Low potency inhibition of $Na_V1.7$ by externally applied QX-314 via a depolarizing shift in the voltage-dependence of activation

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Abstract

QX-314 is a quaternary permanently charged lidocaine derivative that inhibits voltage-gated sodium channels (Na_V). As it is membrane impermeable, it is generally considered that QX-314 applied externally is inactive, unless it can gain access to the internal local anesthetic binding site via another entry pathway. Here, we characterized the electrophysiological effects of QX-314 on $Na_V1.7$ heterologously expressed in HEK293 cells, and find that at high concentrations, external QX-314 inhibited $Na_V1.7$ current (IC_{50} 2.0 $\pm$ 0.3 mM) and shifted the voltage-dependence to more depolarized potentials (ΔV_{50} +10.6 mV). Unlike lidocaine, the activity of external QX-314 was not state- or use-dependent. The effect of externally applied QX-314 on $Na_V1.7$ channel biophysics differed to that of internally applied QX-314, suggesting QX-314 has additional externally accessible site of action. In line with this hypothesis, disruption of the local anesthetic binding site in a [F1748A]$Na_V1.7$ mutant reduced the potency of lidocaine by 40-fold, but had no effect on the potency or activity of externally applied QX-314. Therefore, we conclude using an expression system where QX-314 was unable to cross the membrane, that externally applied QX-314 is able to inhibit $Na_V1.7$ peak current at low millimolar concentrations.

Keywords

QX-314, lidocaine, local anesthetic, voltage-gated sodium channel, $Na_V1.7$, pain

1 Introduction

The local anesthetic QX-314 is a permanently positively charged quaternary lidocaine derivate. QX-314 inhibits voltage-gated sodium channels (Na_V) at an intracellular binding site similar to lidocaine (Frazier et al., 1970). However, unlike lidocaine, which crosses cell membranes via passive diffusion to access the internal local anesthetic binding site, QX-314 is proposed to be membrane impermeable (Frazier et al., 1970; Strichartz, 1973) and thus unable to access the internal binding site following extracellular application. Several studies have explored how QX-314 can enter cells, most of which focus on the pore of the transient receptor potential channel (TRP) family as possible entryways. Accordingly, co-application of QX-314 and the TRPV1 agonist capsaicin led to analgesia that was restricted to TRPV1 expressing C-fibers (Binshtok et al., 2007). Similar effects have been found for the co-application of QX-314 with the TRPA1 agonist carvacrol (Brenneis et al., 2014), the co-application with the TRPM8 agonist menthol (Ongun et al., 2018), and even for the co-application with the Aβ-fiber-specific toll-like receptor 5 (TLR5) agonist flagellin (Xu et al., 2015), albeit the entry pathways in the latter case are less clear.

The ability of QX-314 to selectively silence nerve fibers, depending on the agonist with which it is co-applied, makes it a promising tool for studying the contribution of different nerve fiber types to pain behavior. Indeed, our initial goal was to utilize QX-314-mediated Aβ-fiber silencing to investigate the contribution of Aβ- and Aδ-fibers to $Na_V1.6$-mediated pain behaviors. Surprisingly, we found that QX-314 was able to exert analgesia *in vivo* even without an additional agonist, and that at equipotent concentrations, QX-314 was able to inhibit $Na_V1.7$ heterologously expressed in HEK cells after extracellular application.

We therefore further characterized the electrophysiological effects of externally and internally applied QX-314 on $Na_V1.7$, and report that in addition to a high affinity internal binding site, external QX-314 can also inhibit $Na_V1.7$ peak current at higher concentrations. This extracellular activity is associated with a depolarizing shift in the voltage-dependence of activation, without exhibiting state or use dependence.

2 Materials and Methods

2.1 Chemicals

Cn2 (*Centruroides noxius* toxin 2) and OD1 (*Odontobuthus doriae* toxin 1) were synthesized as previously described (Durek et al., 2013; Israel et al., 2018). QX-314 (Lidocaine *N*-ethyl bromide, purchased on five separate occasions between March 2017 and October 2021; Catalogue No. L5783), lidocaine, and flagellin were purchased from Sigma-Aldrich (Castle Hill, NSW, Australia). Peptides were routinely diluted in 0.1% bovine serum albumin (BSA; Bio-Scientific, Kirrawee, NSW, Australia). All other reagents were purchased from Sigma Aldrich, unless otherwise stated.

2.2 Animals

Ethical approval for *in vivo* experiments was obtained from The University of Queensland Animal Ethics Committee (IMB/PACE/325/15; IMB/PACE/326/15). All experiments were conducted in accordance with the Animal Care and Protection Regulation Qld (2012), the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes, 8th edition (2013), and the International Association for the Study of Pain Guidelines for the Use of Animals in Research.

