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The anti-nociceptive agent ralfinamide inhibits tetrodotoxin-resistant and tetrodotoxin-sensitive Na^+ currents in dorsal root ganglion neurons

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Abstract

Tetrodotoxin-resistant and tetrodotoxin-sensitive Na^+ channels contribute to the abnormal spontaneous firing in dorsal root ganglion neurons associated with neuropathic pain. Effects of the anti-nociceptive agent ralfinamide on tetrodotoxin-resistant and tetrodotoxin-sensitive currents in rat dorsal root ganglion neurons were therefore investigated by patch clamp experiments. Ralfinamide inhibition was voltage-dependent showing highest potency towards inactivated channels. IC_{50} values for tonic block of half-maximal inactivated tetrodotoxin-resistant and tetrodotoxin-sensitive currents were 10 μM and 22 μM. Carbamazepine, an anticonvulsant used in the treatment of pain, showed significantly lower potency. Ralfinamide produced a hyperpolarising shift in the steady-state inactivation curves of both currents confirming the preferential interaction with inactivated channels. Additionally, ralfinamide use and frequency dependently inhibited both currents and significantly delayed repriming from inactivation. All effects were more pronounced for tetrodotoxin-resistant than tetrodotoxin-sensitive currents. The potency and mechanisms of actions of ralfinamide provide a hypothesis for the anti-nociceptive properties found in animal models.

Keywords: Dorsal root ganglion; Sensory neuron; Tetrodotoxin-resistant sodium channel; Tetrodotoxin-sensitive sodium channel; Ralfinamide; NW-1029

1. Introduction

Primary afferent dorsal root ganglion neurons transmit sensory signals into the central nervous system. In normal physiological conditions these signals generally originate at axonal transducer endings in peripheral tissue such as skin and muscle, although sparse spontaneous activity can occur (Bridges et al., 2001). After peripheral nerve injury abnormal spontaneous firing is greatly increased. This hyperexcitability is believed to be a major contributor to neuropathic pain (Bridges et al., 2001). Evidence implies that abnormal neuropathic firing in dorsal root ganglion neurons is accompanied by changes in the expression of voltage-gated Na^+ channels (Lai et al., 2004; Waxman et al., 2002). Since voltage-gated Na^+ channels are critical for action potential generation and impulse propagation, changes in their density are likely to play a major role in the development of neuronal hyperexcitability. In line with this, certain anticonvulsants exhibiting Na^+ blocking properties, e.g. carbamazepine, phenytoin and lamotrigine (Brau and Elliott, 1998; Kuo, 1998) are found to relieve trigeminal neuralgia and central neuropathic pain (Backonja, 2002; Vu, 2004). Thus, Na^+ currents of dorsal root ganglion sensory neurons may provide attractive targets for the development of anti-nociceptive agents.

Two types of voltage-gated Na^+ currents, tetrodotoxin-resistant and tetrodotoxin-sensitive, are expressed in dorsal root ganglion neurons. Two tetrodotoxin-resistant channels exclusively expressed in peripheral sensory neurons ($Na_v1.8$ and 1.9) underlay the tetrodotoxin-resistant currents, while several tetrodotoxin-sensitive channel subtypes contribute to the tetrodotoxin-sensitive currents (Cummins et al., 1999; Lai et al., 2004). Experimental evidence has implicated specific Na^+ channel subtypes with the develop-

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ment of pain conditions. Particularly, redistribution of $Na_v1.8$ channels to the uninjured axons after nerve injury and up-regulation of $Na_v1.3$ channels following axotomy or spinal cord injury appear to correlate with neuropathic pain symptoms and neuronal hyperexcitability (Gold et al., 2003; Hains et al., 2003, 2004; Lai et al., 2004; Waxman et al., 2002). Nevertheless, the relationship between changes in neuronal Na^+ channel densities and firing properties is not fully understood. Accordingly, the relative importance of tetrodotoxin-resistant and tetrodotoxin-sensitive channels for development of neuropathic pain is unclear (Lai et al., 2004; Waxman et al., 2002).

Ralfinamide, previously named NW-1029, was selected from a chemical class of α-aminoamide derivatives blocking Na^+ channels. The drug demonstrates potent anti-hyperalgesic and anti-allodynic properties in animal models of chronic neuropathic pain (Veneroni et al., 2002, 2003). In the present study the inhibitory effects by ralfinamide on Na^+ currents in dorsal root ganglion neurons were investigated in order to explore the mechanism of action of the drug. A previous publication has demonstrated the ability of ralfinamide to block tetrodotoxin-resistant channels in dorsal root ganglion neurons (Faravelli et al., 2003). The present study investigates for the first time the inhibitory effects of ralfinamide on tetrodotoxin-resistant versus tetrodotoxin-sensitive Na^+ currents in rat dorsal root ganglion neurons and compare them to those of the anticonvulsant carbamazepine, which is used to treat trigeminal neuralgia and has shown efficacy in controlled clinical studies of central neuropathic pain (Backonja, 2002; Vu, 2004).

2. Materials and methods

2.1. Cell preparation and culturing

Acutely isolated lumbar dorsal root ganglion neurons were prepared from adult male Wistar rats (150–250 g). Animals were sacrificed under anaesthesia in accordance with the Italian regulations on protection of animals used for experimental and other scientific purpose (D.M. 116192) as well as with the EEC regulation (OJ of E.C. No L 358/1 18.12.86). Ganglia (L1–L5) were dissected from the vertebral column and placed in ice-cold Tyrode (in mM: NaCl 150, KCl 4, $MgCl_2$ 2, Hepes 10, $CaCl_2$ 2, Glucose 10, Phenol red 0.045, pH 7.4 (NaOH)). Connective tissue capsules and nerve trunks were removed and ganglia cut in 2–3 pieces.

Ganglia were enzymatically digested at 37 °C for 30 min in Ca^{2+} free Tyrode containing 1.6 mg/ml collagenase/dispase enzyme (Roche) and for 30 min in Tyrode containing 0.7 mg/ml collagenase (Sigma), 24 mM HCO_3^- and 150 μM Ca^{2+}. After enzymatic digestion the ganglia were mechanically dissociated in Dulbecco's modified Eagle medium (Invitrogen) with 10% FCS using a fire polished Pasteur pipette, centrifuged at 22,000×*g* for 10 min and resuspended in Neurobasal Medium containing 2% B27 (Invitrogen), 2 mM glutamine and 2 mM penicillin streptomycin. Neuronal growth factor (100 ng/ml) was added when tetrodotoxin-resistant currents were to be studied. Cells were plated on poly-D-lysine coated petri dishes and stored at 37 °C in 95% CO_2 and 5% O_2. Patch clamp experiments were performed within 24 h.

2.2. Whole cell patch clamp recordings

Experiments on isolated neurons were carried out using standard whole cell patch clamp methods (Hamill et al., 1981). Membrane currents were recorded and filtered at 5 kHz with an Axon Axopatch 200B amplifier and digitized with an Axon Digidata 1322A (Axon Instruments, CA, USA). Voltage clamping of membrane potentials and data acquisition were controlled online with Axon pClamp9 software. Measuring and reference electrodes were AgCl-Ag electrodes. A Sutter Instrument P-87 Puller (CA, USA) was used for pulling patch clamp pipettes with a resistance of 2–4 MΩ from Harward borosilicate glass tubes. Cells had initial seal resistances of >1 GΩ and access resistances of 4.6±0.2 MΩ. Cells were continuously superfused with extracellular solutions, using a Biologic RSC-200.

