996) and Zhang *et al.* (1999). The midpoint of inactivation used for  $Na_v1.4$  ( $\mu 1$ , SkM1) was  $-75$  mV with a slope of 5.8. For  $Na_v1.5$  (Hh1) the values were  $-95$  mV and 5.8, respectively. When 25% of sodium current was carried by  $Na_v1.5$  the midpoint of inactivation was shifted to  $-79.5$  mV with a slope of 7.9; when 50% of current was carried by  $Na_v1.5$  the midpoint was  $-85$  mV with a slope of 8.8 and when 75% of current was carried by  $Na_v1.5$  the midpoint was  $-90.5$  mV with a slope of 7.9.

to estimate the midpoint and slope of the sodium current carried by the mix of sodium channel isoforms.

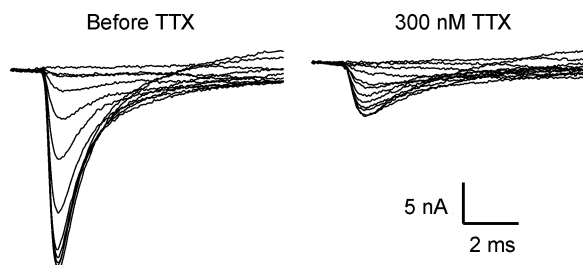
### Results

Both  $Na_v1.4$  and  $Na_v1.5$  sodium channel isoforms are present in steroid-treated, denervated rat muscle (SD muscle) so the voltage dependence of sodium current inactivation is the result of inactivation of both sodium channel isoforms (Rich *et al.* 1999). Studies *in vitro* have found that fast inactivation of  $Na_v1.5$  occurs at potentials 20 mV more hyperpolarized than for  $Na_v1.4$  (Wang *et al.* 1996; Zhang *et al.* 1999). The shift in the voltage dependence of fast inactivation in affected SD muscle that we found previously was slightly more than 10 mV (Rich & Pinter, 2001, 2003). To better understand the effect of mixing two populations of sodium channels with a 20 mV difference in the voltage dependence of inactivation, we modelled the effect of increasing the percentage of  $Na_v1.5$  on gating of the mix of sodium channels. As shown in Fig. 1, increasing the percentage of  $Na_v1.5$  gradually shifts the voltage dependence towards hyperpolarized potentials. In order to shift the voltage dependence of fast inactivation by 10 mV, 50% of sodium current had to be carried by  $Na_v1.5$ . However, increasing the percentage of  $Na_v1.5$  also reduced the slope of the voltage dependence of fast inactivation. In the simulation we used a slope of 5.8 for both  $Na_v1.4$  and  $Na_v1.5$  (Wang *et al.* 1996; Zhang *et al.* 1999). When 50% of the current was carried by  $Na_v1.5$  the slope decreased to 8.8. Even when only 25% of current was carried by  $Na_v1.5$  such that the shift in the midpoint of inactivation was only 4.4 mV, the slope of the Boltzmann fit decreased to 7.9. In our previous studies we found no evidence of a decrease in the slope of fast inactivation in SD fibres with hyperpolarized voltage dependence of fast inactivation (Rich & Pinter, 2001, 2003).



**Figure 2.** Images of control and SD fibres used for repeated formation of seals on the same fibres

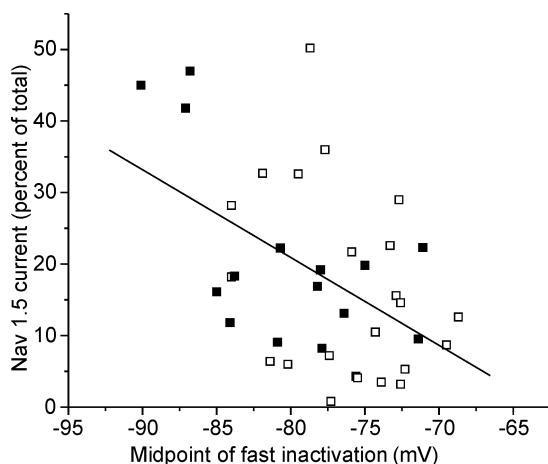
A, three fibres in a control muscle are shown running from left to right. An out of focus endplate stained with rhodamine-conjugated bungarotoxin is present on the left side of fibre 2. A patch electrode is shown contacting fibre 2. B, seven fibres are shown running diagonally in an SD muscle. Fibre diameter is much smaller than in control muscle. A number of endplates stained with rhodamine-bungarotoxin are seen in the field. The presence of endplates is useful in identifying individual fibres when reforming seals.