Male C57BL/6J mice aged 6–8 weeks were housed in groups of 3 or 4 per cage in the animal facility at the Pharmacy Australia Centre of Excellence, University of Queensland, Australia. Mice were maintained at a temperature of 21–23°C under a 12-h light-dark cycle and provided with standard rodent chow and water *ad libitum*.

2.3 Animal behavioural testing

QX-314 (5 mM) and flagellin (0.9 µg) were diluted in phosphate-buffered saline (PBS), and Cn2 (10 nM) and OD1 (300 nM) in PBS/0.1% BSA. Under isoflurane anesthesia (3%), total volumes of 20 µL (Cn2 experiments) or 40 µL (OD1 experiments) containing different combinations of the compounds were administered via shallow subcutaneous intraplantar injection into the left hind paw of mice. Immediately after recovery from anesthesia (which was within minutes for compound injection) mice were placed into polyvinyl boxes (10 × 10 × 10 cm) and spontaneous pain behaviors were counted by a blinded observer from video recordings for 10 min post-injection.

2.4 Cell culture

HEK293 cells stably expressing human $Na_V1.7/\beta1$ or $\beta1/\beta2$ were maintained in MEM (minimal essential media) supplemented with 10% FBS (fetal bovine serum; Assay Matrix, Ivanhoe, VIC, Australia), 2 mM L-glutamine (GlutaMax, Life Technologies, Scoresby, VIC, Australia), and selection antibiotics as recommended by the manufacturer (SB Drug Discovery, Glasgow, UK). All cells were grown in T-75 flasks in a humidified 5% CO_2 incubator at 37°C and passaged every 3–4 days using TrypLE Express (Life Technologies).

2.5 [F1748A]$Na_V1.7$ plasmid and transfection

The WT h$Na_V1.7$ cDNA (NM_002977, a kind gift from Dr James Cox (Cox et al., 2006)) was subjected to *in vitro* site-directed mutagenesis using the QuikChange™ XL mutagenesis kit (Agilent Technologies, Mulgrave, VIC, Australia) following the manufacturer's instructions. A point mutation corresponding to F1748A (based on the numbering of the UniProtKB ID Q15858 splice variant) was created in the h$Na_V1.7$ cDNA at the local anesthetic binding site motif IIISFLVVV resulting in IIISALVVV using the following two oligonucleotides: 5'-GTTATATCATCATATCCGCCCTGGTTGTGGTGAAC-3' and 5'-GTTCACCACAACCAGGGCGGATATGATGATATAAC-3'. The mutation was verified by sequencing by the Australian Genome Research Facility. [F1748A]$Na_V1.7$ was transiently transfected into HEK cells stably expressing $\beta1/\beta2$ using Lipofectamine 2000 (Life Technologies) and used for patch-clamp experiments 48 h after transfection.

2.6 Electrophysiology

All electrophysiology experiments (except for use-dependence) were conducted using a QPatch 16-well automated electrophysiology platform (Sophion Bioscience, Ballerup, Denmark) using 16-well planar patch chip plates (QPlates; Sophion Bioscience) with resistance of 2 ± 0.02 MΩ and patch hole diameter of 1 µm. Positioning pressure was set at −60 mbar, minimum seal resistance at 0.1 GΩ, holding pressure at −20 mbar, and holding potential at −90 mV. Whole-cell currents were filtered at 8 kHz and acquired at 25 kHz and the linear leak was corrected by P/4 subtraction. IV currents were recorded with 70% fast series resistance compensation.

The extracellular solution (ECS) contained (in mM): NaCl (70), choline chloride (70), KCl (4), $CaCl_2$ (2), $MgCl_2$ (1) HEPES (4-(2-hydroxyethyl)-1-piperayineethane-sulfonic acid) (10), and glucose (10). The pH was adjusted to 7.4 with NaOH, and osmolarity was adjusted with sucrose to 305 mOsm. OD1 was diluted in ECS with 0.1% BSA. The intracellular solution (ICS) contained (in mM): CsF (140), EGTA/CsOH (1/5), HEPES (10), NaCl (10). The pH was adjusted to 7.4 with CsOH, and osmolarity was adjusted with sucrose to 320 mOsm. For internal application of QX-314 (100 µM) and lidocaine (5 mM), compounds were added to the ICS.

Two protocols were used to assess state-dependence. For the resting/closed state, a 50-ms test pulse from −90 mV to −20 mV was applied (repetition interval: 20 s). For the partially inactivated/open state, a 20-ms test pulse to −20 mV was applied after an 8000-ms conditioning pulse to −55 mV, with a 50-ms recovery period in between (repetition interval: 12 s). Concentration-response curves were obtained using the above voltage protocols in the presence of increasing concentrations of lidocaine or QX-314 each applied for 2 min.