2.3. Solutions and drugs

External patch clamp solution for studying tetrodotoxin-sensitive currents consisted of (mM): NaCl 40, CholineCl 78, $CaCl_2$ 1.3, $MgCl_2$ 2, KCl 2, $CdCl_2$ 0.4, $NiCl_2$ 0.3, tetraethylammonium chloride 20, HEPES 10, glucose 10 (pH 7.4). When tetrodotoxin-resistant currents were studied 20 mM CholineCl were substituted with NaCl and 250 nM tetrodotoxin added. 250 nM tetrodotoxin completely blocked tetrodotoxin-sensitive currents (not shown). The internal solution contained (mM): CsCl 65, CsF 65, NaCl 10, $CaCl_2$ 1.3, $MgCl_2$ 2, HEPES 10, EGTA 10, MgATP 1 (pH 7.4). Carbamazepine was from Sigma. Stock solutions of ralfinamide (10 mM) and carbamazepine (200 mM) were made in Milli-Q H_2O and dimethylsulfoxide, respectively, and diluted in external solution to final concentrations used.

2.4. Data analysis

Data were analysed using Clampfit 9 (Axon Instruments, CA) and Origin 7.5 (Microcal Inc., Northampton, MA, USA) software. Data points and results are given as arithmetic mean±S.E.M. or when appropriate as geometric mean with 95% confidence limits. *n* indicates the number of cells in each experimental group. Statistical comparisons were made using Student's *t*-test and results considered significantly different when $P<0.05$.

For construction of steady-state inactivation curves, the peak currents (I) elicited from pre-conditioning potentials of −120 to −10 mV were normalised relative to the maximal peak current at −120 mV (I_{max}), and plotted against the respective pre-conditioning potentials. Boltzmann functions were fitted to the steady-state inactivation data, according to the following equation: $I/I_{max}=1/\{1+\exp[(V_{pre}-V_{1/2})/k_h]\}$. V_{pre} is the pre-conditioning potential, $V_{1/2}$ the potential at which half-maximal current inactivation occurs and k_h the corresponding slope factor. A $V_{holding}$ of −120 mV was applied in both tetrodotoxin-resistant and tetrodotoxin-sensitive steady-state inactivation experiment. For all other types of experiments was $V_{holding}$ −120 mV when tetrodotoxin-sensitive currents were studied and −90 mV when tetrodotoxin-resistant currents were studied.

For construction of activation curves, Na^+ conductance (g_{Na}) was calculated according to the equation: $g_{Na}=I/(V_{test}-E_{Na})$. I is the peak current (I) elicited from the pre-conditioning potentials to different test potentials (V_{test}) in 5 mV increments, E_{Na} is the membrane potential at which the peak Na^+ current is reversed. Na^+ conductances were normalised to the maximal Na^+ conductance (g_{Max}) and plotted against test potentials. Boltzmann functions were fitted to the activation curves according to the equation: $g_{Na}/g_{Max}=1/\{1+\exp[(V_{1/2Na}-V_{test})/k_g]\}$. $V_{1/2Na}$ is the potential at which half-maximal Na^+ conductance occurs and k_g is slope factor of the curve.

Tonic blocks were calculated as the decrease in peak current induced by the drug as compared to the current obtained in control/recovery solution immediately before. Drug concentration–inhibition curves were obtained by plotting tonic blocks versus drug concentrations. Dose–response curves were fitted to the tonic block data, according to the logistic equation: $y=A_2+(A_1-A_2)/[1+(x/IC_{50})^p]$. A_1 and A_2 are fixed values of 0 and 1 corresponding to 0% and 100% current inhibition, x is the drug concentration, IC_{50} is the drug concentration resulting in 50% current inhibition and p is the corresponding slope factor.

Repriming was studied applying two pulse protocols composed by an inactivation step, a varying recovery interval and a test pulse. Current amplitudes elicited after the recovery periods were normalised to the peak values of the inactivation steps and plotted against the lengths of the recovery intervals. The equation $y=y_0+A_1(1-e^{(-x/\tau_1)})+A_2(1-e^{(-x/\tau_2)})$ was fitted to the repriming data. τ_1 and τ_1 are the decay factors, A_1 and A_2 are the percentage of repriming occurring in the respective phase and y_0 is the offset.

3. Results

3.1. Slow tetrodotoxin-resistant and fast tetrodotoxin-sensitive currents are present in dorsal root ganglion neurons

Two Na^+ current phenotypes, slow tetrodotoxin-resistant and fast tetrodotoxin-sensitive currents, were identified in dorsal root ganglion neurons on the basis of their kinetics and their sensitivity to tetrodotoxin (250 nM). Tetrodotoxin-resistant current peaks showed considerably slower activation and inactivation kinetics as compared to tetrodotoxin-sensitive as evident from the current traces in Figs. 1A, 2A and 4A versus Figs. 1B, 3A and 5A.

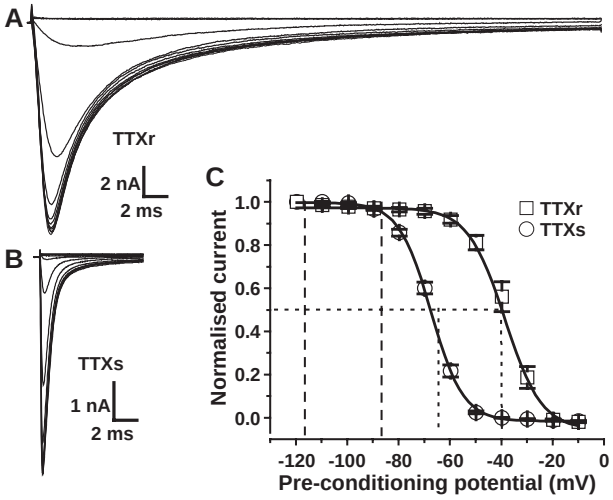

Fig. 1. Steady-state inactivation properties of tetrodotoxin-resistant (TTXr) and tetrodotoxin-sensitive (TTXs) currents were studied applying pre-conditioning potentials ranging from −120 to −10 mV for 2 s followed by −10 mV test potential. (A) and (B) show a tetrodotoxin-resistant and a tetrodotoxin-sensitive steady state inactivation experiment, respectively, illustrating the different activation and inactivation kinetics. (C) Means±S.E.M. are shown for tetrodotoxin-resistant (□) and tetrodotoxin-sensitive (○) experiments. Potentials leading to half-maximal tetrodotoxin-resistant and tetrodotoxin-sensitive currents are −38±2 mV (n=11) and −67±1 mV (n=10), respectively, as indicated by dotted lines. No inactivation is present when tetrodotoxin-resistant currents are elicited from −90 mV and tetrodotoxin-sensitive currents from −120 mV, as indicated by dashed lines. The slope factors of the tetrodotoxin-resistant (k_h=5.8±0.4) and the tetrodotoxin-sensitive (k_h=6.1±0.1) curves were not statistically different.

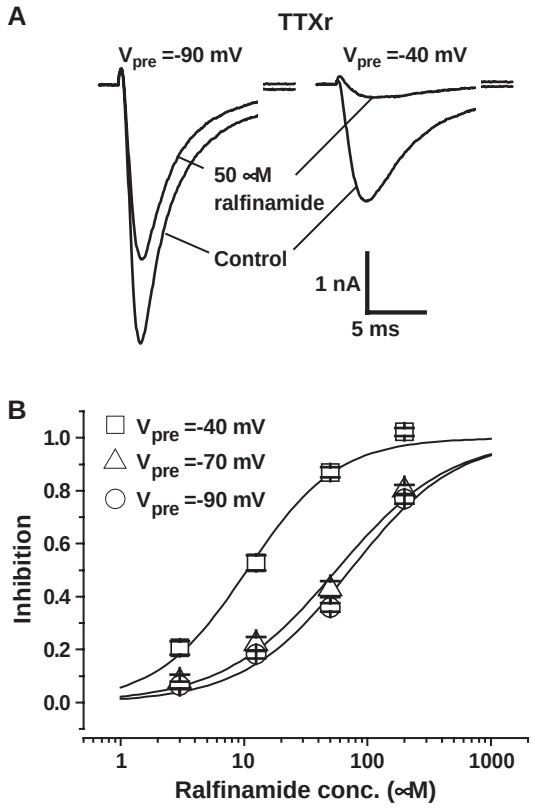

Fig. 2. Ralfinamide concentration- and voltage-dependently inhibits tetrodotoxin-resistant (TTXr) currents. Tonic block by ralfinamide of tetrodotoxin-resistant currents was studied eliciting currents by stepping to −10 mV from 2 s pre-conditioning potentials (V_{pre}) of −90 mV, −70 mV and −40 mV, respectively. (A) Representative experiments showing the tonic block induced by 50 μM ralfinamide of currents evoked from potentials leading to maximal (−90 mV) and half-maximal (−40 mV) tetrodotoxin-resistant currents. (B) Concentration–inhibition curves showing mean±S.E.M. of data for ralfinamide inhibition of tetrodotoxin-resistant currents elicited from the three pre-conditioning potential. IC_{50} values of 10 μM (4–25 μM, n=17), 55 μM (26–117 μM, n=8,) and 72 μM (39–135 μM, n=17) were obtained for currents evoked from −40 mV (□), −70 mV (△) and −90 mV (○), respectively.