**Figure 3. Fast inactivation in an SD fibre before and after application of 300 nM TTX**

After application of 300 nM TTX, close to 70% of the sodium current is blocked. Despite having a relatively high level of  $Na_v1.5$ , the midpoint of inactivation prior to TTX application was  $-72.4$  mV; a value similar to that of control muscle. Furthermore, the voltage dependence of inactivation before and after TTX application was similar (midpoint of inactivation after TTX =  $-70.5$  mV). This suggests that the voltage dependence of inactivation of  $Na_v1.4$  and  $Na_v1.5$  are similar in this fibre.

To examine the role of  $Na_v1.5$  expression in the hyperpolarized shift of sodium current inactivation we measured fast inactivation in 23 SD fibres in the absence and presence of 300 nM TTX. We confirmed that 300 nM TTX blocked nearly all sodium current carried by  $Na_v1.4$  (Lupa *et al.* 1995) in six control fibres (data not shown). In each SD muscle we recorded from several fibres in the absence of TTX and then recorded from the same series of fibres in the presence of TTX (see methods). Fibres were visually re-identified for recording (Fig. 2) so that TTX was only applied once during each experiment.

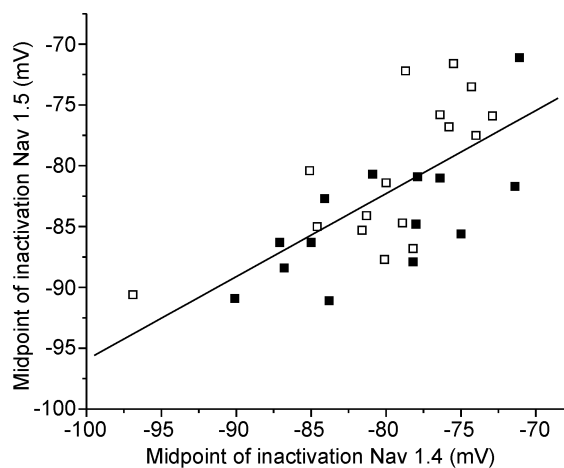


**Figure 4. An increase in the percentage of sodium current carried by  $Na_v1.5$  does not fully account for the shift in the voltage dependence of fast inactivation in SD fibres**

The percentage of  $Na_v1.5$  present in individual fibres is plotted against the midpoint of fast inactivation for the fibre. Superimposed on the graph is the best linear fit of the data ( $R = -0.49$ ,  $P < 0.01$ ). Although there was a correlation between the percentage of current carried by  $Na_v1.5$  and the midpoint of inactivation, there were many fibres with little  $Na_v1.5$  in which there were large shifts in the voltage dependence of inactivation. ■, data from fibres treated with  $\mu$ -conotoxin GIIIB; □, data from fibres treated with TTX.

Based on published data, greater than 50% of sodium current would need to be carried by  $Na_v1.5$  in order to cause a shift of fast inactivation of greater than 10 mV (Fig. 1) (Wang *et al.* 1996; Zhang *et al.* 1999). It is estimated that a dose of 300 nM TTX blocks 97% of  $Na_v1.4$  current and 23% of  $Na_v1.5$  current (Lupa *et al.* 1995). We found that the majority of sodium current in most fibres was blocked by a dose of 300 nM TTX. An example of a fibre in which close to 30% of the current remained after TTX application is shown in Fig. 3. The voltage dependence of fast inactivation in the fibre prior to application of TTX was  $-72.7$  mV with a slope of 4.6. These values are similar to those obtained from control muscle which has no  $Na_v1.5$ , and suggest that, in the fibre shown, having more than a third of the sodium current carried by  $Na_v1.5$  was not sufficient to induce a hyperpolarized shift in fast inactivation of the total current.