Current-voltage (IV) relationships were assessed before and after addition of QX-314 or lidocaine by applying a series of 500-ms conditioning pulses from −100 mV to +55 mV in 5-mV increments and peak currents of each voltage were measured. Afterwards, a 10-ms depolarizing test pulse to 0 mV for QX-314 or to −20 mV for lidocaine followed and peak current was measured to assess voltage-dependence of fast inactivation (repetition interval: 6 s).

To determine the off-rate, QX-314 (5 mM) was added at 60 s recording for 240 s before stepwise washout with ECS every 60 s for 5 min. 50-ms test pulses from –90 mV to –20 mV were applied every 20 s (0.05 Hz).

Use-dependence experiments were conducted on the Patchliner (Nanion Technologies GmbH, Munich, Germany) using an EPC 10 USB Quadro Patch Clamp Amplifier (HEKA Elektronik Dr. Schulze GmbH, Lambrecht/Pfalz, Germany) and the software Patchmaster (version 2x90.3; HEKA Elektronik Dr. Schulze GmbH, Lambrecht/Pfalz, Germany). ECS contained (in mM): NaCl (140), KCl (4), $CaCl_2$ (2), $MgCl_2$ (1), HEPES (10), glucose (5). The pH was adjusted to 7.4 with NaOH, and the osmolarity was 298 mOsm. ICS contained (in mM): CsCl (50), NaCl (10), CsF (60), EGTA (20), HEPES (10). The pH was adjusted to 7.2 with CsOH, and the osmolarity was 285 mOsm. According to the manufacturer's instructions, a seal enhancer was used containing (in mM): NaCl (80), KCl (3), $MgCl_2$ (10), $CaCl_2$ (35), HEPES (Na^+ salt) (10), and washed off prior to running the voltage protocols. The pH was adjusted to 7.4 with HCl, and the osmolarity was 298 mOsm. Cells were included if they had a minimum seal resistance of 2 GΩ and the holding potential was −100 mV. To test for use-dependent inhibition of QX-314 (3 mM) and lidocaine (300 µM), a series of 60 × 20-ms depolarizing pulses to 0 mV with frequencies of 1 Hz or 10 Hz was applied.

2.7 Data analysis and statistics

Data were plotted and analyzed using GraphPad Prism (Version 9.1, GraphPad Software, San Diego, CA, USA). Statistical significance was set at $p < 0.05$ and determined with two-tailed *t*-test or one-way ANOVA with Dunnett's or Šídák's post-test. Data are expressed as the mean ± standard error of the mean (SEM).

For state-dependence, concentration response curves were calculated by dividing the amplitude of the test current (I) by the maximal current elicited (I_0): $I_{Na} = I/I_0$. A four-parameter Hill equation with variable Hill slope was fitted to the data: y = bottom + (top – bottom)/(1 + 10^((LogIC$_{50}$ – x) × HillSlope)).

The current-voltage (IV) relationship was calculated by dividing the amplitude of the test current (I) by the maximal current elicited (I_0): $I_{Na} = I/I_0$. Current in presence of QX-314 or lidocaine was normalized to its control. The voltage-dependence of fast inactivation was calculated by dividing the amplitude of the test current (I) by the maximal current elicited (I_0): $I_{Na} = I/I_0$. The voltage-dependence of activation was obtained from the IV curves as the conductance: $G_{Na} = I_{Na}/(V_{memb} - V_{rev})$, with I_{Na} as the normalized current, V_{memb} as the membrane potential, and V_{rev} as the reversal potential. Steady-state kinetic parameters for activation and fast inactivation were obtained by fitting the data to a Boltzmann equation: y = bottom + (top – bottom)/(1 + *exp*((V_{50} – x)/Slope)).

Use-dependent inhibition was determined by normalizing the amplitude of the test current to the current induced by the first pulse.

3 Results

3.1 Intraplantar injection of QX-314 inhibits pain behavior induced by sodium channel activators

It was recently reported that QX-314 could be co-administered with the TLR5 agonist flagellin to selectively silence Aβ-fibers and reduce mechanical allodynia in multiple pain models *in vivo* (Xu et al., 2015). We initially sought to utilize selective QX-314-mediated Aβ-fiber silencing to explore the contribution of Aβ- and Aδ-fibers to $Na_V1.6$-mediated pain behaviors elicited by intraplantar injection of the $Na_V1.6$-selective β-scorpion toxin Cn2 (Israel et al., 2018). Intraplantar flagellin application alone did not change the number of pain behaviors in 10 minutes induced by Cn2 (**Fig. 1A**; Cn2: 74.5 ± 23.5; Cn2 + flagellin: 73.3 ± 2.9), while the co-application of QX-314 and flagellin abolished pain behaviors (0.4 ± 0.4; $p < 0.01$).

