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ION CHANNELS, TRANSPORTERS

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Pharmacological properties of neuronal TTX-resistant sodium channels and the role of a critical serine pore residue

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Abstract Voltage-gated sodium channels can be characterized by their sensitivity to inhibitors. $Na_v1.5$ is sensitive to block by cadmium and extracellular QX-314, but relatively insensitive to tetrodotoxin and saxitoxin. $Na_v1.4$ is tetrodotoxin- and saxitoxin-sensitive but resistant to cadmium and extracellular QX-314. $Na_v1.8$ and $Na_v1.9$ generate slowly inactivating ($I_{TTXr\text{-}Slow}$) and persistent ($I_{TTXr\text{-}Per}$) currents in sensory neurons that are tetrodotoxin-resistant. Tetrodotoxin sensitivity is largely determined by the identity of a single residue; tyrosine 401 in $Na_v1.4$, cysteine 374 in $Na_v1.5$ and serine 356 and 355 in $Na_v1.8$ and $Na_v1.9$. We asked whether $Na_v1.8$ and $Na_v1.9$ share other pharmacological properties as a result of this serine residue. $I_{TTXr\text{-}Slow}$ and $I_{TTXr\text{-}Per}$ were saxitoxin-resistant and resistant to internal QX-314. $I_{TTXr\text{-}Slow}$ was also resistant to external QX-314 and displayed a approximately fourfold higher sensitivity than $I_{TTXr\text{-}Per}$ to cadmium. The impact of the serine residue was investigated by replacing tyrosine 401 in $Na_v1.4$ with serine (Y401S) or cysteine (Y401C). Both mutants were resistant to tetrodotoxin and saxitoxin. Whereas $Na_v1.4$-Y401C displayed an increased sensitivity to cadmium and extracellular QX-314, the serine substitution did not alter the sensitivity of $Na_v1.4$ to cadmium or QX-314. Our data indicates that while the serine residue determines the sensitivity of $I_{TTXr\text{-}Slow}$ and $I_{TTXr\text{-}Per}$ to tetrodotoxin and saxitoxin, it does not determine their insensitivity to QX-314 or their differential sensitivities to cadmium.

Keywords Sodium channel antagonist · Sodium current · Saxitoxin · Tetrodotoxin · Tetrodotoxin-resistant channels · Sodium channel

Abbreviations $Na_v1.4$: Voltage-gated sodium channel α-subunit from skeletal muscle · $Na_v1.8$: SNS or PN3 · $Na_v1.9$: NaN or SNS2 · NGF: Nerve growth factor · BDNF: Brain-derived neurotrophic factor · NT4/5: Neurotrophin 4/5 · TrkB: Tyrosine kinase B · TTX: tetrodotoxin · STX: Saxitoxin

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Introduction

The TTX-resistant (TTXr) voltage-gated sodium channels $Na_v1.8$ and $Na_v1.9$ are predominantly expressed in spinal sensory neurons [1, 13, 30]. Electrophysiological studies on cultured dorsal root ganglion (DRG) neurons from adult rodents and humans have revealed two distinct TTXr sodium currents, a slowly inactivating current ($I_{TTXr\text{-}Slow}$) and a persistent current ($I_{TTXr\text{-}Per}$) [8, 9, 11, 14, 24]. Studies on transgenic mice lacking $Na_v1.8$ and $Na_v1.9$ respectively have revealed the molecular determinant for $I_{TTXr\text{-}Slow}$ as $Na_v1.8$ [2, 9] and $Na_v1.9$ for $I_{TTXr\text{-}Per}$ [20]. The expression and functional properties of both $I_{TTXr\text{-}Slow}$ and $I_{TTXr\text{-}Per}$ suggest that they can substantially contribute to the electro-responsiveness of nociceptive neurons and thus they are both considered to be attractive targets for the development of new analgesic drugs [4, 12, 18, 23, 33].

$Na_v1.8$ and $Na_v1.9$ are the only sodium channel α subunits that have a serine residue in the pore loop of domain I (IP-loop) at the position known to be crucial for TTX and STX sensitivity of voltage-gated sodium channels (Fig. 1) [1, 3, 13, 27]. The sensitivities to other sodium channel blockers can also be influenced by the identity of the amino acid residue at this position. The cardiac channel $Na_v1.5$, which has a cysteine (C374) at the corresponding position in the IP-loop, is sensitive to block by micromolar levels of cadmium and to external application of the membrane-impermeant quaternary derivative of lidocaine QX-314 [26, 29]. When externally applied, QX-314 can only reach the local anesthetic binding site, which involves specific S6 residues of different domains and is located in the inner portion of the pore, by passing through the sodium channel pore [21, 29, 32]. The cysteine residue located at the TTX-binding site of wild-type $Na_v1.5$ and $Na_v1.4$-Y401C channels is crucial for the ability of extracellular QX-314 to pass through the selectivity pore and block these sodium channels [29]. Substituting this residue in $Na_v1.5$ with tyrosine, which is the corresponding residue in several TTX-sensitive channels including $Na_v1.4$ (Y401), greatly decreases the block of $Na_v1.5$ by cadmium and external QX-314 while increasing the block by STX and TTX. By contrast, substituting Y401 in $Na_v1.4$ with cysteine (Y401C), alanine (Y401A) or aspartate (Y401D) increases block by external QX-314 [29], while decreasing block by STX [15].

The TTX sensitivity of $I_{TTXr\text{-}Per}$ (IC_{50}~40 μM; 9) is similar to that of $I_{TTXr\text{-}Slow}$ (IC_{50}~100 μM; 24) and recombinant $Na_v1.8$ channels (IC_{50}~60 μM; 1). However, relatively little is known about the sensitivity of $I_{TTXr\text{-}Per}$ to other agents that can block sodium channels. Interestingly, it has been purported that $Na_v1.9$ generates a TTX-resistant but saxitoxin (STX)- sensitive neurotrophin-evoked depolarizing current within CNS neurons [5]. In the present study we asked whether $I_{TTXr\text{-}Slow}$and $I_{TTXr\text{-}Per}$ share further pharmacological properties that might be determined by the serine pore residue of Nav1.8 and Nav1.9. We compared the sensitivity of $I_{TTXr\text{-}Per}$and $I_{TTXr\text{-}Slow}$ to block by STX, extracellular cadmium and QX-314. We also determined whether the serine residue is a critical determinant for the sensitivity to these blockers by using site-directed mutagenisis. Since both $Na_v1.8$ and $Na_v1.9$ generally express very poorly in heterologous expression systems, we examined wild-type $Na_v1.4$ ($Na_v1.4$-WT) channels and mutants where tyrosine 401 was replaced by a serine ($Na_v1.4$-Y401S) or a cysteine ($Na_v1.4$-Y401C).

Our data indicate that the pore serine residue plays an important role in determining the sensitivity of $I_{TTXr\text{-}Slow}$ and $I_{TTXr\text{-}Per}$ to STX and TTX but not to cadmium and QX-314.