To examine the effect of differing amounts of  $Na_v1.5$  on the voltage dependence of inactivation, we plotted the percentage of current carried by  $Na_v1.5$  versus the midpoint of fast inactivation in SD fibres and found a correlation (Fig. 4,  $R = -0.49$ ,  $P < 0.01$ ). However, in a number of fibres there were hyperpolarized shifts in the voltage dependence of inactivation in the absence of significant expression of  $Na_v1.5$ . This suggests that a shift in the voltage dependence of  $Na_v1.4$  may also contribute to the shift in voltage dependence of inactivation. We thus determined the voltage dependence of both  $Na_v1.4$  and  $Na_v1.5$  in SD muscle. In 7/23 fibres too little current remained after application of TTX to determine the voltage



**Figure 5. The voltage dependence of inactivation of both  $Na_v1.4$  and  $Na_v1.5$  is shifted towards hyperpolarized potentials in affected SD fibres**

The plot of midpoint of inactivation of  $Na_v1.5$  versus the midpoint of inactivation of  $Na_v1.4$  in individual SD fibres reveals a significant correlation between the midpoints of inactivation of the two sodium channel isoforms ( $R = 0.68$ ,  $P < 0.01$ ). ■, data from fibres treated with  $\mu$ -conotoxin GIIIB; □, data from fibres treated with TTX.

**Table 1. Voltage dependence of inactivation of Nav1.4 and Nav1.5**

	Control	Steroid treated	Denervated	SD
Nav1.4	-72.6 ± 1.0	-72.4 ± 1.2	-76.4 ± 1.2	-80.0 ± 1.1
	6.2 ± 0.4	5.7 ± 1.0	6.5 ± 0.4	5.0 ± 0.9
	n = 19	n = 11	n = 15	n = 30
Nav1.5	No current	No current	-79.3 ± 1.6	-82.3 ± 1.1
			9.5 ± 0.7	11.8 ± 0.7
			n = 15	n = 30

Values shown for the voltage dependence of inactivation in control, steroid-treated innervated, denervated, and steroid-treated denervated muscle. The top value in each block represents the midpoint of inactivation (mV), the second value is the slope of inactivation (mV), and the third value is the number of fibres studied. Values shown represent the means ± S.E.M.

dependence of Nav1.5. However, in 16 fibres we were able to measure the voltage dependence of sodium current inactivation after application of 300 nM TTX. In these fibres, the current carried by Nav1.5 was subtracted from the total current to leave only current carried by Nav1.4 (see Methods). This allowed for comparison of inactivation of Nav1.4 relative to that of Nav1.5 in individual fibres. There was no significant difference between the calculated midpoint of fast inactivation of Nav1.4 and Nav1.5. This finding differed dramatically from previous studies that have compared gating of Nav1.4 and Nav1.5 *in vitro* (Wang *et al.* 1996; Zhang *et al.* 1999). We thus further examined inactivation of Nav1.4 relative to Nav1.5 in skeletal muscle *in vivo*.

Although TTX is an excellent tool for distinguishing between Nav1.4 and Nav1.5, at doses needed to block Nav1.4 there is block of a portion of Nav1.5 channels. Since Nav1.5 currents are generally small to begin with, this partial block makes measurement of Nav1.5 inactivation difficult. To further evaluate inactivation of Nav1.5 we performed a set of experiments in which  $\mu$ -conotoxin GIIIB was used to selectively block Nav1.4.  $\mu$ -Conotoxin GIIIB is selective for Nav1.4 (Cruz *et al.* 1985; Li *et al.* 2003), and thus allows for measurement of larger Nav1.5 currents. We confirmed that we could block greater than 99% of sodium current carried by Nav1.4 using 600 nM  $\mu$ -conotoxin in 10 control muscle fibres (data not shown). When we blocked Nav1.4 using 600 nM  $\mu$ -conotoxin GIIIB in SD muscle, slightly more current remained than after block with TTX (21.4% versus 14.9%). The estimate of the amount of sodium current carried by Nav1.5 was similar in the two groups (21.4% versus 16.2%,  $P = 0.25$ ). Thus, data following application of 300 nM TTX was pooled with data following application of 600 nM  $\mu$ -conotoxin GIIIB.