Surprisingly, although treatment with QX-314 alone with comparable doses was previously shown to lack efficacy in paclitaxel-induced neuropathy (Xu et al., 2015), we observed a similar striking inhibition of Cn2-mediated pain behaviors when QX-314 was administered without flagellin (1.8 ± 0.9; $p < 0.01$), which did not significantly differ from the co-application. To assess whether the surprising analgesic effects of QX-314 were specific to $Na_V1.6$-mediated pain, we next assessed nociceptive behaviors induced by the $Na_V1.7$-selective α-like toxin OD1 (Deuis et al., 2016), reasoning that the presence of toxins may have permitted intracellular access of QX-314. Similar to our observations with Cn2, intraplantar injection of OD1 caused spontaneous pain behaviors (**Fig. 1B**; pain behaviors/10 min: 154.4 ± 11.8), which were again significantly decreased by QX-314 alone (6.3 ± 3.9; $p < 0.0001$).

3.2 External QX-314 inhibits $Na_V1.7$ without state- and use-dependence

To explore the putative mechanisms of this unexpected analgesic activity in greater detail, we next turned to HEK293 cells heterologously expressing $Na_V1.7$ to assess whether the presence of OD1 could permit access for QX-314 to the intracellular local anesthetic binding site. Surprisingly, we observed that the presence of OD1 did not significantly affect the potency of external QX-314 (**Fig. 2A**; IC_{50}: QX-314, 2.0 ± 0.3 mM; QX-314 + OD1, 2.4 ± 0.4 mM). Indeed, concentrations of QX-314 that were analgesic *in vivo* (5 mM; **Fig. 1A** and **B**) led to substantial inhibition of $Na_V1.7$-mediated currents both in the presence and absence of OD1 (**Fig. 2A**), suggesting that analgesic activity could be mediated by direct effects on $Na_V1.7$.

Because OD1 inhibits inactivation by stabilizing the open state of $Na_V1.7$, we next determined the effects of externally applied QX-314 on $Na_V1.7$ channels at different holding potentials of -90 mV (where the majority of channels are in the closed/resting state) and -55 mV (where approximately half of the channels are in the open/inactivated state) (**Fig. 2C** and **D**). External

QX-314 did not show state-dependence with an IC_{50} of 2.0 ± 0.3 mM in the closed/resting state and an IC_{50} of 2.3 ± 0.4 mM in the open/inactivated state (**Fig. 2E**). In contrast, externally applied lidocaine had a significantly higher potency at $Na_V1.7$ in the open/inactivated state (**Fig. 2E**; IC_{50} 32.5 ± 9.0 µM) than in the closed/resting state (IC_{50} 681.6 ± 23.8 µM; $p < 0.0001$), consistent with its known mechanism of action. To exclude the possibility of use-dependent inhibition by QX-314, which could lead to selective silencing of rapidly-firing neurons, we next assessed the effect of QX-314 during a series of depolarizing pulses with frequencies of 1 Hz and 10 Hz. External lidocaine addition caused a reduction of current to 57.0 ± 10.3 % of the initial current at 10 Hz but not 1 Hz frequency ($p < 0.05$), whereas external QX-314 did not display use-dependence (**Fig. 2F** and **G**).

3.3 External and internal QX-314 inhibit $Na_V1.7$ via different mechanisms

Since external QX-314 displayed neither state-dependence nor use-dependence, we next assessed its effects on channel gating. Interestingly, external QX-314 caused striking effects on the voltage-dependence of channel gating that were distinct from the biophysical effects of external lidocaine at approximately equipotent concentrations (**Fig. 3A**, **B**, **D** and **E**, **Table 1**). Specifically, external QX-314 caused a small (ΔV_{50} 4.2 mV) but statistically significant depolarizing shift in the voltage-dependence of fast inactivation (**Fig. 3C**; **Table 1**) and a pronounced (ΔV_{50} 10.6 mV) shift in the voltage-dependence of activation to more depolarized potentials (**Fig. 3C**; $p < 0.001$). Consistent with activity at the intracellular local anesthetic binding site and the inability of QX-314 to cross membranes, QX-314 applied in the intracellular solution was significantly more potent than external QX-314, with a concentration of 5 mM leading to complete current block at all test voltages using the IV protocol shown in insert Fig. 3A (data not shown). At a concentration causing approximately

50% inhibition (100 µM) (Stueber et al., 2016), internal QX-314 no longer affected the voltage-dependence of activation (Δ V_{50} 0.86 mV) but now shifted voltage-dependence of inactivation to more negative membrane potentials (**Fig. 3C**; Δ V_{50} 17.0 mV, $p < 0.0001$). Similar effects were also observed for $Na_V1.4$, where external QX-314 reduced current by shifting the voltage-dependence of activation (**Fig. S1**).