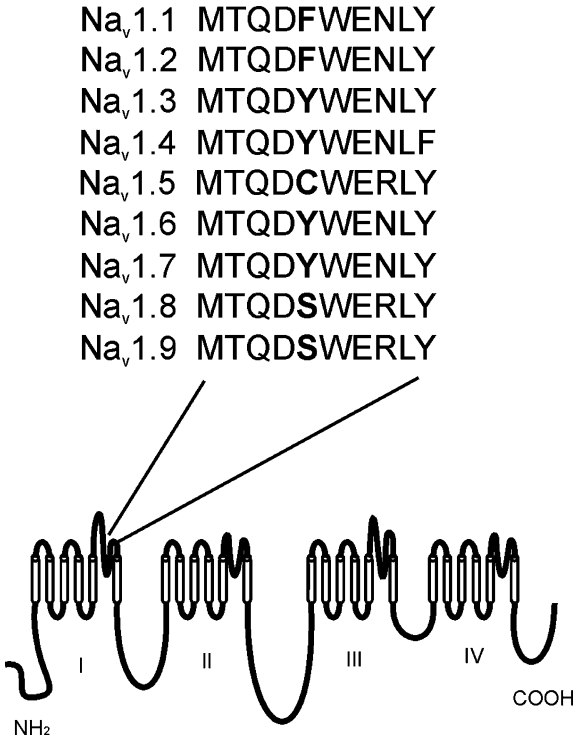

Fig. 1 Schematic showing the location of the critical residue for sodium channel sensitivity to guanidinium toxins. The residues surrounding the critical residue (Y401 in $Na_v1.4$, shown in bold) in the IP-loop are shown for the known voltage-gated sodium channels $Na_v1.1$–1.9

Material and methods

Construction of mammalian expression vectors encoding rat $Na_v1.4$

The construction of wild-type $Na_v1.4$-RBG4 construct for expression in mammalian cells has been described previously [31]. $Na_v1.4$-Y401C and $Na_v1.4$-Y401S cDNAs in pBluescript SK^+ [15] were gifts from Edward Moczydlowski and Laurent Schild, respectively. The $Na_v1.4$ coding sequence was cut out of these plasmid with the restriction endonuclease EcoR1, purified and ligated into the RBG4 expression vector at the EcoR1 site.

Transfection of HEK 293 Cells

Transfections were carried out using the calcium phosphate precipitation method as described previously [10]. HEK293 cells were grown under standard tissue culture conditions (5% CO_2, 37°C) in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum. The calcium phosphate-DNA mixture (channel constructs and a green fluorescent protein reporter plasmid) was added to the cell culture medium for 1–2 h, after which the cells were washed with fresh medium. Cells exhibiting green fluorescent protein

fluorescence were selected for whole-cell patch-clamp recordings after 1–2 days in culture.

Culture of DRG neurons

DRG cells were studied after short-term culture (< 24 h). Cultures of DRG neurons were prepared as previously described [11]. Briefly, the L4 and L5 DRG ganglia were harvested from adult male Sprague Dawley rats. The DRG tissue was treated with collagenase A (1 mg/ml) for 25 min and collagenase D (1 mg/ml) and papain (30 U/ml) for 25 min. The suspension was dissociated in DMEM and Ham's F12 medium supplemented with 10% fetal bovine serum, and DRG neurons were plated on glass coverslips coated with Poly-L-Lysine and Laminin. Electrophysiological recordings were made within 24 h of dissociation.

Whole-cell patch-clamp recordings

Whole-cell patch-clamp recordings were conducted at room temperature (~21°C) with an EPC-9 amplifier. Data were acquired on a PC with the pulse program (v 8.1, HEKA Electronics, Lambrecht, Germany). Fire-polished electrodes (0.8–2 MΩ) were fabricated from 1.7 mm capillary glass, using a Sutter P-97 puller (Novato, CA, USA). Cells were not considered for analysis if the initial seal resistance was < 2 GΩ, if they had high leakage currents (holding current > 0.1 nA at −80 mV for HEK 293 cells; > 0.5 nA for DRG neurons), or an access resistance > 4 MΩ . The average access resistance was 1.9 ± 0.1 MΩ for HEK 293 cells ($n = 99$) and 2.0 ± 0.1 MΩ for DRG neurons ($n = 59$) (mean ± S.E). Voltage errors were minimized using 80–90% series resistance compensation, and the capacitance artifact was cancelled by using the computer-controlled circuitry of the patch-clamp amplifier. Linear leak subtraction, based on resistance estimates from four to five hyperpolarizing pulses applied before the depolarizing test potential, was used for all voltage-clamp recordings. Membrane currents were usually filtered at 2.5 kHz and sampled at 10 kHz. The pipette solution contained (in mM) 140 CsF, 1 EGTA, 10 NaCl and 10 HEPES, pH 7.3. The standard bathing solution was (in mM) 140 NaCl, 3 KCl, 1 $MgCl_2$, 1 $CaCl_2$, and 10 HEPES, pH 7.3. For experiments on DRG neurons, 250 nM TTX was added to the bathing solution to block all TTX-sensitive currents. The liquid junction potential for these solutions was < 8 mV; data were not corrected to account for this offset. The osmolarity of all solutions was adjusted to 310 mOsm (Wescor 5500 osmometer, Logan, UT). The offset potential was zeroed before the cells were patched.

Toxin solutions and bath application

TTX was obtained from Alomone Laboratories Jerusalem, Israel, STX and QX-314 were obtained from Sigma-Aldrich St. Louis, MO, USA. Toxins were applied through a gravity- driven system allowing a rapid perfusion of the recording chamber. Toxins were washed off in specific experiments using the same perfusion system. The IC_{50} was calculated based on the single-site Langmuir inhibition isotherm using the following function: $IC_{50} = (I_{toxin}/I_0) \times [toxin]/(1\text{-}I_{toxin}/I_0)$, where I_0 and I_{toxin} are the peak sodium currents measured before and during application of toxin, respectively, and [toxin] is the concentration of toxin. The sensitivity of sodium currents to specific blockers was usually tested with several different blocker concentrations. However, for estimation of the IC50, a single concentration was chosen. The IC50s estimated with this approach are in good agreement with IC50s calculated from more complete dose-response curves. For example, Backx et al. [3] determined that the IC50 of Nav1.4 for TTX is 16 nM, the IC50 of Nav1.4-Y401C for TTX is > 50 μM and the IC50 of Nav1.4-Y401C for cadmium is 29 μM, which are very close to our estimates (Table 1). Results are presented as mean ± SEM.

Data analysis

Data were analyzed with Pulsefit (HEKA El ectronics) and Origin (Microcal Software, Northampton, MA, USA) software programs. Unless otherwise noted, statistical significance was determined at $P < 0.05$, using an unpaired Student's t test. Results are presented as mean ± SEM and error bars in the figures represent SE. The curves in the figures are drawn to guide the eye unless otherwise noted.