Not all SD fibres have a hyperpolarized shift in the voltage dependence of inactivation (Rich & Pinter, 2001, 2003). The reason for the variation between fibres is unknown, but it provided a way to compare the voltage dependence of inactivation of Nav1.4 to that of Nav1.5 in

both mildly and severely affected fibres. We found that the voltage dependence of inactivation of Nav1.4 and Nav1.5 were shifted in parallel in more severely affected SD fibres (Fig. 5,  $R = 0.68$ ,  $P < 0.01$ ). This suggests that the process underlying the shift in inactivation affects both Nav1.4 and Nav1.5.

To determine whether the similarity in the voltage dependence of inactivation of Nav1.4 and Nav1.5 was caused by SD treatment we measured inactivation of Nav1.4 and Nav1.5 in both SD and denervated muscle (Table 1, Fig. 6). We have previously found that both denervation and SD treatment of muscle shift inactivation in a subset of fibres (Rich & Pinter, 2003). The primary difference is that SD treatment causes the shift in a greater percentage of fibres. Steroid treatment had no effect on the voltage dependence of inactivation of Nav1.4 in innervated muscle. In control and steroid-treated innervated muscle, there was very little current carried by Nav1.5, so the effect of steroid treatment on inactivation of Nav1.5 in innervated muscle could not be studied. Following SD treatment, there was a shift in the voltage dependence of inactivation of Nav1.4 ( $P < 0.01$ ). Denervation alone did not cause a statistically significant shift in the gating of Nav1.4 ( $P = 0.10$ ), suggesting that both denervation and treatment with steroids contribute to the shift. This agrees with our previous finding that steroid treatment amplifies the effect of denervation on the midpoint of inactivation (Rich & Pinter, 2003).

In neither denervated nor SD fibres was there a statistically significant difference between the voltage dependence of inactivation of Nav1.4 and Nav1.5 (Table 1). There was also no significant difference in the voltage dependence of inactivation of Nav1.5 between denervated and SD fibres. Thus it is unclear whether steroid treatment contributes to the shift of inactivation of Nav1.5 that occurs in more severely affected fibres (Fig. 5). It does appear, however, that the voltage dependence of inactivation of Nav1.4 and Nav1.5 is similar in both denervated and SD fibres.

## Discussion

We report that a hyperpolarized shift in the voltage dependence of fast inactivation of both  $\text{Na}_v1.4$  and  $\text{Na}_v1.5$  is present in affected fibres in an animal model of critical illness myopathy. Surprisingly, we found that the voltage dependence of inactivation of  $\text{Na}_v1.4$  is similar to that of  $\text{Na}_v1.5$  in skeletal muscle *in vivo*. Our data suggest that modulation of the voltage dependence of inactivation of both  $\text{Na}_v1.4$  and  $\text{Na}_v1.5$  contributes to muscle fibre electrical inexcitability in the animal model of critical illness myopathy.

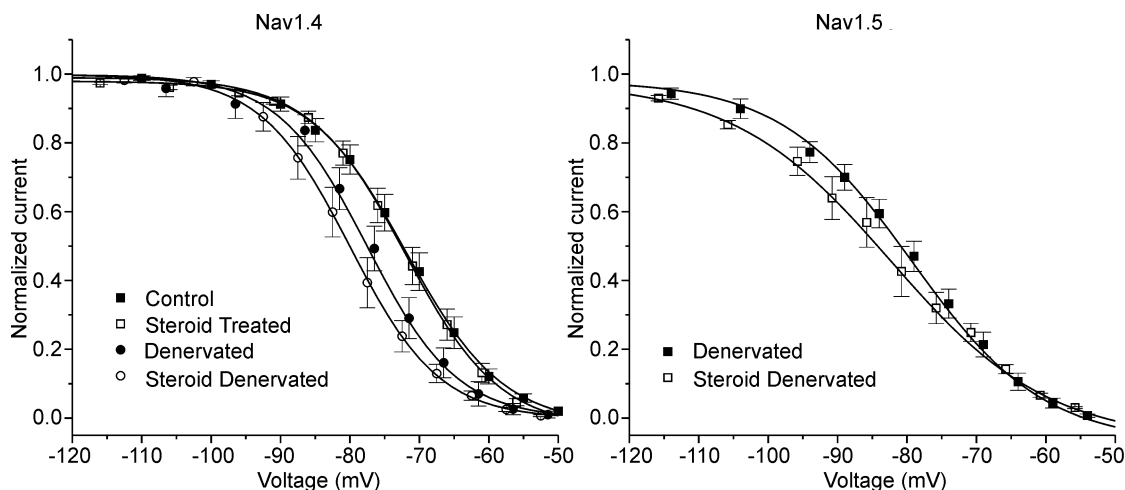
### The role of $\text{Na}_v1.5$ expression on excitability of SD muscle