The effect of internal, but not external, QX-314 was therefore qualitatively similar to the effects of lidocaine, which caused a shift of the voltage-dependence of fast inactivation to more negative membrane potentials after both internal and external application (**Fig. 3F**; **Table 1**; external lidocaine, Δ V_{50} 12.5 mV, $p < 0.0001$; internal lidocaine, Δ V_{50} 9.6 mV, $p < 0.01$). Interestingly, and in contrast to external QX-314, the voltage-dependence of activation was not affected by either external or internal lidocaine at equipotent concentrations (external lidocaine 600 µM, Δ V_{50} 1.9 mV; internal lidocaine 5 mM, Δ V_{50} 0.6 mV), or by external lidocaine at approximately IC_{90} (5 mM, data not shown).

Thus, the effects of internally applied QX-314 resemble those of lidocaine, while external QX-314 exhibited distinct effects on voltage-dependent activation and fast inactivation, suggesting that internal but not external QX-314 targets the local anesthetic binding site. Moreover, inhibition of $Na_V1.7$ current by externally applied QX-314 was readily reversible with current returning to control levels within minutes of washout (**Fig. 4**). To further investigate if the similar effects of lidocaine and internal QX-314 were both mediated at the same intracellular binding site in $Na_V1.7$ channels, we next assessed the pharmacology of QX-314 and lidocaine at the local anesthetic binding mutant [F1748A]$Na_V1.7$.

	V_{50} of voltage-dependence of activation	*V_{50} of voltage-dependence of fast inactivation*
$Na_V1.7$		
Control	–24.4 ± 1.3 mV	–59.3 ± 0.8mV
QX-314 ECS	–13.8 ± 0.8 mV*	–55.1 ± 1.6 mV*
QX-314 ICS	–23.5 ± 1.5 mV	–76.3 ± 0.8 mV*
Control	–24.2 ± 1.1 mV	–64.5 ± 1.0 mV
Lidocaine ECS	–26.1 ± 2.1 mV	–77.0 ± 1.3 mV*
Lidocaine ICS	–23.6 ± 2.3 mV	–74.1 ± 2.0 mV*
[F1748A]$Na_V1.7$		
Control	–21.4 ± 1.1 mV	–60.2 ± 1.4 mV
QX-314 ECS	–10.2 ± 2.8 mV*	–60.7 ± 0.7 mV
QX-314 ICS	–25.7 ± 2.5 mV	–60.3 ± 2.9 mV

*Table 1: V_{50} values of voltage-dependent activation and fast inactivation (QX-314 ECS: 5 mM; QX-314 ICS: 100 µM; lidocaine ECS: 600 µM; lidocaine ICS: 5 mM). Data reported as mean ± SEM. * indicates statistically significant shifts from controls tested with one-way ANOVA and Dunnett's post-test.*

3.4 Mutation of the intracellular located local anesthetic binding site abolishes the internal but not the external effects of QX-314

The local anesthetic binding site mutant [F1748A]$Na_V1.7$ had a significantly depolarized V_{50} of voltage-dependence of fast inactivation compared to WT $Na_V1.7$ (**Fig. S2A**; **Table S1**; Δ V_{50}

14.1 mV, $p < 0.01$), while the voltage-dependence of activation was not affected by the F1748A mutation.

Consistent with previous reports highlighting the importance of F1748 for local anesthetic activity (Ragsdale et al., 1994), the potency of external lidocaine was significantly decreased at the [F1748A]$Na_V1.7$ mutant in both the open/inactivated state (**Fig. 5A**; IC_{50}: WT, 42.3 ± 9.0 µM; F1748A, 1.7 ± 0.3 mM, $p < 0.001$) as well as in the closed/resting state (IC_{50}: WT, 637.4 ± 23.4 µM; F1748A, 4.8 ± 1.1 mM, $p < 0.05$, unpaired t-test; data not shown). In contrast, and consistent with an alternative binding site, the potency of external QX-314 was not affected by the F1748A mutation, neither in the open/inactivated state (**Fig. 5B**; IC_{50}: WT, 2.3 ± 0.3 mM; F1748A, 2.1 ± 0.4 mM) nor in the closed/resting state (IC_{50}: WT, 2.0 ± 0.3 mM; F1748A, 1.7 ± 0.4 mM; data not shown). In addition, the effect of external QX-314 on the voltage-dependence of activation was also not impacted. As in WT $Na_V1.7$ (**Fig. 3C**), external QX-314 shifted voltage-dependence of activation in [F1748A]$Na_V1.7$ to more depolarizing potentials (**Fig. 5C**; **Fig. S2B**; ΔV_{50} 11.2 mV, $p < 0.01$). However, internal QX-314 lost activity at the [F1748A]$Na_V1.7$ mutant and no longer affected voltage-dependence of fast inactivation (ΔV_{50} 0.1 mV), suggesting that it interacted with the local anesthetic binding site in WT $Na_V1.7$.