Tetrodotoxin-resistant currents were studied in small dorsal root ganglion neurons (the mean membrane capacitance was 30±1 pF, n=63) as these neurons are thought to be more numerous in nociceptors than larger neurons (Djouhri et al., 1998). Sizes of cells used for different types of tetrodotoxin-resistant experiments were not significantly different. Most small neurons expressed tetrodotoxin-sensitive and tetrodotoxin-resistant currents in variable proportions. All tetrodotoxin-resistant experiments were therefore performed in presence of tetrodotoxin in order to isolate tetrodotoxin-resistant currents.

In agreement with other observations (Cummins and Waxman, 1997; Weiser and Wilson, 2002; Zhou and Zhao, 2000), very few small dorsal root ganglion neurons expressing only tetrodotoxin-sensitive current could be found in the present study (<3%). Currently, no pharmacological tools are available to inhibit tetrodotoxin-resistant but not tetrodotoxin-sensitive currents. Medium sized neurons, expressing tetrodotoxin-sensitive, but not tetrodotoxin-resistant currents, with a mean membrane capacitance of 56±1 pF (n=46) were therefore used for tetrodotoxin-sensitive experiments. Sizes of cells used for different types of tetrodotoxin-sensitive experiments were not significantly different.

3.2. Steady-state inactivation properties of tetrodotoxin-resistant and tetrodotoxin-sensitive currents

Classical Na^+ channel blockers (lamotrigine, phenytoin, carbamazepine, lidocaine) cause a larger inhibition of partly inactivated Na^+ currents than of non-inactivated currents (Brau et al., 2001; Kuo, 1998). Drug inhibition of tetrodotoxin-resistant and tetrodotoxin-sensitive peak current amplitudes (tonic block) were therefore studied on currents evoked from pre-conditioning potentials keeping the channels in the resting state and resulting in maximal currents, and from depolarised pre-conditioning potentials inactivating channels and leading to half-maximal currents.

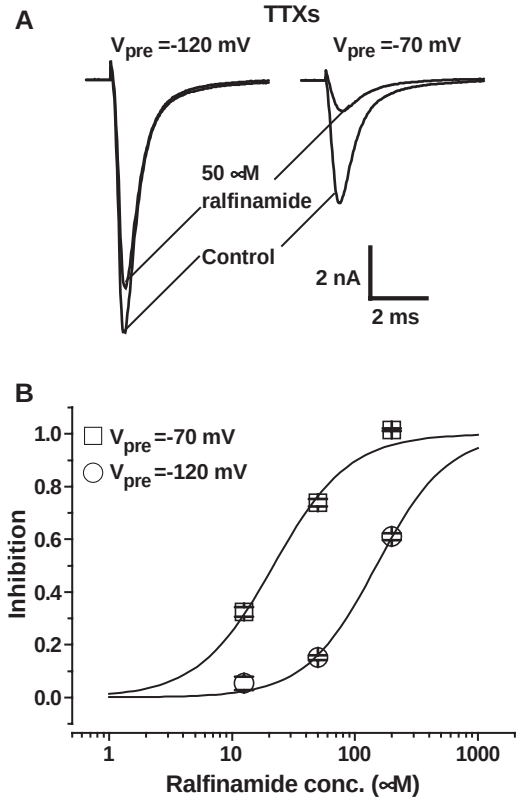

Fig. 3. Ralfinamide inhibits tetrodotoxin-sensitive currents (TTXs) in a concentration and voltage-dependent manner with depolarised conditions favouring the inhibition. Tonic block experiments were performed eliciting tetrodotoxin-sensitive currents by stepping to −10 mV from 2 s pre-conditioning potentials (V_{pre}) of −120 mV (n=9) and −70 mV (n=6), respectively. (A) illustrates representative experiments investigating the tonic inhibition by 50 μM ralfinamide of tetrodotoxin-sensitive currents evoked from potentials leading to maximal (−120 mV) and half-maximal (−70 mV) currents. (B) shows mean±S.E.M. of data for ralfinamide inhibition of tetrodotoxin-sensitive currents elicited from the two pre-conditioning potentials. The concentration–inhibition curves gave IC_{50} values of 22 μM (17–28 μM, n=6) and 151 μM (120–186 μM, n=9) for currents evoked from −70 mV (□) and −120 mV (○), respectively.

Table 1
IC_{50} values for ralfinamide and carbamazepine block of tetrodotoxin-resistant (TTXr) and tetrodotoxin-sensitive (TTXs) currents evoked from different pre-conditioning potentials (V_{pre})

V_{pre} (mV)	Ralfinamide (μM)		Carbamazepine (μM)	
	TTXr	TTXs	TTXr	TTXs
−120		151 (*n*=9)		>800 (*n*=10)
−90	72 (*n*=17)		>800 (*n*=14)	
−70	55 (*n*=8)	22 (*n*=6)	>800 (*n*=6)	85 (*n*=8)
−40	10 (*n*=17)		123 (*n*=14)	

IC_{50} values obtained using pre-conditioning potentials of −90 and −40 mV are the values for inhibition of maximal and half-maximal tetrodotoxin-resistant currents. Using pre-conditioning potentials of −120 and −70 mV results in IC_{50} values for inhibition of maximal and half-maximal tetrodotoxin-sensitive currents. Results are given as geometric means. *n* indicates the number of cells.

The study of the steady-state inactivation properties of tetrodotoxin-resistant and tetrodotoxin-sensitive currents in dorsal root ganglion neurons revealed that membrane potentials leading to half-maximal currents ($V_{1/2}$) were −38 mV and −67 mV, respectively (Fig. 1C). No inactivation was present when tetrodotoxin-resistant currents were elicited from −90 mV and tetrodotoxin-sensitive currents from −120 mV (Fig. 1C). Pre-conditioning potentials of −90 and −40 for tetrodotoxin-resistant currents and −120 and −70 for tetrodotoxin-sensitive currents were therefore used in the tonic block experiments in order to have maximal and half-maximal current availabilities. In addition, tonic block of tetrodotoxin-resistant currents elicited from −70 mV was studied in order to be able to compare tonic blocks of currents elicited from the same potential.

3.3. Ralfinamide voltage-dependently inhibits tetrodotoxin-resistant and tetrodotoxin-sensitive currents

Ralfinamide (3–200 μM) inhibition of tetrodotoxin-resistant and tetrodotoxin-sensitive peak current amplitudes (tonic block) was strictly concentration dependent (Figs. 2 and 3). The inhibition was maximal within 2 min after extracellular application of the drug and currents recovered within few min of washout with drug-free external solution.