We have previously demonstrated that loss of electrical excitability in patients with critical illness myopathy can be replicated by combining denervation and corticosteroid treatment in rat muscle *in vivo* (steroid-denervated; SD) (Rich *et al.* 1998; Rich & Pinter, 2001). In this model there are a number of factors that contribute to reduced excitability of muscle. Depolarization of the resting potential following denervation is one of the most important factors since it increases inactivation of sodium channels (Rich & Pinter, 2003). However, changes in the specific membrane resistance as well as a reduction in sodium current density are also contributing factors (Rich *et al.* 1998). Finally, there is a 10–15 mV hyperpolarized shift in the voltage dependence of fast inactivation that

appears to play a central role in loss of excitability (Rich & Pinter, 2001, 2003). The current study was aimed at determining the cause of this shift.

In normal muscle fibres, only  $\text{Na}_v1.4$  is present (Yang *et al.* 1991). In SD muscle, however, mRNA for both the  $\text{Na}_v1.4$  and  $\text{Na}_v1.5$  is present at high levels (Rich *et al.* 1999). The voltage dependence *in vitro* of fast inactivation of the  $\text{Na}_v1.5$  has been found to be close to 20 mV more negative than for  $\text{Na}_v1.4$  (Wang *et al.* 1996; Zhang *et al.* 1999). Thus, it seemed reasonable to expect that higher levels of  $\text{Na}_v1.5$  might underlie the hyperpolarized shift in the voltage dependence of fast inactivation in affected SD fibres. However, we found that higher levels of  $\text{Na}_v1.5$  correlated only loosely with a more hyperpolarized voltage dependence of inactivation.

Comparison of the voltage dependence of inactivation of  $\text{Na}_v1.4$  to that of  $\text{Na}_v1.5$  *in vivo* revealed a similar voltage dependence of inactivation. These data suggest that the voltage dependence of inactivation of  $\text{Na}_v1.5$  may be different *in vivo* from what has been reported *in vitro*. One explanation for the difference is that most studies of the voltage dependence of  $\text{Na}_v1.5$  have used traditional gigaseal patch recording whereas we are using loose patch. It has been found that gigaseal patch recording shifts the voltage dependence of  $\text{Na}_v1.5$  in isolated cardiac myocytes by close to -20 mV whereas loose patch does not (Eickhorn *et al.* 1994). If  $\text{Na}_v1.5$  activates and inactivates at similar potentials to  $\text{Na}_v1.4$ , its re-expression in denervated and SD muscle might be expected to have little effect on excitability. However,  $\text{Na}_v1.5$  has been found to be less



**Figure 6. The voltage dependence of fast inactivation of  $\text{Na}_v1.4$  and  $\text{Na}_v1.5$  following denervation and steroid treatment**

On the left, the normalized current carried by  $\text{Na}_v1.4$  is plotted *versus* the pre-pulse potential for control, denervated, steroid-treated and SD muscle fibres. The inactivation curves of  $\text{Na}_v1.4$  from control and steroid-treated fibres are nearly superimposed. The inactivation curve for denervated fibres is moderately shifted towards more hyperpolarized potentials; the curve for SD fibres is the most shifted. On the right, the normalized current carried by  $\text{Na}_v1.5$  is plotted *versus* the pre-pulse potential for denervated and SD muscle fibres. Too little  $\text{Na}_v1.5$  current was present to study in control and steroid treated muscle. Error bars represent the s.e.m. Control ( $n = 19$ ), steroid treated ( $n = 11$ ), denervated ( $n = 15$ ), steroid denervated ( $n = 30$ ).

susceptible to slow inactivation (Richmond *et al.* 1998; Vilin *et al.* 2001). Since both denervated and SD muscle fibres have relatively depolarized resting potentials, the resistance of Na<sub>v</sub>1.5 to slow inactivation may serve to increase excitability.