Results

$I_{TTXr\text{-}Slow}$ and $I_{TTXr\text{-}Per}$ are TTX- and STX-resistant

The majority of small (< 25 μm in diameter) and medium-size (25–35 μm in diameter) DRG neurons from adult rat displayed two TTXr sodium currents, $I_{TTXr\text{-}Slow}$ and $I_{TTXr\text{-}Per}$, with distinct kinetic properties (Fig. 2A-C). Both of these TTXr currents are known to be highly resistant to TTX: the IC_{50} of TTX for $I_{TTXr\text{-}Slow}$ is ~60 μM [1, 24] and for $I_{TTXr\text{-}Per}$ ~40 μM [9]. We asked whether $I_{TTXr\text{-}Slow}$ and $I_{TTXr\text{-}Per}$ also have similar sensitivities to STX. To examine $I_{TTXr\text{-}Slow}$, cells were held at −80 mV and depolarizing pulses to 0 mV were applied (Fig. 2D). The IC_{50} value of 6.0 ± 0.9 μM ($n = 4$) for STX-induced block of $I_{TTXr\text{-}Slow}$ is in agreement with a previous report investigating $Na_v1.8$ expressed in *Xenopus* oocytes [1]. To examine the STX sensitivity of $I_{TTXr\text{-}Per}$, cells were held at −120 mV, and test pulses to −50 mV were applied (Fig. 2E). $I_{TTXr\text{-}Per}$ displayed resistance to micromolar concentrations of STX (IC_{50} 13.7 ± 3.4 μM, $n = 5$) similar to that of $I_{TTXr\text{-}Slow}$ ($P > 0.05$). By contrast, the cardiac sodium channel ($Na_v1.5$) displays an intermediate sensitivity to STX

Table 1 Functional and Pharmacological properties of investigated sodium channels

Channel	V_m(mV)	V_h(mV)	TTX(IC_{50})	STX(IC_{50})	Cd^{2+} (IC_{50})	QX-314 out(1.0 mM)% remaining	QX-314 in(200 μM)% remaining
$I_{TTXr\text{-}Slow}$(DRG)	−21.2 ± 1.2 [a]	−35.7 ± 1.5	~60 [c] μM	6.0 ± 0.9 μM (5 μM)	0.3 ± 0.03 mM (0.5 mM)	97 ± 5	71 ± 3
$I_{TTXr\text{-}Per}$(DRG)	−54.2 ± 1.3 [a]	−44 ± 1 [b]	39 ± 9 [b] μM	13.7 ± 3.4 μM (5 μM)	1.2 ± 0.2 mM (1 mM)	n.d.	89 ± 7
$Na_v1.4$-WT(HEK293)	−29.2 ± 1.9	−71.9 ± 1.3	13.5 ± 1.0 nM (25 nM)	1.8 ± 0.2 nM (2 nM)	1.0 ± 0.1 mM (1 mM)	97 ± 2	41 ± 2
Y401S(HEK293)	−26.2 ± 1.9	−72.7 ± 1.1	> 100μM (20 μM)	5.6 ± 0.4μM (5 μM)	1.1 ± 0.2 mM (1 mM)	102 ± 6	38 ± 2
Y401C(HEK293)	−25.2 ± 0.8	−72.1 ± 0.8	48 ± 13μM (10 μM)	0.4 ± 0.06μM (0.5 μM)	10.5 ± 3.0μM (20 μM)	56 ± 8	42 ± 2

[a] 28, [b] 9, [c] 1, n.d. not determined. Values in parenthesis indicate the blocker concentration used to estimate the sensitivity of the specific sodium currents to specific blockers.

(IC_{50} = 91 nM; 26) and TTX sensitive channels such as Nav1.2 and Nav1.4 display high sensitivities to STX (IC_{50} ~2 nM; 15, 17). Thus, both of the TTXr sodium currents in DRG neurons display high resistance to STX as well as to TTX, a unique pharmacology not described for any other known sodium channel isoform.

The role of the serine residue of $Na_v1.8$ and $Na_v1.9$ in determining the sensitivity to TTX and STX was examined on $Na_v1.4$-WT channels and the mutant channels $Na_v1.4$-Y401S and $Na_v1.4$-Y401C. Robust, fast-inactivating inward currents were recorded from HEK293 cells transfected with all three constructs (Fig. 3). Cells were held at −100 mV and functional parameters were determined. All three constructs displayed nearly identical voltage dependence of activation and steady-state inactivation (Fig. 3D, E; Table 1). To examine the pharmacology, cells were held at −100 mV and depolarizing pulses from −100 mV to 0 mV were applied. $Na_v1.4$-WT was blocked by nanomolar concentrations of both TTX (IC_{50} = 13.5 ± 1.0 nM, n = 4) and STX (IC_{50} = 1.8 ± 0.2 nM, n = 4) (Fig. 4a, D). In contrast, TTX was much less effective (P < 0.05) at blocking the two mutant constructs $Na_v1.4$-Y401S (Fig. 4b; IC_{50} > 100 μM, n = 4) and $Na_v1.4$-Y401C (Fig. 4e; IC_{50} = 48 ± 13 μM, n = 4). $Na_v1.4$-Y401S displayed a ~3000-fold lower sensitivity to STX compared to $Na_v1.4$-WT channels (Fig. 4c; IC_{50} = 5.6 ± 0.4 μM, n = 4), similar to the sensitivities determined for $I_{TTXr\text{-}Slow}$ and $I_{TTXr\text{-}Per}$. The sensitivity of $Na_v1.4$-Y401C to STX was ~200-fold lower when compared to $Na_v1.4$-WT (Fig. 4f; IC_{50} = 0.4 ± 0.06 μM, n = 4), but higher than for $Na_v1.4$-Y401S. Thus, the serine residue in the IP-loop of sodium channels confers a pronounced resistance to both of the guanidinium toxins TTX and STX.

$I_{TTXr\text{-}Slow}$ and $I_{TTXr\text{-}Per}$ sodium currents have different sensitivities to cadmium

We determined the sensitivity of $I_{TTXr\text{-}Slow}$ and $I_{TTXr\text{-}Per}$ in DRG neurons to extracellular cadmium, using the same pulse-protocols as described above (Fig. 5 a, b). In the presence of 250-nM TTX in the extracellular solution, which was needed to block all TTX-sensitive currents, $I_{TTXr\text{-}Slow}$ (Fig. 5a) displayed a significantly (P < 0.005) higher sensitivity to cadmium (IC_{50} = 0.3 ± 0.03 mM, n = 6) compared to $I_{TTXr\text{-}Per}$ (Fig. 5b; IC_{50} = 1.2 ± 0.2 mM μM, n = 10).

