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Effects of ralfinamide, a Na⁺ channel blocker, on firing properties of nociceptive dorsal root ganglion neurons of adult rats

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Abstract

Recent studies revealed that ralfinamide, a Na⁺ channel blocker, suppressed tetrodotoxin-resistant Na⁺ currents in dorsal root ganglion (DRG) neurons and reduced pain reactions in animal models of inflammatory and neuropathic pain. Here, we investigated the effects of ralfinamide on Na⁺ currents; firing properties and action potential (AP) parameters in capsaicin-responsive and -unresponsive DRG neurons from adult rats in the presence of TTX (0.5 μM). Ralfinamide inhibited TTX-resistant Na⁺ currents in a frequency and voltage dependent manner. Small to medium sized neurons exhibited different firing properties during prolonged depolarizing current pulses (600 ms). One group of neurons fired multiple spikes (tonic), while another group fired four or less APs (phasic). In capsaicin-responsive tonic firing neurons, ralfinamide (25 μM) reduced the number of APs from 10.6 ± 1.8 to 2.6 ± 0.7 APs/600ms, whereas in capsaicin-unresponsive tonic neurons the drug did not significantly change firing (8.4 ± 0.9 in control to 6.6 ± 2.0 APs/600ms). In capsaicin-responsive phasic neurons, substance P and 4-aminopyridine induced multiple spikes, an effect that was reversed by ralfinamide (25 μM). In addition to effects on firing, ralfinamide increased the threshold, decreased the overshoot, and increased the rate of rise of the AP.

To conclude, ralfinamide suppressed afferent hyperexcitability selectively in capsaicin-responsive, presumably nociceptive neurons, but had no measurable effects on firing in CAPS-unresponsive neurons. The action of ralfinamide to selectively inhibit tonic firing in nociceptive neurons very likely contributes to the effectiveness of the drug in reducing inflammatory and neuropathic pain as well as bladder overactivity.

Keywords

Na⁺ currents; firing; dorsal root ganglia; capsaicin; nociceptive neurons; tetrodotoxin

Introduction

Ralfinamide (NW-1029, Stummann et al., 2005), is a Na⁺ channel blocker that suppresses tetrodotoxin (TTX)-resistant Na⁺ currents in small to medium size (C-type) dorsal root ganglia (DRG) neurons about twice as selectively as it blocks TTX-sensitive currents in the same neurons (Stummann et al., 2005). The blocking action of ralfinamide shows frequency and voltage dependence (Stummann et al., 2005). In experimental animal models of inflammatory and neuropathic pain, systemic administration of ralfinamide elicits anti-nociceptive effects (Veneroni et al., 2003). Recent studies in our laboratory also revealed that ralfinamide

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suppressed cyclophosphamide- and acetic acid-induced bladder hyperactivity without affecting normal voiding function (Geisselman et al., 2005; Artim et al., 2005).

Hyperexcitability of primary afferent pathways is a common feature of nociceptive sensitization after nerve injury or tissue inflammation (Hillsley et al., 2006; Moore et al., 2002; Yoshimura and de Groat, 1999). In C-fiber type bladder afferent neurons identified by retrograde dye transport, systemic administration of cyclophosphamide (CYP) which caused cystitis and hyperreflexia of urinary bladder converted phasic firing (1–3 action potentials) to tonic firing (multiple action potentials, Yoshimura and de Groat, 1999). Voltage clamp experiments revealed that the increased afferent neuron excitability was related to a decrease in A-type K^+ channel currents. Several other studies investigating the mechanisms underlying afferent hyperexcitability have provided support for these observations. In capsaicin (CAPS)-responsive DRG neuron, substance P acutely converts phasic firing to tonic firing (Sculptoreanu et al. 2004), an effect which was mimicked by 4-aminopyridine, an A-type K^+ -channel inhibitor. In addition KW-7158, a K^+ channel opener, reversed the effect of substance P or 4-aminopyridine, suggesting that a reduction in K^+ currents was responsible in part for the increased excitability of the afferent neurons.