4 Discussion

Here, we describe for the first time the effect of externally applied QX-314 on $Na_V1.7$ heterologously expressed in HEK cells, without the co-expression of TRP channels, and find that at high concentrations, external QX-314 is able to inhibit $Na_V1.7$ peak current and shift the voltage-dependence of activation to more depolarized potentials. Given the distinct differences following external and internal application of QX-314 on $Na_V1.7$ biophysics it is

clear that QX-314 was not able to cross the membrane to access the internal local anesthetic binding site in our expression system. Therefore, we postulate that QX-314 has an additional externally accessible, albeit low affinity (IC_{50} = 2.0 mM), site of action at $Na_V1.7$.

To investigate the activity of external QX-314 on $Na_V1.7$ further, we disrupted the internal local anesthetic binding site, located in the inner cavity of the Na_V channel pore formed by residues in the S6 segments of DI, DIII, and DIV (Catterall et al., 2005; Catterall and Swanson, 2015). To do this, we generated an [F1748A]$Na_V1.7$ mutant, as the corresponding mutation in r$Na_V1.2$ has previously been demonstrated to reduce the affinity of etidocaine and internally applied QX-314 (Ragsdale et al., 1994). Consistent with the known pharmacology of lidocaine and QX-314, internal QX-314 (100 μM) no longer shifted the voltage-dependence of inactivation, and the potency of external lidocaine was decreased by 40-fold, confirming the importance of this residue for local anesthetic activity at the intracellular binding site. However, when QX-314 was applied externally, the potency and the depolarizing shift in the voltage-dependence of activation remained unaffected, providing further evidence that the externally accessible site of action does not require access to the internal local anesthetic binding site.

While the molecular mechanisms and structural features contributing to $Na_V1.7$ block by high concentrations of QX-314 remain to be determined, the decrease in peak current and depolarizing shift in voltage-dependence of activation seen in the presence of external QX-314 is reminiscent to that of protonation of Na_V channels (Jones et al., 2011). This suggests that the positively charged QX-314 may form multiple interactions with negatively charged amino acid residues in the pore and/or voltage-sensing domains of Na_V channels, to both block the pore and restrict the movement of the S4 voltage-sensing segments (Peters et al., 2018). In line with this, external QX-314 inhibited $Na_V1.4$ currents in a similar manner to

$Na_V1.7$, suggesting its effects are unlikely to be selective for particular Na_V subtype(s), although selectivity of external QX-314 at $Na_V1.1$–$Na_V1.9$ remains to be assessed.

QX-314 administered by intraplantar injection (5 mM) alone was able to attenuate nocifensive behaviors induced by the $Na_V1.6$ activator Cn2 and the $Na_V1.7$ activator OD1 *in vivo*. While the injected concentration of QX-314 is high enough to inhibit Na_V channels externally, there is uncertainty over the effective concentration reached at the site of action (nerve terminals) at the time of the behavioral measurements, which may have been lower than the external IC_{50} (< 2.0 mM). Therefore, we cannot preclude the possibility that QX-314 was able to access the internal binding site in our *in vivo* experiments to elicit anti-nociception, especially in native neurons expressing TRP channels. While it is unlikely the toxins themselves promoted Na_V channel states more amenable to entry of QX-314, given our results showing co-application of OD1 did not change the potency of external QX-314 at $Na_V1.7$, membrane depolarization caused by Cn2 and OD1 may have indirectly activated TRP channels by shifting the voltage-dependence of activation, allowing QX-314 to cross the membrane and access the intracellular binding site (Karashima et al., 2009; Vetter et al., 2012; Voets et al., 2004). Alternatively, QX-314 itself may have activated TRP channels, as QX-314 has previously been reported to activate TRPV1 channels at concentrations above 10 mM (Rivera-Acevedo et al., 2011). In addition, isoflurane has been demonstrated to activate TRP channels (Cornett et al., 2008; Matta et al., 2008), and as our ethics approval stipulates use of this inhalation anesthetic during intraplantar injections, we cannot exclude that intracellular entry of QX-314 via TRP channels was favored in our *in vivo* experiments. In support of this, studies that do not report any anti-nociceptive activity following intraplantar injection of QX-314 alone at comparable concentrations do not describe the use of anesthetic during intraplantar

injections (Binshtok et al., 2007; Xu et al., 2015). Therefore, it is difficult to ascertain how much the external QX-314 activity contributes to Na_V inhibition in native neurons *in vivo*.