The blocking effect of extracellular cadmium on the $Na_v1.4$-WT, $Na_v1.4$-Y401C and $Na_v1.4$-Y401S was investigated. One-way ANOVAs were used to test for differences between the cadmium sensitivity of the currents generated by the $Na_v1.4$ constructs, $I_{TTXr\text{-}Slow}$ and $I_{TTXr\text{-}Per}$. ANOVA indicated that there were significant differences between the groups. The difference between pairs of individual data sets was tested by Fisher's least significant difference (LSD) multiple comparison test and P values < 0.05 were considered to be significant. While $Na_v1.4$-WT channels were relatively insensitive to

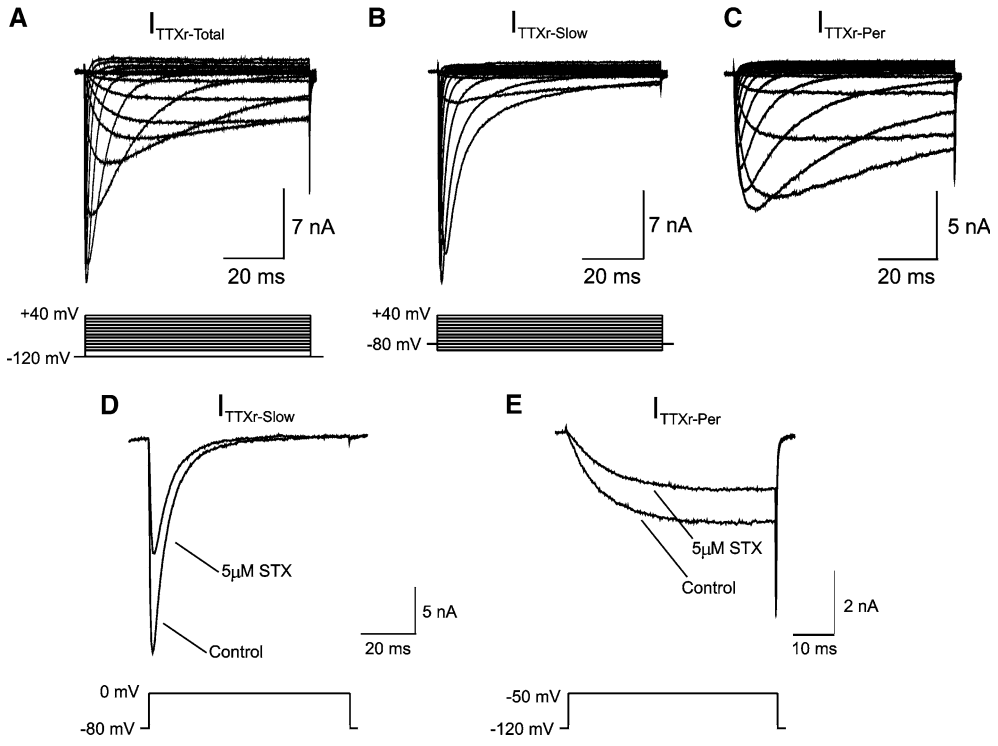

Fig. 2 Representative current traces of $I_{TTXr-Slow}$ and $I_{TTXr-Per}$ in DRG neurons. Currents were recorded in presence of 250 nM TTX and activated by 100 ms test pulses from −100 mV to +40 mV in steps of 10 mV. When held at −120 mV, cells exhibited both $I_{TTXr-Slow}$ and $I_{TTXr-Per}$ (A). When the holding potential was reduced to −80 mV, only $I_{TTXr-Slow}$ was activated (B). $I_{TTXr-Per}$ (C) is revealed by subtraction of $I_{TTXr-Slow}$ (B) from the total current (A). (For functional parameters, see Table 1). (D, E) Representative DRG TTXr current traces from experiments performed with STX (5 μM). Holding the cells at −80 mV, $I_{TTXr-Slow}$ was activated by test pulses from to 0 mV (D). Holding the cells at −120 mV, $I_{TTXr-Per}$ was activated by 100 ms test pulses to −50 mV (E). Because of a different voltage-dependence of activation of $I_{TTXr-Slow}$ and $I_{TTXr-Per}$, this protocol allows isolation of $I_{TTXr-Per}$. Whereas $I_{TTXr-Per}$ is fully activated by the step to −50 mV, $I_{TTXr-Slow}$ is activated only at more depolarized potentials (Table 1, 9, 28). Both $I_{TTXr-Slow}$ and $I_{TTXr-Per}$ were resistant to STX

cadmium (Fig. 5c; $IC_{50} = 1.0 \pm 0.1$ mM, $n = 6$), $Na_v1.4$-Y401C channels displayed a 100-fold enhanced cadmium sensitivity (Fig. 5e; $IC_{50} = 10.5 \pm 3.0$ μM, $n = 4$; $P < 0.05$, Fisher's LSD). This enhanced sensitivity of the $Na_v1.4$-Y401C construct confirms previous reports that a cysteine in the critical TTX-binding position in the IP-loop has a significant effect on the sensitivity to cadmium [17, 26]. In contrast, the cadmium sensitivity of $Na_v1.4$-Y401S channels (Fig. 5d; $IC_{50} = 1.1 \pm 0.2$ mM, it n = 4) was not different ($P > 0.05$) from that of $Na_v1.4$-WT channels, showing that the serine residue at this position in the pore does not alter the cadmium sensitivity of this sodium channel isoform. Because the cadmium-induced block of sodium channels is thought to occur through an interaction with pore residues near the TTX-binding site, it is possible that the presence of 250 nM TTX in these experiments might have altered the cadmium IC_{50} values for one or both of these currents. In order to estimate the influence of TTX on the cadmium-induced block of $I_{TTXr-Slow}$ and $I_{TTXr-Per}$, the Cd^{++} sensitivities of $Na_v1.4$-Y401S and $Na_v1.4$-Y401C were also investigated in the presence of 250 nM TTX in the extracellular solution. The cadmium sensitivity of $Na_v1.4$-Y401C was slightly decreased in the presence of TTX ($IC_{50} = 17.3 \pm 5.0$ μM, $n = 5$). Similarly, TTX caused a small reduction of cadmium-induced block of $Na_v1.4$-Y401S ($IC_{50} = 2.1 \pm 0.4$ mM, $n = 6$). These results indicate that the presence of 250 nM TTX slightly attenuates the resistance of $I_{TTXr-Slow}$ and $I_{TTXr-Per}$ to cadmium. However, irrespective of TTX, pair-wise comparisons revealed that while the cadmium sensitivity of $I_{TTXr-Per}$ was not different from that of $Na_v1.4$-WT or $Na_v1.4$-Y401S currents, the cadmium sensitivities of $I_{TTXr-Slow}$, $I_{TTXr-Per}$ and $Na_v1.4$-Y401S currents were significantly different from those of $Na_v1.4$-Y401C currents and the cadmium sensitivities of $I_{TTXr-Per}$ and $Na_v1.4$-Y401S currents were significantly different from those of $I_{TTXr-Slow}$. This indicates that, unlike cysteine in the IP loop, the serine pore residue of $Na_v1.8$ and $Na_v1.9$ does not enhance the cadmium sensitivity of these isoforms. The difference between the cadmium sensitivities of $Na_v1.8$ and $Na_v1.9$ currents is likely to be caused by other pore residues.