The finding that the voltage dependence of inactivation of Na<sub>v</sub>1.5 can be modified in a model of critical illness myopathy raises the question as to whether patients with critical illness myopathy may have cardiac problems caused by increased inactivation of Na<sub>v</sub>1.5. We previously found that severe sepsis can cause critical illness myopathy (Rich *et al.* 1997; Bird & Rich, 2002) as well as abnormalities on ECG that are consistent with a reduction in cardiac sodium current (Rich *et al.* 2002). Such a reduction in cardiac sodium current may contribute to the decrease in cardiac contractility that is found in sepsis (Parker *et al.* 1984; Ognibene *et al.* 1988). It is thus possible that an alteration in the voltage dependence of inactivation of Na<sub>v</sub>1.5 contributes to cardiac dysfunction in septic patients.

#### Possible factors modulating voltage dependence of inactivation of Na<sub>v</sub>1.4 and Na<sub>v</sub>1.5

There is evidence suggesting that gating of Na<sub>v</sub>1.4 is modulated *in vivo* from work studying the voltage dependence of sodium channel gating in different fibre types. It has been found that slow twitch fibres have a more positive voltage dependence of inactivation (Ruff & Whittlesey, 1993; Ruff, 1996). Since Na<sub>v</sub>1.4 is the only sodium channel present in adult muscle (Yang *et al.* 1991; Rich *et al.* 1999), this suggests that gating of Na<sub>v</sub>1.4 is different in fast and slow fibres. We used the extensor digitorum longus muscle in which the vast majority of fibres are fast twitch (Ruff *et al.* 1982). Thus, in our study the shift we see in fast inactivation is occurring in fibres that had a fast phenotype prior to denervation and steroid treatment.

A number of secondary modifications have been found to modulate sodium channel function *in vitro*. Phosphorylation by PKA and PKC has been shown to reduce peak sodium conductance (Cantrell & Catterall, 2001) through a process that is similar to slow inactivation (Carr *et al.* 2003). However, in general, phosphorylation does not cause major changes in the voltage dependence of fast inactivation (Cantrell & Catterall, 2001). Glycosylation shifts the voltage dependence of fast inactivation of both Na<sub>v</sub>1.4 and Na<sub>v</sub>1.5 toward hyperpolarized potentials through what appears to be a surface charge mechanism (Bennett *et al.* 1997; Zhang *et al.* 1999). However, the shift in the voltage dependence of fast inactivation caused by glycosylation appears modest. Nitric oxide has also been found to inhibit neuronal sodium currents through hyperpolarized shifts in both fast and slow inactivation (Li *et al.* 1998; Bielefeldt *et al.* 1999). It is not known whether

nitric oxide can affect gating of Na<sub>v</sub>1.4 or Na<sub>v</sub>1.5. It has recently been found that calcium-dependent binding of calmodulin enhances slow inactivation of Na<sub>v</sub>1.5 sodium channels (Tan *et al.* 2002). Calmodulin binds Na<sub>v</sub>1.4 in a calcium-independent manner, but appears to have little effect on the voltage dependence of activation and fast inactivation (Herzog *et al.* 2003; see, however, Deschenes *et al.* 2002). Further studies in SD muscle will be necessary to determine whether any of these mechanisms are involved in the altered voltage dependence of Na<sub>v</sub>1.4 and Na<sub>v</sub>1.5 inactivation in this disorder.

Our work suggests that a hyperpolarized shift in the voltage dependence of inactivation of Na<sub>v</sub>1.4 and Na<sub>v</sub>1.5 contribute to muscle fibre inexcitability in the animal model of critical illness myopathy. Other situations in which abnormal regulation of excitability occurs in the setting of genetically normal sodium channels include neuropathic pain and neuronal response to demyelination (Waxman *et al.* 2000; Lai *et al.* 2003). However, in these situations it appears that regulation of sodium channel isoform expression may be the most important change. It thus appears that loss of muscle fibre excitability in critical illness myopathy represents a new kind of ion channel disease in which the defect is caused by neither a mutation in the channel nor a change in isoform expression.

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