Despite these caveats, our study challenges the dogma that QX-314 has no inhibitory effect on Na_V channels when applied externally, and results from studies using co-application of QX-314 with an agonist to silence specific neuronal populations need to be interpreted with caution, especially when using QX-314 at a high concentration and/or the appropriate QX-314 control alone is omitted. Indeed, the concentration of QX-314 used in many published studies often ranges from 0.2 to 2% (or 5–50 mM), which is well above the IC_{50} required to inhibit $Na_V1.7$ when applied externally (for review including table overview: (Wang et al., 2021)). Therefore, while the permanently charged lidocaine derivative QX-314 may be considered membrane impermeable, it should not be considered inactive when applied extracellularly, particularly at doses frequently used in *in vivo* experiments.

5 Conclusion

In conclusion, using an expression system in which QX-314 could not access the intracellular local anesthetic binding site, we identified that at high concentrations, external QX-314 is able to inhibit sodium current by shifting the voltage-dependence of activation to more depolarized potentials.

Acknowledgements

This work was funded by the Australian National Health and Medical Research Council (NMHRC) through a NHMRC Career Development Fellowship (APP1162503) awarded to I.V. and a NHMRC Early Career Fellowship (APP1139961) awarded to J.R.D.

Declaration of interest

None.

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Figure 1. Analgesic effects of QX-314 on sodium channel activator-induced pain. (**A**) Flagellin (0.9 µg) did not affect spontaneous pain caused by 10 nM Cn2 but co-application of flagellin and QX-314 (5 mM) as well as QX-314 on its own significantly reduced spontaneous pain (Cn2, n = 4; Cn2 + flagellin, n = 3; Cn2 + flagellin + QX-314, n = 5; Cn2 + QX-314, n = 4; $p < 0.01$, one-way ANOVA, Šídák's post-test). (**B**) QX-314 (5 mM) significantly decreased spontaneous pain caused by 300 nM OD1 (OD1, n = 5; OD1 + QX-314, n = 4; $p < 0.0001$, unpaired t-test). Data are presented as mean ± SEM.

Figure 2. External QX-314 is neither state-dependent nor use-dependent. (**A**) Concentration response curve of external QX-314 elicited by a 50-ms pulse to −20 mV from a holding potential of −90 mV with and without OD1 (300 nM). The presence of OD1 did not affect the potency of external QX-314 control (IC_{50}: control (blue), 2.0 ± 0.3 mM, n = 9; OD1 (pink), 2.4 ± 0.4 mM, n= 10). (**B**) Representative traces of sodium current before (black) and after addition of 300 nM OD1 (grey) or 300 nM OD1 and 5 mM QX-314 (pink). (**C**) Voltage protocol used to assess the closed/resting state, repetition interval 20 s. (**D**) Voltage protocol used to assess the open/inactivated state, repetition interval 12 s. (**E**) IC_{50} values of external QX-314 and lidocaine obtained from concentration response curves. Current was elicited by a 50-ms pulse to −20 mV from a holding potential of 90 mV or by a 20-ms pulse to −20 mV from an 8-s conditioning voltage step of −55 mV. External QX-314 had no preference for the closed/resting state (IC_{50} 2.0 ± 0.3 mM, n = 9) or the open/inactivated state (IC_{50} 2.3 ± 0.4 mM, n = 8). External lidocaine displayed significant preference for the open/inactivated state (IC_{50} 32.5 ± 9.0 µM, n = 5) compared to the closed/resting state (IC_{50} 681.6 ± 23.8 µM, n = 3); $p < 0.0001$, unpaired t-test. (**F**) $Na_V1.7$ pulsed from −100 mV to 0 mV at 10 Hz in absence (black, n = 12) and presence of 3 mM QX-314 (blue, n = 9) or 300 µM lidocaine (orange, n = 3). Inset shows voltage protocol, frequency 10 Hz. (**G**) Normalized current of last pulse at frequencies of 1 Hz and 10 Hz for external QX-314 (3 mM) and external lidocaine (300 µM). Unlike lidocaine (1 Hz, 103.3 ± 2.4%, n = 3; 10 Hz, 57.0 ± 10.3%, n = 3, $p < 0.05$, unpaired t-test), external QX-314 did not display use-dependence (1 Hz, 85.9 ± 5.7%, n = 6; 10 Hz, 84.7 ± 3.5%, n = 9). Data are presented as mean ± SEM.