Intracellular, but not extracellular, block by QX-314 of $I_{TTXr-Slow}$ and $I_{TTXr-Per}$

We determined whether or not a serine residue in the pore allows QX-314 to pass through the pore and block

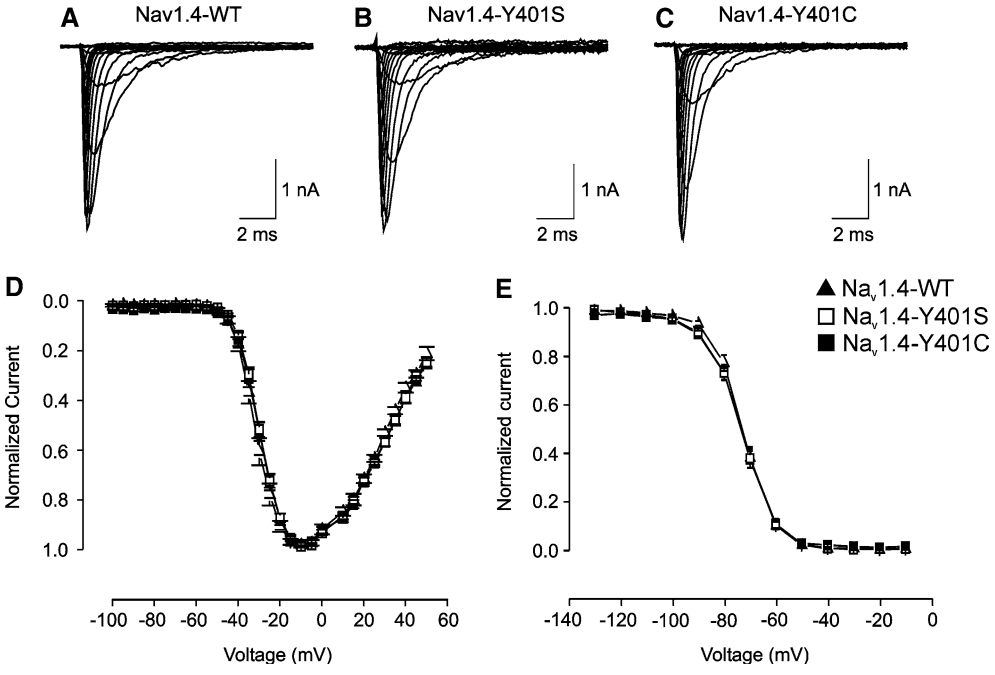

Fig. 3 Representative current traces from Na$_v$1.4-WT (n = 9) (A), Na$_v$1.4-Y401S (n = 20) (B) and Na$_v$1.4-Y401C (n = 14) (C) channels expressed in HEK293 cells. Cells were held at −100 mV. Current traces were activated by 100 ms test pulses from −100 mV to +50 mV in steps of 10 mV. All three constructs displayed nearly identical voltage dependence of activation (D). To examine the steady-state inactivation relations, currents were activated by 20 ms test pulses to −10 mV after 500 ms prepulses to potentials from −130 mV to −10 mV. Steady-state inactivation curves were identical for all three constructs (E). (For functional parameters, see Table 1)

$I_{TTXr\text{-}Slow}$or $I_{TTXr\text{-}Per}$. First, QX-314 was applied extracellularly to determine the accessibility of QX-314 to Na$_v$1.8 and Na$_v$1.9. The extracellular solution contained 250 nM TTX in order to block all TTX-sensitive currents. Currents were activated every 30 s for 12 min. During this protocol 1 mM QX-314 induced very little reduction of the $I_{TTXr\text{-}Slow}$ peak amplitude (to 97 ± 5%, $n = 4$ with QX-314 versus 101 ± 5%, $n = 4$ under control conditions; $P > 0.05$) (Fig. 6a). The effects of extracellular QX-314 on $I_{TTXr\text{-}Per}$ were hard to measure because this current exhibited substantial run-down in the whole-cell mode when activated every 30 s for 12 min without QX-314 (Fig. 6c), making it impossible to determine whether $I_{TTXr\text{-}Per}$ is blocked by extracellular QX-314. The ability of QX-314 to block $I_{TTXr\text{-}Slow}$ and $I_{TTXr\text{-}Per}$ when applied intracellularly was investigated using 200 µM QX-314 in the pipette solution. Experiments were started 3 min after whole-cell mode had been established and currents were activated at 0.75 Hz for 45 s. As shown in Fig. 6b, intracellular QX-314 blocks $I_{TTXr\text{-}Slow}$ (to 71 ± 3%, $n = 5$ with QX-314 versus 95 ± 1%, $n = 5$ under control conditions; $P < 0.001$). Intracellular QX-314 also seemed to block $I_{TTXr\text{-}Per}$ compared to the control experiments (to 89 ± 7%, $n = 6$ with QX-314 versus 98 ± 10%, $n = 8$ under control conditions) (Fig. 6d). However the $I_{TTXr\text{-}Per}$ was relatively

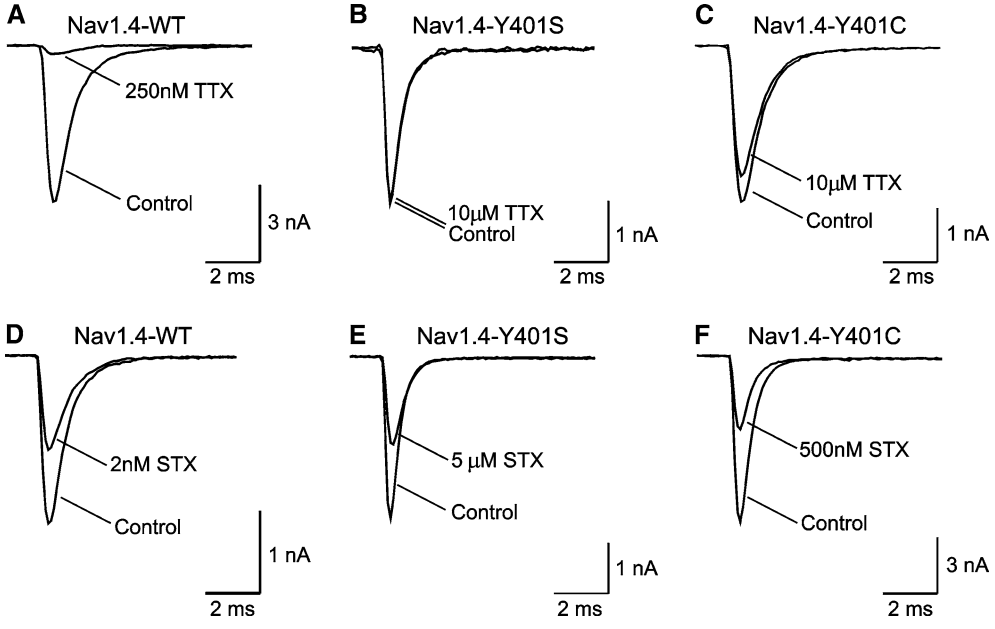

Fig. 4 Representative Na$_v$1.4 current traces from experiments performed with STX and TTX. Holding cells at −100 mV, the Na$_v$1.4-constructs were activated by 100 ms voltage steps to 0 mV. Na$_v$1.4-WT was blocked by nanomolar concentrations of both TTX (A) and STX (D). The two mutant Na$_v$1.4 channels were insensitive to both toxins (Na$_v$1.4-Y401S: B and E; Na$_v$1.4-Y401C: C and F) (For IC_{50}-values, see Table 1)