Tetrodotoxin-resistant currents, evoked from a pre-conditioning potential giving maximal current availability (−90 mV), were inhibited by ralfinamide with an IC_{50} value of 72 μM (Fig. 2, Table 1). The IC_{50} value for tonic block by ralfinamide of tetrodotoxin-resistant currents elicited from a slightly depolarised pre-conditioning potential of −70 mV was lower, but not significantly different from the IC_{50} for tonic block of the maximal peak currents (55 μM, Fig. 2, Table 1). Strong voltage-dependent increase of ralfinamide inhibition was obtained when a depolarised pre-conditioning potential of −40 mV ($V_{1/2}$ for tetrodotoxin-resistant currents) was applied, yielding an IC_{50} value of 10 μM (Fig. 2, Table 1).

Ralfinamide inhibited tetrodotoxin-sensitive currents evoked from a resting state (V_{pre}=−120 mV) showing an IC_{50} value for the tonic block of 151 μM (Fig. 3, Table 1). Significant voltage-dependent increase of the sensitivity to ralfinamide was found, as the IC_{50} value for inhibition of tetrodotoxin-sensitive current elicited from $V_{1/2}$ (−70 mV) was 22 μM (Fig. 3, Table 1).

3.4. Carbamazepine inhibits tetrodotoxin-resistant and tetrodotoxin-sensitive currents with less potency than ralfinamide

Tonic block by carbamazepine (50–800 μM) of tetrodotoxin-resistant and tetrodotoxin-sensitive in dorsal root ganglion neurons was strictly concentration dependent (Figs. 4 and 5). The effect was stable within 2 min after extracellular application of the drug and current recovery occurred after few min of washout with drug-free external solution.

Carbamazepine had only weak inhibitory effect on tetrodotoxin-resistant currents evoked from a pre-conditioning potential giving maximal current availability (V_{pre}=−90 mV, IC_{50}>800 μM, Fig. 4, Table 1) and from the slightly

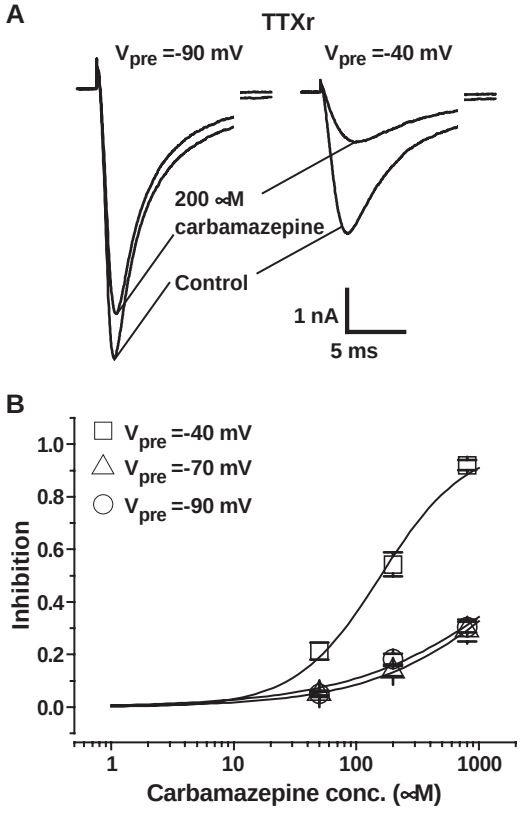

Fig. 4. Tonic block of tetrodotoxin-resistant currents (TTXr) by carbamazepine is concentration and voltage dependent. Currents were evoked by stepping to −10 mV from pre-conditioning potentials (V_{pre}) of −90 mV (*n*=14), −70 mV (*n*=6) and −40 mV (*n*=14), respectively. (A) Representative experiments of carbamazepine inhibition of tetrodotoxin-resistant currents evoked from potentials leading to maximal (−90 mV) and half-maximal (−40 mV) currents. (B) Concentration–inhibition curves showing mean±S.E.M. of data for carbamazepine inhibition of tetrodotoxin-resistant currents elicited from the three pre-conditioning potential. In depolarised conditions (−40 mV, □) an IC_{50} value of 123 μM (16–977 μM, *n*=14) was obtained. For currents evoked from −70 mV (△) and −90 mV (○) the IC_{50} values were >800 μM (*n*=6 and *n*=14, respectively).

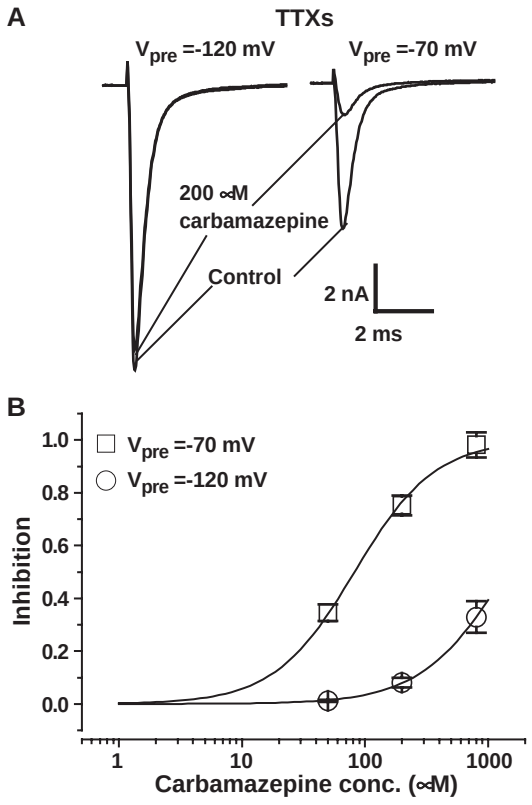

Fig. 5. Tonic block of tetrodotoxin-sensitive (TTXs) currents by carbamazepine is concentration and voltage dependent. Currents were elicited by stepping to −10 mV from pre-conditioning potentials (V_{pre}) of −120 mV (n=10) and −70 mV (n=8), respectively. (A) illustrates representative experiments investigating the tonic inhibition by 200 μM carbamazepine of tetrodotoxin-sensitive currents evoked from potentials leading to maximal (−120 mV) and half-maximal (−70 mV) currents. (B) Mean±S.E.M. of data for carbamazepine inhibition of tetrodotoxin-sensitive currents elicited from the two pre-conditioning potentials is shown in the concentration–inhibition curves. An IC_{50} value of 85 μM (45–158 μM, n=8) was obtained for currents evoked from −70 mV (□). The IC_{50} for block of maximal currents (−120 mV, ○) was >800 μM (n=10).

depolarised pre-conditioning potential of −70 mV (IC_{50}>800 μM, Fig. 4, Table 1). Further depolarising the pre-conditioning potential to −40 mV ($V_{1/2}$) yielded an IC_{50} value of 123 μM (Fig. 4, Table 1) and demonstrated voltage-dependence of carbamazepine inhibition of tetrodotoxin-resistant currents.

Tetrodotoxin-sensitive currents evoked from a resting state were only weakly inhibited by carbamazepine (V_{pre}=−120 mV, IC_{50}>800 μM, Fig. 5, Table 1). An IC_{50} of 85 μM (Fig. 5, Table 1) was determined for tonic block by carbamazepine of tetrodotoxin-sensitive currents elicited from $V_{1/2}$ (−70 mV) demonstrating voltage-dependence.

3.5. Ralfinamide shifts the steady-state inactivation curves of tetrodotoxin-resistant and tetrodotoxin-sensitive currents, without affecting the activation curves

The effects of ralfinamide on tetrodotoxin-resistant and tetrodotoxin-sensitive activation and steady-state inactivation were investigated in order to characterise the mechanism of action of ralfinamide inhibition of Na^+ currents in dorsal root ganglion neurons. Ralfinamide shifted the steady-state inactivation curves of both currents in hyperpolarising directions, but without changing the slopes of the curves (P<0.01). A concentration of 50 μM shifted $V_{1/2}$ for tetrodotoxin-resistant currents −11 mV, from −38 mV in control conditions to −49 mV (Fig. 6A). The same concentration shifted the $V_{1/2}$ of tetrodotoxin-sensitive currents −15 mV from −67 mV in control condition to −82 mV (Fig. 6B). The difference between the shifts induced by ralfinamide of the tetrodotoxin-resistant and the tetrodotoxin-sensitive steady-state inactivation curves were statistically significant (0.01<P<0.05).