An alteration in Na^+ channels is also important in the sensitization of visceral nociceptive afferent neurons (Hillsley et al., 2006; Lai et al., 2004). $Na_v1.8$ is a predominant subtype of TTX-resistant Na^+ channels expressed in primary afferent neurons innervating the urinary bladder (Black et al., 2003). Patch-clamp studies on bladder afferent neurons demonstrated that 70% of the neurons were CAPS-responsive and exhibited TTX-resistant Na^+ currents and action potentials (Yoshimura et al., 1996). Intrathecal administration of a $Na_v1.8$ antisense oligodeoxy-nucleotide decreased the amplitude of TTX-resistant currents in bladder afferent neurons and reduced the frequent voiding evoked by irritation of the bladder by intravesical infusion of acetic acid (Yoshimura et al., 2001). Pretreatment with CAPS that desensitizes CAPS-responsive afferents has been shown to prevent acetic acid- and CYP- induced bladder hyperactivity (Maggi et al., 1992). From these findings, it is reasonable to assume that the TTX-resistant Na^+ current in CAPS-responsive neurons might be involved in afferent hyperexcitability induced by bladder irritation and inflammation. A recent study using $Na_v1.8$ (–/–) mice has also shown the importance of $Na_v1.8$ in generating chronic hyperexcitability of afferent neurons innervating the colon inflamed by *Nippostrongylus brasiliensis* (Hillsley et al., 2006).

In this study, we investigated the effects of ralfinamide on: (1) TTX-resistant Na^+ currents evoked after depolarizing prepulses and at different frequencies of stimulation in DRG neurons of adult rats, (2) the phasic and tonic firing patterns of CAPS-responsive and CAPS-unresponsive neurons and (3) the facilitation of firing elicited by substance P and 4-aminopyridine. The results indicate that in CAPS-responsive neurons activity-dependent suppression of TTX-resistant Na^+ currents induced by ralfinamide contributes to inhibition of tonic firing, a marker for sensitization of nociceptive afferent pathways.

Methods

Cell preparation and culturing

Adult male Sprague-Dawley rats (200–250 g) were treated in accordance with guidelines approved by the University of Pittsburgh Institutional Animal Care and Use Committee. Neurons were isolated from DRG using methods previously described (Sculptoreanu et al. 2004). Briefly, freshly removed ganglia were minced and enzymatically digested at 37°C for 10 min in DMEM containing 2.5 mg/ml trypsin, followed by 50 min in DMEM containing 2.5 mg/ml collagenase D (Boehringer-Mannheim) and 4 mg/ml trypsin inhibitor type 2S (Sigma). The DRG were dissociated mechanically by titration using siliconized Pasteur pipette. The cell

suspension was layered on DMEM containing 50 % bovine serum and centrifuged at 800 rpm ($150 \times g$) at room temperature to remove most of the debris and broken cells. After withdrawing the supernatant, the pellet containing neurons was resuspended in DMEM containing 10% heat-inactivated horse serum and 5% fetal bovine serum, and plated on collagen-coated 35 mm petri dishes (Biocoat; Becton Dickinson) and kept in 95% air and 5% CO₂ incubators at 37°C until recording.