unstable under control conditions and the extent of current inhibition was relatively small and not significant ($P > 0.05$).

To determine the role of a serine at the crucial site of TTX binding on the QX-314 sensitivity of sodium channels, QX-314 experiments were also performed with the $Na_v1.4$-WT, $Na_v1.4$-Y401S and $Na_v1.4$-Y401C constructs. As with $I_{TTXr\text{-}Slow}$ and $I_{TTXr\text{-}Per}$, extracellular block of 1 mM QX-314 was determined on currents activated every 30 s for 12 min. In these experiments TTX was not added to the extracellular solution. Whereas the peak current amplitudes of both $Na_v1.4$-WT ($97 \pm 2\%$, $n = 4$), and $Na_v1.4$-Y401S ($102 \pm 6\%$, $n = 4$) were not significantly reduced by extracellular QX-314, the $Na_v1.4$-Y401C current was reduced by extracellular QX-314 (to $56 \pm 8\%$, $n = 4$) (Fig. 6e). While these data are in good agreement with a recent report from Sunami et al. [29], demonstrating that the cysteine residue allows extracellular QX-314 to block sodium channels, they also show for the first time that a serine residue at this site in the pore does not allow extracellular QX-314 to travel through the pore. We wanted to estimate whether the presence of TTX in extracellular solution might contribute to the insensitivity of $I_{TTXr\text{-}Slow}$ to extracellular QX-314. Therefore, the same QX-314 experiments were performed for $Na_v1.4$-Y401C with 250 nM TTX in the extracellular solution. $Na_v1.4$-Y401C was still blocked by extracellular QX-314 (~60%, $n = 2$; data not shown), indicating that the lack of QX-314-induced extracellular block of $I_{TTXr\text{-}Slow}$ was not caused by the presence of 250 nM TTX in the extracellular solution. We also asked whether the serine residue in the TTX-binding site plays a role in determining the resistance of $I_{TTXr\text{-}Slow}$ and $I_{TTXr\text{-}Per}$ to intracellular block by QX-314. As demonstrated in Fig. 6f, all three $Na_v1.4$ constructs were equally blocked by 200 µM intracellular QX314 ($Na_v1.4$-WT: to $41 \pm 2\%$, $n = 4$; $Na_v1.4$-Y401S: to $38 \pm 2\%$, $n = 4$; $Na_v1.4$-Y401C: to $42 \pm 2\%$, $n = 5$). Thus, this pore residue does not seem to interfere with the intracellular binding of QX-314.

One-way ANOVA was used to test for differences between the sensitivity of the currents generated by the $Na_v1.4$ constructs, $I_{TTXr\text{-}Slow}$ and $I_{TTXr\text{-}Per}$ to intracellular QX-314. ANOVA indicated that there were significant differences between the groups. The difference between pairs of individual data sets was tested by Fisher's least significant difference (LSD) multiple comparison test and P values < 0.05 were considered to be significant. Pair-wise comparisons revealed that although there was no significant difference between the QX-314 sensitivity of the currents generated by the $Na_v1.4$ constructs, $I_{TTXr\text{-}Slow}$ and $I_{TTXr\text{-}Per}$ were significantly less sensitive to intracellular QX-314. This analysis also indicated that $I_{TTXr\text{-}Per}$ is less sensitive to intracellular QX-314 than $I_{TTXr\text{-}Slow}$.

Discussion

The signature of the TTX-binding residue in the IP loop is known to be critical for the sensitivity of sodium

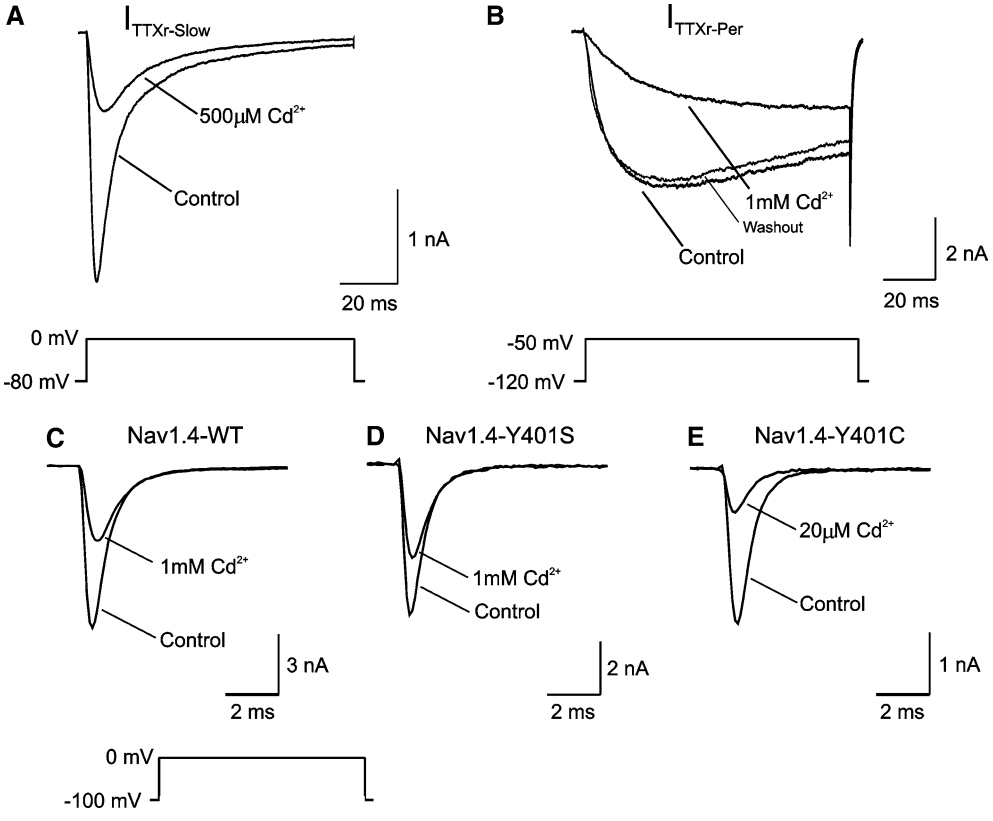

Fig. 5 Current traces from representative experiments performed with Cd^{++}. All currents were examined as described in Figures 1 and 4. $I_{TTXr\text{-}Slow}$(A) was more sensitive to block by Cd^{++} than $I_{TTXr\text{-}Per}$(B). The Cd^{++}-sensitivities for $Na_v1.4$-WT (C) and $Na_v1.4$-Y401S (D) were similar, but increased for $Na_v1.4$-Y401C (E). (For IC_{50}-values, see Table 1)