In contrast, ralfinamide did not induce a significant change in neither the half-maximal voltage for activation nor the slope values of the tetrodotoxin-resistant (Fig. 6C, P<0.01) and tetrodotoxin-sensitive activation curves (Fig. 6D). Thus, the drug does not shift the voltage range across which tetrodotoxin-resistant and tetrodotoxin-sensitive activation occur.

3.6. Use and frequency dependent inhibition by ralfinamide of tetrodotoxin-resistant and tetrodotoxin-sensitive currents

Use (the number of stimulations) and frequency dependence of ralfinamide block of tetrodotoxin-resistant and tetrodotoxin-sensitive currents were evaluated applying 30 consecutive pulses at frequencies of 0.5 and 5 Hz (Fig. 7). Since peak current amplitudes were generally decreased at the 30th pulse in the control experiments, additional fractional blocks were obtained by subtracting the normalised peak currents of the 30th pulses in the presence of ralfinamide from that of the control experiments.

The inhibition by ralfinamide of tetrodotoxin-resistant currents evoked by repetitive stimuli was strongly use and frequency dependent. Ralfinamide (50 μM) caused a significant additional fractional block at the 30th pulse of 11% when pulses were delivered at 0.5 Hz (P<0.01, Fig. 7A) and 29% when delivered at 5 Hz (P<0.01, Fig. 7B). In contrast, the use and frequency dependence of ralfinamide inhibition of tetrodotoxin-sensitive currents was weaker. Ralfinamide (50 μM) did not produce an additional inhibition at low frequency (0.5 Hz) stimulated tetrodotoxin-sensitive currents (P>0.05, Fig. 7C). The same concentration resulted in an additional fractional block of 11% of the 30th peak amplitudes of tetrodotoxin-sensitive currents evoked at 5 Hz (0.01<P<0.05, Fig. 7D).

3.7. Ralfinamide delays repriming of tetrodotoxin-resistant and tetrodotoxin-sensitive currents

Double-pulse protocols were used to study the effect of ralfinamide on recovery from inactivation of tetrodotoxin-resistant and tetrodotoxin-sensitive Na^+ currents. Repriming curves for both currents demonstrated two recovery

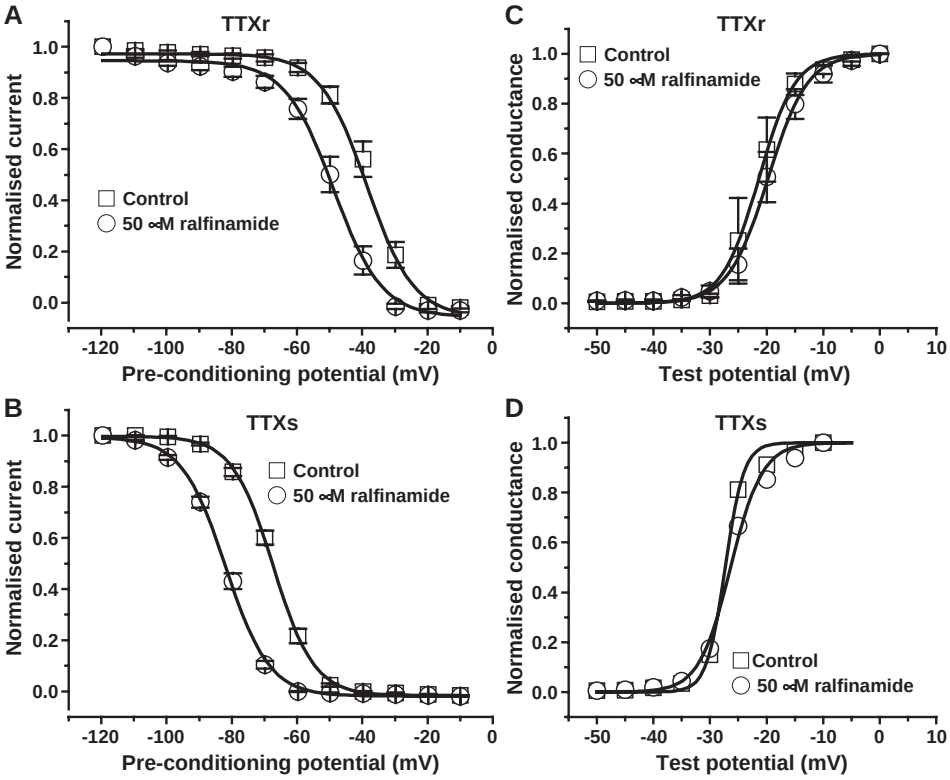

Fig. 6. Ralfinamide shifts the steady-state inactivation properties without affecting the activation curves of tetrodotoxin-resistant (TTXr) and tetrodotoxin-sensitive (TTXs) currents. Steady-state inactivation protocols as well as the steady-state properties of tetrodotoxin-resistant and tetrodotoxin-sensitive currents in control conditions are described in the figure text of Fig. 1. Applying 50 μM ralfinamide shifts the potential leading to half-maximal tetrodotoxin-resistant currents (A) by −11 mV from −38±2 mV (□, n=11) to −49±2 mV (○, n=11). The slope factor of the curve in the presence of ralfinamide (k_h=6.3±0.2) was not statistically different from that in control (k_h=5.8±0.4). Similarly, 50 μM ralfinamide shifts the potentials leading to half-maximal of tetrodotoxin-sensitive (B) currents by −15 mV from −67±1 mV (□, n=10) to −82±1 mV (○, n=8). The slope factor of the control curve (k_h=6.1±0.1) was not affected by the presence of ralfinamide (k_h=6.5±0.1). Steady-state inactivation data are shown as mean±S.E.M. in both panels. Activation properties were studied eliciting the currents from the holding potentials (−120 mV for tetrodotoxin-sensitive and −90 mV for tetrodotoxin-resistant currents) to different test potentials in 5 mV increments. (C) illustrates the tetrodotoxin-resistant activation curves in control ($V_{1/2Na}$=−21±2 mV, k_g=2.2±0.5, n=5) and in the presence of 50 μM ralfinamide ($V_{1/2Na}$=−19±1 mV, k_g=3.2±0.5, n=5). (D) shows the tetrodotoxin-sensitive activation curves in control ($V_{1/2Na}$=−27 mV, k_g=1.6, n=2) and in the presence of 50 μM ralfinamide ($V_{1/2Na}$=−26 mV, k_g=2.7, n=2). In all panels data are shown as mean±S.E.M. when n>2 and mean when n=2.

components: a fast and a slow (Fig. 8). In control conditions, repriming of tetrodotoxin-resistant currents (τ_1=0.15 s, τ_2=1.94 s) was slower than repriming of tetrodotoxin-sensitive currents (τ_1=0.05 s, τ_2=0.83 s). Applying 50 μM ralfinamide significantly slowed the rate of recovery of tetrodotoxin-resistant currents (τ_1=0.76 s, τ_2=8.10 s, P<0.01), increasing τ_1 by a factor 5 and τ_2 by a factor 4. The same concentration also decreased the rate of tetrodotoxin-sensitive repriming (τ_1=0.29 s, τ_2=1.55 s, P<0.01), increasing τ_1 and τ_2 6- and 2-folds, respectively.

4. Discussion

Peripheral nerve damage leading to abnormal spontaneous firing in sensory neurons can induce neuropathic pain. Relocalisation and changed expression of tetrodotoxin-resistant and/or tetrodotoxin-sensitive voltage-gated Na^+ channels in sensory dorsal root ganglion neurons are assumed to play a significant role for the development of this hyperexcitability (Lai et al., 2004). Ralfinamide has demonstrated potent anti-hyperalgesic and anti-allodynic activities when tested in animal models of neuropathic pain (Veneroni et al., 2002, 2003). In the present study, the effects of ralfinamide on tetrodotoxin-resistant versus tetrodotoxin-sensitive currents in rat dorsal root ganglion neurons were studied in order to investigate the mechanisms of inhibition.