Whole cell patch clamp recording

Na⁺ currents, membrane potentials and firing in DRG neurons after 1–3 days in culture were recorded with whole cell patch clamp techniques, using an Axopatch 200A amplifier (Axon Instruments, Foster City, California). All experiments shown here were done in the presence of 0.5 μM tetrodotoxin to exclude the contribution of TTX-sensitive currents in the effects of ralfinamide. Pulse stimulation and data acquisition were controlled with pClamp software (Axon Instruments). Signals were filtered at 2 kHz and digitized. Patch pipettes were pulled from borosilicate glass capillary tubing (Warner Instruments Inc.) and had resistances of 1.0–2.5 MΩ when filled with internal solution. Immediately before recording Na⁺ currents, the culture media was replaced with an external solution with reduced Na⁺ and zero K⁺ concentrations containing (in mM): 50 N-methyl D-glucamine (NMDG), 18 TEA-Cl, 65 NaCl, 2 CaCl₂, 2 MgCl₂, 0.05 CdCl₂, 0.05 LaCl₃, 0.05 NiCl₂, 0.05 4-AP, 0.005 nitrendipine, 0.0005 TTX, 10 HEPES, pH 7.4 adjusted with NaOH. For membrane potential and action potential measurements, Dulbecco's phosphate buffered saline (Invitrogen) was used, which contains (in mM): 138 NaCl, 2.6 KCl, 0.9 CaCl₂, 0.5 MgCl₂, 1.5 KH₂PO₄, 8.1 Na₂HPO₄ to which 0.5 μM TTX was added. Internal solution for Na⁺ current measurements contained (mM): 125 NMDG, 5 NaCl, 2.5 MgCl₂, 10 EGTA, 10 HEPES, 3 Mg-ATP, 0.3 cAMP, 0.5 tris-GTP, pH 7.2 adjusted with NaOH. Internal solution for membrane potential and action potential measurements was (mM): 120 KCl, 10 K₂HPO₄, 10 NaCl, 1 MgCl₂, 1 EGTA, 10 HEPES, 3 Mg-ATP, 0.3 cAMP, 0.5 tris-GTP, pH adjusted to 7.4 with HCl. All recordings were made at room temperature and the bath was grounded using AgCl/Ag agar bridge.

Na⁺ currents and membrane potentials were recorded from small to medium-sized DRG neurons with diameters <40 μm. TTX (0.5 μM) was applied for recording of TTX-resistant Na⁺ currents and action potentials. CAPS responsiveness was determined by adding CAPS (0.5 μM) at the end of the recording of Na⁺ currents or firing. Results are presented as means ± SEM. Paired t-test was used for statistical analysis.

For analysis of single action potential parameters, a series of 5 ms depolarizing current pulse was applied with 50 pA increments from 0 to 700 pA. Measurements of action potential parameters (threshold, duration, rate of rise) were determined for stimuli 50–80 pA in intensity which were chosen just above firing threshold (75 ± 20 pA). To examine firing characteristics, action potentials were elicited by depolarizing current pulses with a duration of 600 ms at intensities, increasing in 50 pA increments from 0 to 700 pA. Prior to recordings, holding potential was adjusted to −58 mV. The steady-state effects of ralfinamide were confirmed by repeated recordings every 10 s for 3–5 min.

Ralfinamide (NW-1029, Stummann et al., 2005) was kindly provided by Newron Pharmaceuticals SpA (Italy). Collagenase B was purchased from Boehringer-Mannheim, TTX from Alomone Labs, and CAPS from Calbiochem. All other chemicals were purchased from Sigma.

Results

Effects of Ralfinamide on TTX-resistant Na⁺ currents

The effects of ralfinamide on TTX-resistant Na⁺ currents were studied in small to medium-sized neurons with diameters <40 μm. To evaluate the activity-dependent blocking action of the drug, experiments were conducted in presence of 0.5 μM TTX using depolarizing prepulses to condition the Na⁺ channels or different frequencies of depolarizing stimulation to activate the channels. In prepulse experiments, Na⁺ currents were elicited by 20 ms duration step depolarizations to −10 mV following 2 s prepulses to different potentials, −90 mV, −70 mV and −40 mV. Ralfinamide inhibited the peak amplitude of TTX-resistant Na⁺ currents by 7%, 21% and 58% following −90 mV, −70 mV and −40 mV prepulses, respectively (Fig. 1A). To examine frequency-dependent inhibition, TTX-resistant currents were evoked by 40 consecutive pulses (5 ms in duration) to −10 mV from a −90 mV holding potential at frequencies of 5 Hz and 14 Hz in presence of TTX (0.5 μM). During 14 Hz stimulation, ralfinamide inhibited the peak current of the 40th pulse by 17%, while at 5 Hz, it only inhibited the currents by 2% (Fig. 1 B).