Figure 3. Effect of QX-314 and lidocaine on electrophysiological properties of $Na_V1.7$. (**A**) Representative traces of sodium current before (control) and after addition of 5 mM QX-314. Inset shows voltage protocol used to determine voltage-dependence of activation and voltage-dependence of inactivation, repetition interval 6 s. For clarity, only currents elicited by every other voltage step is shown; current at –10 mV shown in blue. (**B**) Current-voltage relationship in absence (black) and presence of 5 mM external QX-314 (blue, n = 5). Current in presence of QX-314 was normalized to buffer control. (**C**) Voltage-dependence of activation (filled symbols) and fast inactivation (clear symbols) in absence (black, n = 5) and presence of 5 mM external (blue, n = 5; added to ECS) and 100 µM internal QX-314 (orange, n = 5; added to ICS). Currents of each group (buffer and QX-314) are normalized to themselves. External QX-314 shifted activation to more depolarizing potentials (V_{50}: control, –24.4 $\pm$ 1.3 mV; QX-314 ECS, –13.8 $\pm$ 0.8 mV, $p < 0.001$) and caused a slight shift in inactivation (V_{50}: control, –59.3 $\pm$ 0.8 mV; QX-314 ECS, –55.1 $\pm$ 1.6 mV, $p < 0.05$). Internal QX-314 shifted inactivation to more hyperpolarizing potentials (V_{50} –76.3 $\pm$ 0.8 mV, $p < 0.0001$) and did not affect activation (V_{50} –23.5 $\pm$ 1.5 mV). (**D**) Representative traces of sodium current before (control) and after addition of 600 µM lidocaine. Inset shows voltage-protocol used to determine voltage-dependence of activation and voltage-dependence of inactivation, repetition interval 6 s. For clarity, only currents elicited by every other voltage step are shown; current at –10 mV shown in orange. (**E**) Current-voltage relationship in absence (black) and presence of 5 mM (white, n = 8) and 600 µM external lidocaine (orange, n = 6). Current in presence of lidocaine was normalized to buffer control. (**F**) Voltage-dependence of activation (filled symbols) and fast inactivation (clear symbols) in absence (black, n = 6) and presence of 600 µM external (blue, n = 6; added to ECS) and 5 mM internal lidocaine (orange, n = 3; added to ICS). Currents of each group (buffer and lidocaine) are normalized to themselves. Both external and internal

lidocaine shifted inactivation to more hyperpolarizing potentials (V_{50}: control, –64.5 $\pm$ 1.0 mV; lidocaine ECS, –77.0 $\pm$ 1.3 mV, $p < 0.0001$; lidocaine ICS, –74.1 $\pm$ 2.0 mV, $p < 0.01$) but did not affect voltage-dependence of activation (V_{50}: control, –24.2 $\pm$ 1.1 mV; lidocaine ECS, –26.1 $\pm$ 2.1 mV; lidocaine ICS, –23.6 $\pm$ 2.3 mV). Data are presented as mean $\pm$ SEM. Statistical significance was determined using one-way ANOVA with Dunnett's post-test.

Figure 4. Effect of external QX-314 is reversible. (**A**) Repeated wash steps (grey arrows, repetition interval 60 s) reversed the effect of 5 mM external QX-314 (blue arrow), n = 5. (**B**) Voltage protocol used during washout, repetition interval 20 s.

Figure 5. Effects of QX-314 at [F1748A]$Na_V1.7$. Concentration response curves of lidocaine (**A**) and QX-314 (**B**) at WT and [F1748A]$Na_V1.7$ in the open/inactivated state. The F1748A mutation reduced the potency of external lidocaine (IC_{50}: WT, 42.3 $\pm$ 9.0 µM, n = 5; F1748A, 1.7 $\pm$ 0.3 mM, n = 5; $p < 0.001$, unpaired t-test) but not external QX-314 (IC_{50}: WT, 2.3 $\pm$ 0.3 mM, n = 8; F1748A, 2.1 $\pm$ 0.4 mM, n = 5). (**C**) Voltage-dependence of activation (filled symbols) and fast inactivation (clear symbols) of [F1748A]$Na_V1.7$ in absence (black) and presence of 5 mM external (blue; added to ECS) and 100 µM internal QX-314 (orange; added to ICS). Currents of each group (buffer and QX-314) are normalized to themselves. External QX-314 had no effect on voltage-dependence of fast inactivation (V_{50}: control, –60.2 $\pm$ 1.4 mV, n = 6; QX-314 ECS –60.7 $\pm$ 0.7 mV, n = 3) but shifted voltage-dependence of activation to more depolarized potentials (V_{50}: control, –21.4 $\pm$ 1.1 mV, n = 5; QX-314 ECS, –10.2 $\pm$ 2.8 mV, n = 4; $p < 0.01$, one-way ANOVA, Dunnett's post-test). Internal QX-314 had no effect on voltage-dependence of inactivation (V_{50}: QX-314 ICS, –60.3 $\pm$ 2.9 mV, n = 4) and activation (V_{50}: QX-314 ICS, –25.7 $\pm$ 2.5 mV, n = 4). All data are presented as mean $\pm$ SEM.