On the basis of current kinetics and sensitivity to tetrodotoxin (250 nM) dorsal root ganglion neurons were demonstrated to possess two Na^+ current phenotypes, slow tetrodotoxin-resistant and fast tetrodotoxin-sensitive currents. Activation and inactivation kinetics of tetrodotoxin-resistant current peaks were notably slower than those of tetrodotoxin-sensitive currents traces in Figs. 1A, 2A and 4A versus Figs. 1B, 3A and 5A. Potentials leading to half-maximal inactivation of the two current types were also significantly different (Fig. 1C). These findings are in agreement with previous studies (see e.g. Cummins and Waxman, 1997).

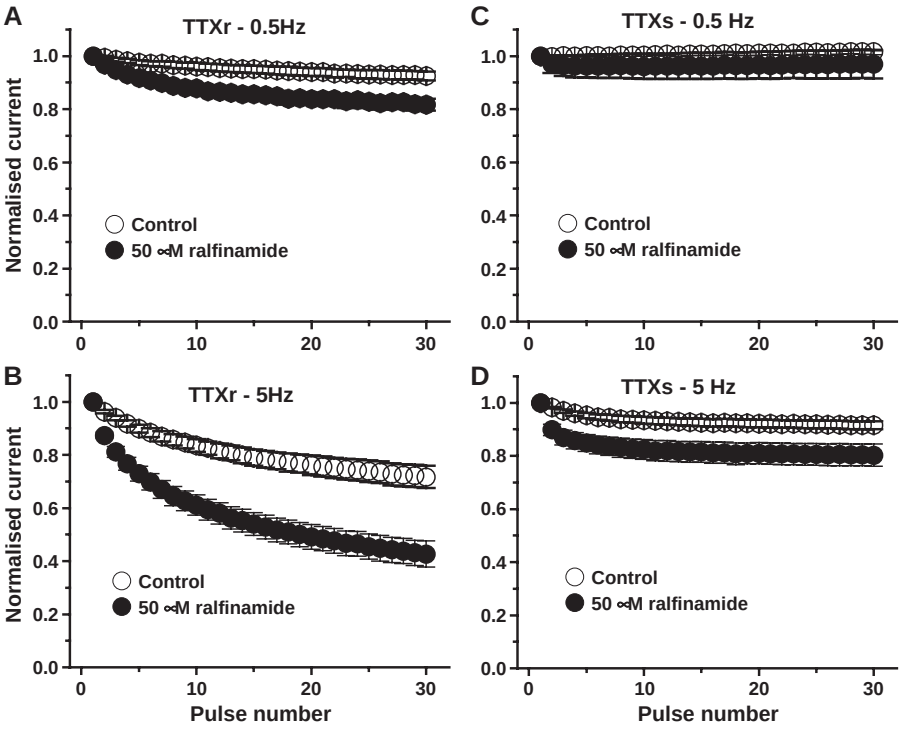

Fig. 7. Use dependent block by 50 μM ralfinamide of tetrodotoxin-resistant (TTXr) and tetrodotoxin-sensitive (TTXs) currents were studied applying 30 consecutive 40 ms pulses to −10 mV at frequencies of 0.5 Hz and 5 Hz. Tetrodotoxin-resistant currents were evoked from holding potentials of −90 and tetrodotoxin-sensitive currents from −120 mV. Current amplitudes were normalised to the amplitude of the first pulse and blotted as function of the pulse number. Data are shown as mean±S.E.M. in all panels. Ralfinamide induced additional fractional blocks of 11±2% (n=8) of tetrodotoxin-resistant currents elicited at 0.5 Hz (A) and 29±2% (n=10) of tetrodotoxin-resistant currents elicited at 5 Hz (B). No statistically significant use dependent block (n=6) was generated by ralfinamide of tetrodotoxin-sensitive currents elicited at 0.5 Hz (C), but an additional fractional block of 11±3% (n=9) was obtained with a frequency of 5 Hz (D).

Molecular biology and patch clamp experiments indicate that at least two tetrodotoxin-resistant Na^+ channel subtypes, $Na_v1.8$ and $Na_v1.9$, are expressed in small dorsal root ganglion neurons (Cummins et al., 1999). The tetrodotoxin-resistant currents in uninjured neurons may occur from both channels, but $Na_v1.8$ channels are the major contributors to peak tetrodotoxin-resistant currents evoked in whole cell patch clamp experiments (Cummins et al., 1999). The persistent low-threshold tetrodotoxin-resistant current described by Cummins et al. (1999) and mediated by $Na_v1.9$ channels were not detected with the voltage clamp protocols used in the present study (Fig. 1A). Thus, the tetrodotoxin-resistant current recorded in small neurons reflects the activation of $Na_v1.8$. Also, other groups did not detect persistent tetrodotoxin-resistant currents in dorsal root ganglion neurons applying their protocols (Akopian et al., 1999; Gold and Thut, 2001).

In the present study total tetrodotoxin-sensitive sodium currents were studied in medium sized neurons (see Section 3.1). Small primary afferent dorsal root ganglion neurons are thought to be more numerous in nociceptors than medium and large neurons, but also a substantial proportion of medium and large neurons are nociceptive (Djouhri et al., 1998). In normal animals dorsal root ganglion neurons of all sizes express the same four tetrodotoxin-sensitive channel subtypes, $Na_v1.1$, $Na_v1.2$, $Na_v1.6$ and $Na_v1.7$ (Black et al., 1996, 2004; Djouhri et al., 2003; Hong et al., 2004). Neither electrophysiological nor pharmacological tools make it possible to separate the different tetrodotoxin-sensitive current subtypes and can therefore not be used to determine the relative proportions of functional subtype expressions contributing to the total tetrodotoxin-sensitive current. This information must be extrapolated from in situ hybridisation and immunocytochemistry studies indicating some differences in expression levels (Black et al., 1996, 2004; Djouhri et al., 2003; Hong et al., 2004), which, however, do not always correlate with the amount of plasma membrane expression (see e.g. Djouhri et al. (2003)). Overall the present literature does not lead to expectation of strong variation between results obtained with differently sized dorsal root ganglion neurons.

In the present study ralfinamide was demonstrated to inhibit tetrodotoxin-sensitive and tetrodotoxin-resistant currents of dorsal root ganglion neurons in a concentration and voltage dependent manner, exhibiting 7-fold higher tonic block when currents were evoked from depolarised potentials inactivating the channels (IC_{50} values are listed in Table 1). Comparing IC_{50} values for ralfinamide inhibition of tetrodotoxin-resistant and tetrodotoxin-sensitive currents evoked from potentials leading to the same biophysical conditions of the two currents reveals 2-fold higher potency (P<0.01) towards maximal and half-maximal tetrodotoxin-