Effects of ralfinamide on tonic and phasic firing

Current clamp measurements of evoked action potentials were performed in rat DRG neurons with a diameter less than 40 μm. Prior to recordings, TTX (0.5 μM) was added to isolate TTX-resistant action potentials. CAPS responsiveness was determined by adding CAPS (0.5 μM) at the end of the series of recording protocols. In the presence of TTX, prolonged depolarizing current pulses (600 ms duration) elicited two types of firing. Firing was defined as tonic if the neuron fired more than 4 action potentials (range 5–26, mean 9.6 ± 1.4 , $n=20$) and phasic if it produced 4 or less action potentials (range 1–4, mean 1.6 ± 0.3 , $n=11$) even at maximal stimulus intensities. When ralfinamide (6.25–50 μM) was applied to CAPS-responsive tonic neurons, the number of action potentials was suppressed in concentration-dependent and reversible manner (Fig. 2). Typically the effect of ralfinamide which was maximal at 50 μM, consisted of a suppression of all action potentials except the first one in the train (Fig. 3A), although in some cells 2–3 spikes remained after the highest concentration of the drug (Fig. 2). In control experiments, the half maximal firing rate was 5.3 ± 2.0 spikes/600 ms at 220 pA stimulus intensity in CAPS-responsive tonic neurons, and 4.2 ± 1.3 spikes/600 ms at 230 pA stimulus intensity in CAPS-unresponsive tonic neurons (Fig. 3B, D). The effect of ralfinamide on maximal evoked firing was more prominent in CAPS-responsive than in CAPS-unresponsive tonic neurons. Firing in CAPS-responsive neurons elicited at 500 pA current intensity was reduced from an average of 10.6 ± 1.8 spikes/600 ms in control to 2.6 ± 0.7 ($p<0.05$) and 1.2 ± 0.9 spikes/600 ms ($p<0.01$) after 25 and 50 μM ralfinamide, respectively (Fig. 3B). On the other hand, in CAPS-unresponsive neurons 25 and 50 μM ralfinamide reduced firing from 7.8 ± 1.1 spikes/600 ms in control to 5.7 ± 1.7 (NS) and 4.1 ± 2.1 spikes/600 ms ($p<0.05$) after 25 and 50 μM ralfinamide, respectively (Fig. 3D). In CAPS-responsive and -unresponsive phasic neurons which exhibited one action potential in response to prolonged depolarizing current pulses, ralfinamide did not block the first spike (Fig. 4).

Effects of ralfinamide on substance P and 4-aminopyridine enhancement of firing in phasic neurons

Substance P and 4-aminopyridine which were shown previously to facilitate firing in CAPS-responsive DRG neurons (Sculptoreanu et al., 2004; Sculptoreanu and de Groat, 2007; Yoshimura et al., 1996) were used in the present experiments to activate multiple spikes in phasic neurons. Substance P (0.5 μM) increased the number of action potentials in CAPS-responsive neurons from 1.0 ± 0.1 spikes/600 ms at 500 pA of current intensity in control to 3.7 ± 0.3 and 6.2 ± 0.3 spikes/600 ms at 500 pA and 700 pA of current intensity, respectively (Figs. 4A and B), but did not alter the firing in CAPS-unresponsive neurons (Figs. 4C and D).

Ralfinamide (25 and 50 μM) significantly inhibited substance P-enhanced firing in phasic, CAPS-responsive neurons (Figs. 4A and B) but did not affect the single action potentials in CAPS-unresponsive neurons following treatment with substance P (Figs. 4C and D). 4-Aminopyridine (50 μM), an A-type K^+ channel blocker, mimicked the effects of substance P, increasing the number of action potentials in phasic, CAPS-responsive neurons. Ralfinamide (25 μM) significantly suppressed 4-aminopyridine enhancement of firing (Fig. 5).