channels to several sodium channel blockers [3, 26, 29]. In this study we present novel data showing that a serine at this position in voltage-gated sodium channels 1) imparts a unique high resistance to block by STX and TTX, 2) does not account for the enhanced sensitivity of $I_{TTXr\text{-}Slow}$ to cadmium, and 3) does not allow extracellular QX-314 to pass through the pore.

$I_{TTXr\text{-}Slow}$ and $I_{TTXr\text{-}Per}$ are resistant to guanidinium toxins

When expressed in Xenopus oocytes, $Na_v1.8$ displays TTX- and STX resistance similar to that of $I_{TTXr\text{-}Slow}$ from DRG neurons [1, 24, 27]. By contrast, mutant $Na_v1.8$ channels where a phenylalanine was substituted for the serine residue at the critical position in the IP loop produce currents with high sensitivity to TTX and STX [27]. The high resistance of $I_{TTXr\text{-}Slow}$ and $I_{TTXr\text{-}Per}$ in DRG neurons to block by TTX and STX is a pharmacology not described for any other sodium channel isoform. $I_{TTXr\text{-}Slow}$, $I_{TTXr\text{-}Per}$ and $Na_v1.4$-Y401S channels displayed strikingly similar resistance to TTX and STX (Table 1). Because $Na_v1.8$ and $Na_v1.9$ are the only known sodium channel isoforms where the critical TTX-binding residue in the IP loop is serine, our data here comparing the pharmacology of $I_{TTXr\text{-}Slow}$ and $I_{TTXr\text{-}Per}$ with $Na_v1.4$-Y401S currents are in agreement with previous studies suggesting that $Na_v1.8$ and $Na_v1.9$ are the molecular substrates for $I_{TTXr\text{-}Slow}$ and $I_{TTXr\text{-}Per}$ [2, 9, 20]. Interestingly, $Na_v1.9$ has been proposed to act as a ligand-mediated sodium rather than as a voltage-gated sodium channel. Together with trkB receptors, Nav1.9 might be the molecular determinant for rapid BDNF or NT4/5 evoked depolarizing sodium currents observed in CNS neurons [5]. This neurotrophin-evoked sodium current was insensitive to TTX but was completely blocked by 10 nM STX. The authors of this study argued

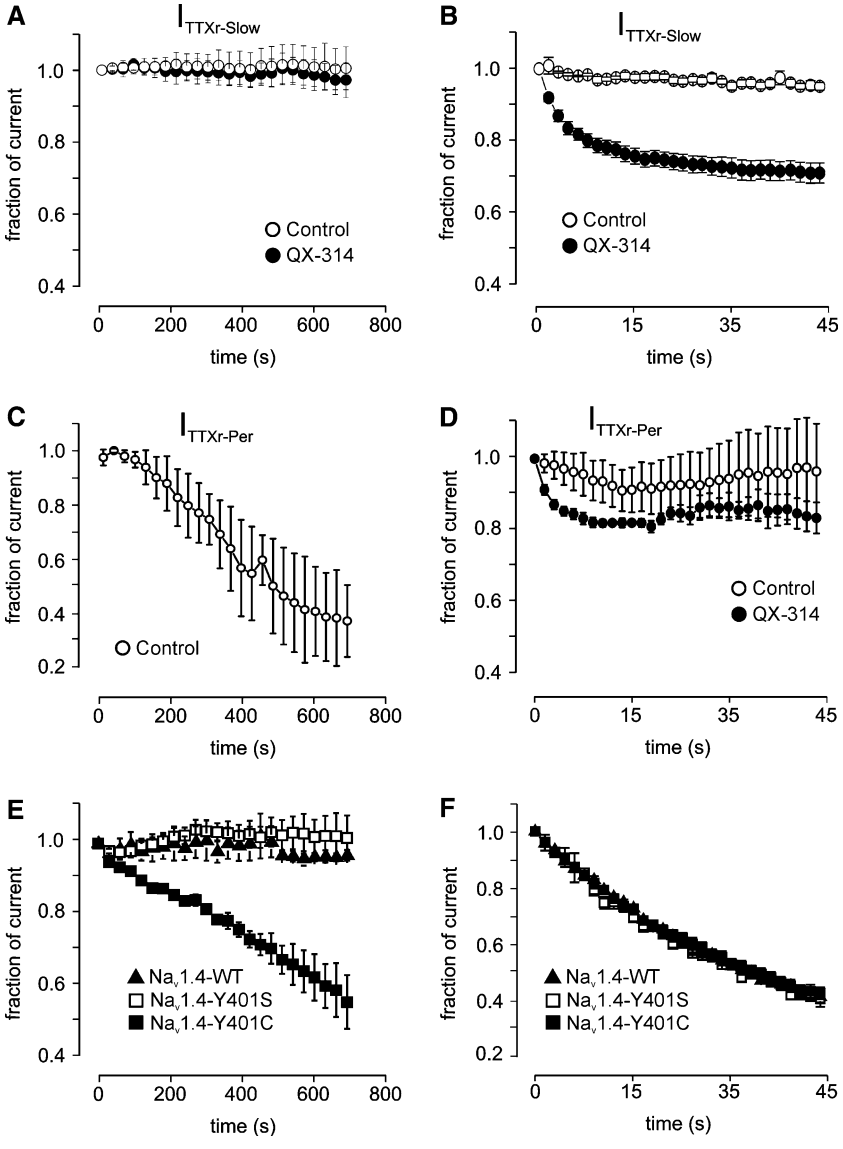

Fig. 6 Block of TTXr currents by extracellular (A,C,E) and intracellular (B,D,F) application of QX-314. (A) $I_{TTXr\text{-}Slow}$ was not blocked by extracellular QX-314 (1 mM). Currents were activated with test pulses from −80 mV to 0 mV every 30 s for 12 minutes. (B) $I_{TTXr\text{-}Slow}$ was sensitive to intracellular QX-314 (200 μM). Currents were activated after 3 min in the whole-cell mode with test pulses from −80 mV to 0 mV applied at 0.75 Hz for 45 s. (C) $I_{TTXr\text{-}Per}$, activated with test pulses from −120 mV to −50 mV every 30 s for 12 minutes, displayed a substantial rundown under control conditions making it difficult to examine the effect of extracellular QX-314. (D) $I_{TTXr\text{-}Per}$ was relatively insensitive to block by intracellular QX-314 (200 μM). Currents were activated after 3 min in the whole-cell mode with test pulses from −120 mV to −50 mV applied at 0.75 Hz for 45 s. (E) $Na_v1.4$-Y401C, but not $Na_v1.4$-WT or $Na_v1.4$-Y401S, channels were blocked by 1mM extracellular QX-314. Currents were evoked with test pulses from −100 mV to 0 mV every 30 s. In contrast, the three constructs were identically blocked by 200 μM intracellular QX-314 (F). Currents were evoked with test pulses from −100 mV to 0 mV at 0.75 Hz