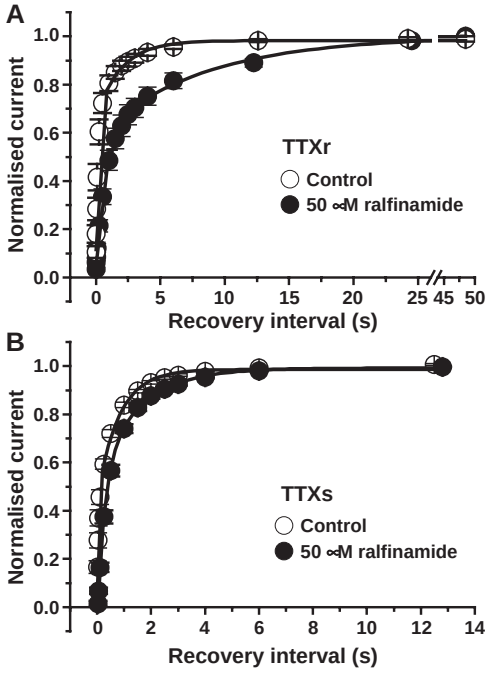

Fig. 8. Effects of 50 μM ralfinamide on repriming of (A) tetrodotoxin-resistant (TTXr) currents and (B) tetrodotoxin-sensitive (TTXs) currents from slow inactivation were studied using two pulse protocols composed by an inactivation step of 2 s at −10 mV, varying repriming intervals at the holding potentials (−120 mV for tetrodotoxin-sensitive and −90 mV for tetrodotoxin-resistant) and a test pulse of 50 ms to −10 mV. Data are shown as mean±S.E.M. in all panels. Fractional recovery (peak current evoked with test pulse divided by peak current evoked during conditioning pulse) was plotted as a function of interpulse duration. The best fits to the data were obtained with functions having two exponential terms (see Materials and methods), as the curves exhibited two recovery components: a fast and a slow. (A) For tetrodotoxin-resistant repriming the function of best fit had τ_1=0.15±0.02 s and τ_2=1.94±0.33 s (A_1=62±4%, A_2=31±3%, y_0=6±3%, n=6) in control conditions and τ_1=0.76±0.12 s and τ_2=8.10±1.38 s (A_1=53±6%, A_2=42±5%, y_0=4±1%, n=5) in the presence of 50 μM ralfinamide. (B) For tetrodotoxin-sensitive repriming the function of best fit had τ_1=0.05±0.01 s and τ_2=0.83±0.04 s (A_1=36±1%, A_2=52±2%, y_0=10±2 n=6) in control conditions and τ_1=0.29±0.04 s and τ_2=1.55±0.09 s (A_1=60±2%, A_2=44±3%, y_0=−5±1%, n=5) in the presence of 50 μM ralfinamide.

resistant currents than towards maximal and half-maximal tetrodotoxin-sensitive currents, respectively.

The anticonvulsant carbamazepine has been used for more than five decades to relieve trigeminal neuralgia, and controlled clinical studies have shown efficacy in treatment of central neuropathic pain (Backonja, 2002; Vu, 2004). Carbamazepine exhibits Na^+ channel blocking properties (Brau et al., 2001; Kuo, 1998) and was therefore chosen as the reference standard of this study. Carbamazepine showed much lower potency towards maximal and half-maximal tetrodotoxin-sensitive and tetrodotoxin-resistant currents than ralfinamide (IC_{50} values are listed in Table 1). Carbamazepine showed increased potency towards both currents evoked from depolarised potentials, differing from ralfinamide by exhibiting preferential activity towards tetrodotoxin-sensitive channels (2 fold difference in IC_{50} values for tetrodotoxin-sensitive versus tetrodotoxin-resistant, $0.01<P<0.05$).

The tetrodotoxin-resistant steady-state inactivation curve is about 30 mV more depolarised than the tetrodotoxin-sensitive curve (Fig. 1C). Consequently, in physiological conditions a higher proportion of tetrodotoxin-sensitive than tetrodotoxin-resistant channels will be inactivated at any given membrane potential at which inactivation occurs. Since resting membrane potentials of dorsal root ganglion neurons are in the range of −50 to −80 mV (Amir et al., 1999; Blair and Bean, 2002; Renganathan et al., 2001) more tetrodotoxin-sensitive than tetrodotoxin-resistant channels are inactivated. Consequently, a larger part of tetrodotoxin-sensitive channels are not available for activation and are accessible for ralfinamide or carbamazepine binding further favouring inhibition. This is in agreement with our results demonstrating higher tonic block by ralfinamide and carbamazepine of tetrodotoxin-sensitive than tetrodotoxin-resistant currents when both currents are evoked from −70 mV (Table 1). To sum up, the tonic block effects of Na^+ channel inhibitors on neurons, having Na^+ currents consisting of a combination tetrodotoxin-sensitive and tetrodotoxin-resistant currents, depends both on the potency of the drug towards each channel subtype as well as the channel availabilities.

In physiological conditions tetrodotoxin-resistant and tetrodotoxin-sensitive channels expressed in the same neuron are subjected to the same membrane potentials in resting conditions as well as during neuronal firing. As mentioned above, ralfinamide and carbamazepine cause a significant higher tonic block of tetrodotoxin-sensitive than of tetrodotoxin-resistant currents when the two currents are evoked from the same pre-conditioning potential (−70 mV, Table 1). Of interest is the fact that ralfinamide cause a strong block of both current types evoked from −70 mV, while carbamazepine has almost no effect on the tetrodotoxin-resistant current and is 4 times less potent for tetrodotoxin-sensitive current inhibition (Table 1). In more depolarised conditions (pre-conditioning potentials of −40 mV in Table 1) carbamazepine exhibits a stronger block of tetrodotoxin-resistant currents, but the effect is still small as compared to that of ralfinamide. These observations become even more important in consideration of the plasma levels clinically obtained. Clinical studies with ralfinamide have shown that relevant doses result in plasma levels of 4 μg/ml corresponding to 10 μM (company communication). Hence, assuming that tissue exposure levels are in the same range as plasma levels, attenuation of both tetrodotoxin-resistant and tetrodotoxin-sensitive currents may be expected. Carbamazepine administrated in typical clinical doses gives plasma concentrations of 4–12 μg/ml, which is equivalent to 16–48 μM (Macdonald, 1995). Thus, tetrodotoxin-resistant currents can only be very weakly modulated, while tetrodotoxin-sensitive attenuation probably occurs. In conclusion, clinical doses of ralfinamide are expected to reduce both tetrodotoxin-resistant and tetrodotoxin-sensitive currents in dorsal root ganglion neurons, while clinical relevant doses

of carbamazepine may mainly influence tetrodotoxin-sensitive currents.

The net effect of Na^+ currents inhibitors on neuronal firing depends not only on the strength of the tonic block. Important is also the ability of the drug to induce further inhibition in high frequency repetitive conditions (use and frequency dependent block) and to stabilize channel inactivation. The last can be achieved by either increasing the probability of inactivation or by slowing repriming. Increased drug potency with increased membrane depolarisation has previously been described for several Na^+ channel inhibitors, notably local anaesthetics such as lidocaine and certain anticonvulsants such as carbamazepine, phenytoin and lamotrigine (Brau and Elliott, 1998; Kuo, 1998). Such state-dependence is often explained by the hypothesis that the drug has a higher affinity for Na^+ channels in the inactivated state than for Na^+ channels at rest, resulting in an increase of the overall affinity of the drug as more channels inactivate at depolarised potentials (Hille, 2001).

Ralfinamide caused a tonic block of Na^+ currents in dorsal root ganglion neurons evoked from potentials not inactivating the channels (Figs. 2 and 3) demonstrating affinity for tetrodotoxin-resistant and tetrodotoxin-sensitive channels in their resting state. The block was further increased in a voltage dependent manner when currents were evoked from depolarised potentials inactivating the channels (Figs. 2 and 3). In order to characterise the mechanism of voltage dependence of ralfinamide, the effect of the drug on tetrodotoxin-resistant and tetrodotoxin-sensitive steady-state inactivation were investigated. Ralfinamide shifted the steady-state inactivation curves of both currents in hyperpolarising directions demonstrating that the voltage dependent mechanism of action is due to relatively stronger inhibition of currents elicited from inactivated states. The hyperpolarising shift (−15 mV) by ralfinamide of the tetrodotoxin-sensitive steady-state inactivation curve was larger than the shift (−11 mV) of the tetrodotoxin-resistant steady-state inactivation curve ($0.01<P<0.05$), indicating higher affinity of ralfinamide towards inactivated tetrodotoxin-sensitive than inactivated tetrodotoxin-resistant channels. In the tonic block experiments the voltage dependence of ralfinamide inhibition was the same for the two channel types (7-fold increase in the potency). This implies that at least for tetrodotoxin-resistant channels, the voltage dependence cannot simply result from inhibition by binding to inactivated channels, but may be due to other mechanisms of inhibition such as binding to pre-open or open channels, slowing down the rate of recovery or making "slow inactivation" faster so that the channels need less time to inactivate (Grant, 2002; Xie et al., 1995). Single channel and mutation studies are needed to convincingly resolve the molecular mechanisms underlying the observed phenomena.

Repetitive stimulations at two different frequencies (0.5 and 5 Hz) of both tetrodotoxin-resistant and tetrodotoxin-sensitive currents were used to assess the use (additional fractional/phasic) and frequency dependent block induced by ralfinamide. Use dependent block of tetrodotoxin-resistant currents was stronger than the block of tetrodotoxin-sensitive currents at both 0.5 and 5 Hz. The most evident effect was obtained for tetrodotoxin-resistant currents elicited at 5 Hz, where an additional fractional phasic block of 29% over the tonic block was measured at the last pulse of the train. Also the frequency dependence of ralfinamide inhibition of tetrodotoxin-resistant currents were stronger than that of tetrodotoxin-sensitive currents, as increasing the frequency from 0.5 to 5 Hz lead to larger increase of the additional fractional block for tetrodotoxin-resistant than tetrodotoxin-sensitive currents.

The use and frequency dependent mechanism of action of ralfinamide inhibition is consistent with the finding that ralfinamide has high affinity for the inactivated state of the channels. Brief repetitive membrane depolarisations make the channels cycle through states of activation, inactivation and recovery. When the stimulation frequency is high enough, the period preceding each test pulse is not long enough to allow full recovery from inactivation, generating progressive accumulation of channel inactivation. The result is that an increasing fraction of channels are trapped in the inactivated state allowing higher degree of ralfinamide binding, an effect that is more pronounced for the slowly activating tetrodotoxin-resistant current.

The ability of ralfinamide to increase the amount of inactivated channels by delaying recovery from inactivation was investigated directly. Na^+ channels exhibit at least two distinct kinetic classes of inactivation, termed slow and fast (Goldin, 2003). In order to generate slow inactivation, long inactivation steps (2 s) were used. Repriming of tetrodotoxin-resistant and tetrodotoxin-sensitive currents occurred in two phases with distinct decay factors (Fig. 8). Ralfinamide significantly delayed the two repriming components of both currents. The most evident effect was on tetrodotoxin-resistant currents where the drug leads to a higher percentage of channels repriming in the slow phase. The ability of ralfinamide to delay the recovery from inactivation reduces the current availability during repetitive firing and therefore may reduce high frequency firing in dorsal root ganglion neurons. The present study for the first times demonstrates that ralfinamide delays repriming from slow inactivation. The effect of ralfinamide on tetrodotoxin-resistant repriming from fast (100 ms inactivation steps) inactivation has been investigated previously showing that also recovery from fast inactivation of tetrodotoxin-resistant channels is delayed by ralfinamide (Faravelli et al., 2003), further strengthening the prospect that ralfinamide reduces high frequency repetitive firing.

The ability of ralfinamide to inhibit tetrodotoxin-resistant and tetrodotoxin-sensitive currents in dorsal root ganglion neurons may explain the anti-neuropathic activity in animal models demonstrated by Veneroni et al. (2002, 2003). The same feature of ralfinamide may also explain why ralfinamide shows much stronger anti-hyperalgesic

and anti-allodynic effects in the animal models than carbamazepine (De Vry et al., 2004; Gonzalez et al., 2000). Models of neuropathic pain show that mechanical injury of peripheral nerves result in changes of localisation and expression of tetrodotoxin-resistant and/or tetrodotoxin-sensitive voltage-gated Na^+ channels in dorsal root ganglion sensory neurons as well as in spontaneous ectopic discharge. Since voltage-gated Na^+ channels are essential for generation of action potentials, their relocalisation and changed density are likely to underlay the hyperexcitability. Nevertheless, the relationship between changes in neuronal Na^+ channel densities and firing properties is not fully understood (Lai et al., 2004; Waxman et al., 2002). Thus, the relative importance of tetrodotoxin-resistant and tetrodotoxin-sensitive channels for development of neuropathic pain is also unclear.

Tetrodotoxin-sensitive channels most likely contribute to action potential firing in dorsal root ganglion neurons of healthy animals (Blair and Bean, 2002; Gold et al., 2003). The density and voltage dependence of tetrodotoxin-sensitive currents are unchanged after nerve axotomy, but their recovery from inactivation becomes faster (Cummins and Waxman, 1997). This, to a certain extent, seems attributable to up-regulation of tetrodotoxin-sensitive $Na_v1.3$ channels, which are absent in normal physiological conditions (Cummins et al., 2001). The acceleration of the repriming properties may reduce the firing threshold or enable the higher frequency of action potential firing after nerve injury. In line with this, tetrodotoxin-sensitive channels are apparently essential for the increased membrane oscillations apparently underlying ectopic firing after nerve injury (Amir et al., 1999) and for action potential generation in injured neurons (Zhang et al., 2004). Furthermore, $Na_v1.3$ antisense "knock-down" experiments demonstrates correlation between increased expression of tetrodotoxin-sensitive $Na_v1.3$ channels and hyperexcitability in dorsal horn neurons as well as neuropathic pain symptoms after peripheral nerve and spinal cord injury (Hains et al., 2003, 2004). Thus one possibility is that ralfinamide leads to pain relief via inhibition of tetrodotoxin-sensitive channels.

In normal physiological conditions tetrodotoxin-resistant currents in dorsal root ganglion neurons play an important role for generation of action potentials and repetitive firing (Blair and Bean, 2002; Gold et al., 2003; Renganathan et al., 2001; Zhang et al., 2004). Antisense decrease of $Na_v1.8$ protein effectively prevents development of hyperalgesia and allodynia, providing evidence for a role of $Na_v1.8$ in the development of neuropathic pain (Lai et al., 2002; Porreca et al., 1999). Several groups find a significant down-regulation of tetrodotoxin-resistant channels in dorsal root ganglion soma after nerve injury implying that the site of action of $Na_v1.8$ is not the cell bodies of injured neurons (Cummins and Waxman, 1997; Decosterd et al., 2002; Gold et al., 2003; Zhang et al., 2004). In the soma of adjacent uninjured dorsal root ganglion nerves, $Na_v1.8$ protein and functional channels do not significantly change according to some groups (Decosterd et al., 2002; Gold et al., 2003) or up-regulate but without affecting the action potential of the soma according to Zhang et al. (2004). However, along the distal axons of the uninjured neurons there is an up-regulation of $Na_v1.8$ immunoreactivity correlating with an increased tetrodotoxin-resistant component in the action potential (Gold et al., 2003). Moreover, tetrodotoxin-resistant currents appear crucial for spontaneous activity after axonal injury (Roza et al., 2003). Altogether, these observations suggest that the ability of ralfinamide to block tetrodotoxin-resistant channels is crucial for relief from neuropathic pain. The hypothesis is in line with the demonstration of stronger anti-hyperalgesic and anti-allodynic effects in animal models of ralfinamide, inhibiting tetrodotoxin-resistant (and tetrodotoxin-sensitive) currents, than of carbamazepine, largely modulating tetrodotoxin-sensitive currents.

The overall conclusion is that the voltage-dependent inhibitory properties of ralfinamide against tetrodotoxin-resistant and tetrodotoxin-sensitive currents in dorsal root ganglion neurons provide a hypothesis to explain the anti-neuropathic properties demonstrated in animal models. The stronger anti-hyperalgesic and anti-allodynic effects in animal models of ralfinamide as compared to carbamazepine may arise from the ability of ralfinamide in clinical relevant doses to strongly attenuate both tetrodotoxin-resistant and tetrodotoxin-sensitive currents in dorsal root ganglion neurons, while clinical doses of carbamazepine may mainly influence tetrodotoxin-sensitive currents. The tetrodotoxin-sensitive and especially the tetrodotoxin-resistant blocking properties of ralfinamide are further increased when the currents are subjected to high frequency repetitive activation, as in hyperexcitability conditions.

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