that because of the serine in the IP loop, $Na_v1.9$ carries a typical sequence motif for a TTX-resistant/STX-sensitive sodium channel. This is contradicted by the present study and other reports [1, 15, 24, 27] which present clear evidence that the serine pore residue is accompanied with a high resistance to both TTX and STX. It was recently demonstrated that $I_{TTXr\text{-}Per}$ is abolished in $Na_v1.9$-null mutant mice [20]. Therefore, we feel it is very likely that $Na_v1.9$ generates the TTX- and STX-resistant persistent current described in the present study, and it is not clear what accounts for the STX-sensitive current attributed to $Na_v1.9$ in the report from Blum et al. [5].

The serine pore residue does not determine the sensitivity to cadmium and QX-314

Sodium channels can be differentially blocked by cadmium [16, 17]. Because of an interaction with the cysteine residue in the IP loop, cadmium is a high-affinity blocker of $Na_v1.5$. This is in agreement with site-directed mutagenesis experiments where cysteine substitution for phenylalanine or tyrosine strongly enhanced cadmium-sensitivities (this study and 3, 15, 26). A previous study on DRG neurons demonstrated that the cadmium sensitivity of slow TTXr currents is higher than the cadmium sensitivity of TTX-sensitive currents [24]. Interestingly, despite the common serine in their pore, $I_{TTXr\text{-}Slow}$ was more sensitive to cadmium block than $I_{TTXr\text{-}Per}$. Moreover, we show that the cadmium-sensitivity of $Na_v1.4$-Y401S was not altered compared to $Na_v1.4$-WT channels, suggesting that the serine pore residue of $Na_v1.8$ and $Na_v1.9$ does not control the cadmium sensitivity of these isoforms. The higher cadmium sensitivity described for $I_{TTXr\text{-}Slow}$ currents in DRG neurons (24; this study) cannot be explained by the serine pore residue but may be due to the cysteine residue at position 833 (C833) in the IIP loop of $Na_v1.8$. Rat $Na_v1.9$ has an aspartic acid (D740) and $Na_v1.4$ has an asparagine (N739) at the corresponding site. Carbonneau et al. [7] recently reported that a cysteine substitution at a nearby tryptophan residue (W736C) in the amino-terminus of the IP loop of $Na_v1.4$ channels increased the cadmium sensitivity of $Na_v1.4$ (IC_{50} ~150 μM). Interestingly, while both rat and mouse $Na_v1.8$ have a cysteine residue at position 833, human and dog $Na_v1.8$ have histidines at this location. The sensitivity of these nonrodent $Na_v1.8$ channels to cadmium has not yet been tested.

When compared to TTX-sensitive sodium currents, $I_{TTXr\text{-}Slow}$ is relatively resistant to commonly used sodium channel blockers such as lidocaine, mexiletine and carbamazepine [2, 6, 11, 24, 25]. The sensitivity of $I_{TTXr\text{-}Per}$ to these agents is not known. Local anesthetics block sodium channels by binding to specific residues in the S6 segments that form part of the inner pore of the channel [19, 22, 32]. The quaternary lidocaine derivative QX-314 is membrane-impermeable and can only reach the inner pore by passing through the pore of the channel [21, 29]. $Na_v1.5$ and cardiac sodium currents are sensitive to extracellular QX-314. Qu et al. [21] reported that a threonine residue in S6 of domain IV of $Na_v1.5$ was an important determinant of sensitivity to extracellular QX-314 [21]. This threonine is conserved in S6 of domain IV of $Na_v1.8$. Sunami et al. [29] reported that the size of the residue at the crucial TTX-binding site in domain I was an even more crucial determinant of sensitivity to extracellular QX-314. They reported that large residues such as phenyalanine and tyrosine prevented QX-314 from passing through the pore, but that alanine, cysteine and even a negatively charged but relatively small amino acid such as aspartate allowed external QX-314 to pass through the pore and block the channel. Since a serine is even smaller than cysteine, we expected that $I_{TTXr\text{-}Slow}$, $I_{TTXr\text{-}Per}$ and $Na_v1.4$-Y401S channels would be sensitive to extracellular QX-314. Surprisingly, we report in this study that while $Na_v1.4$-Y401C channels are efficiently blocked by extracellular QX-314, $Na_v1.4$-Y401S channels are not. External QX-314 did not produce significant block of the $I_{TTXr\text{-}Slow}$ in DRG neurons. Due to a substantial rundown of $I_{TTXr\text{-}Per}$ during electrophysiological recordings under control conditions (see Fig. 6c), it was not possible to examine the effect of external QX-314 on $I_{TTXr\text{-}Per}$. Our finding that the serine pore residue in the IP loop does not allow extracellular QX-314 to pass through the pore helps explain why $I_{TTXr\text{-}Slow}$ was poorly blocked by extracellular QX-314. However, both $I_{TTXr\text{-}Slow}$ and $I_{TTXr\text{-}Per}$ were also relatively poorly blocked by intracellularly applied QX-314 compared to the $Na_v1.4$ constructs. The fact that block of $Na_v1.4$-Y401S by intracellular QX-314 was identical to that of $Na_v1.4$-WT and $Na_v1.4$-Y401C suggests the resistance of $I_{TTXr\text{-}Slow}$ and $I_{TTXr\text{-}Per}$ to local anesthetics cannot be explained by the serine pore residue in the IP loop of $Na_v1.8$ and $Na_v1.9$. It is not clear what determines this relative resistance of $I_{TTXr\text{-}Slow}$ and $I_{TTXr\text{-}Per}$ to local anesthetics, but it may be related to changes in residues in the S6 regions involved in local anesthetics binding or to differences in inactivation properties of these currents.

Our data show that $I_{TTXr\text{-}Slow}$ and $I_{TTXr\text{-}Per}$ display unique pharmacological properties. Functional properties of both $I_{TTXr\text{-}Slow}$ and $I_{TTXr\text{-}Per}$ suggest that they can play critical roles in action potential electrogenesis of nociceptive neurons. These isoforms are both attractive targets for the development of new analgesic drugs. Therefore, the present results may be valuable for the development of selective blockers of $Na_v1.8$ and $Na_v1.9$.

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