Effects of ralfinamide on single action potentials

In presence of TTX (0.5 μM), single action potentials were elicited by 5 ms duration depolarizing current pulses incremented in 50 pA steps every 10 sec. Various parameters of the action potential generation were measured on responses elicited by current pulses just above the threshold (Fig. 6, Table 1). Action potential threshold was significantly increased by ralfinamide (25 and 50 μM) in CAPS-responsive tonic neurons ($p < 0.01$, compared to control). In CAPS-responsive phasic neurons, substance P decreased the threshold by 14% ($p < 0.001$). This effect was reversed by the subsequent administration of ralfinamide (25 and 50 μM , $p < 0.01$).

Ralfinamide decreased the rate of rise of action potentials (dV/dt_{max}) significantly ($p < 0.05$) at concentrations of 25 μM and 50 μM in CAPS-responsive phasic neurons treated with substance P and in untreated CAPS-responsive tonic neurons. Ralfinamide (50 μM) significantly decreased the amplitude of action potential overshoot in all groups of neurons. The effects of ralfinamide on TTX-resistant single action potential parameters were considerably larger in CAPS-responsive (tonic and phasic) than CAPS-unresponsive neurons (Table 1). Rate of spike repolarization and amplitude of spike after-hyperpolarization were not changed by addition of ralfinamide in concentrations up to 50 μM in all groups of neurons.

Discussion

The present study revealed that ralfinamide, a drug that produces both frequency- and voltage-dependent blockade of TTX-resistant Na^+ channels, had more prominent suppressant effects on CAPS-responsive DRG neurons than on CAPS-unresponsive neurons. Ralfinamide increased the threshold, decreased the overshoot, decreased the rate of rise of APs and reduced tonic firing induced by long duration depolarizing current pulses. Ralfinamide also reversed the facilitation of firing induced by agents (substance P, 4-aminopyridine) that block A-type K^+ currents.

The major Na^+ current contributing to firing in CAPS-responsive nociceptive neurons is mediated by the TTX-resistant $\text{Na}_v1.8$ channel (Renganathan et al., 2001) although a TTX-resistant slowly inactivating $\text{Na}_v1.9$ channel may also be involved, particularly in pathological conditions such as inflammation (Priest et al., 2005). Both channels are presently of interest as targets for the development of drugs to treat inflammatory and neuropathic pain (Lai et al., 2003; Amir et al., 2006; Attal and Bouhassira, 2006). Ralfinamide (Stummann et al., 2005) is thought to interact with a site that is conserved among several members of the Na_v family of ion channels (Chevrier et al., 2004; Ragsdale et al., 1996; Zymanyi et al., 1989). Many classes of drugs, including local anesthetics, antiarrhythmics and anticonvulsants may act at this site to block Na^+ channels (Ragsdale et al., 1996; Lai et al., 2003; Amir et al., 2006). The blockade of Na^+ channels by local anesthetics which is both frequency and voltage-dependent, was initially explained using the modulated receptor hypothesis in which the drug has different affinities for the open and inactivated states of the Na^+ channel (Braun et al., 2001; Hille, 1977; Hondeghem and Katzung, 1977). In more recent studies, it has become apparent that binding of Na^+ channel blockers to slowly inactivated states of Na^+ channels (Lenkey et al., 2006) or slow binding to rapid inactivated states (Kuo and Bean, 1994) may be responsible for the frequency and voltage-dependence of channel blockade. In this study we confirmed that

ralfinamide produces a frequency- and voltage- dependent suppression of TTX-resistant Na^+ currents as in the previous report by Stummann et al. (2005). This action is likely to be an important factor in the suppression of tonic firing in CAPS-responsive afferent neurons as well as in the anti-nociceptive effect of ralfinamide.

A major question arising from the present data is how does ralfinamide selectively inhibit firing in CAPS-responsive as compared to CAPS-unresponsive neurons even though the Aps in both groups of neurons were mediated by the TTX-resistant $\text{Na}_v1.8$ channel, since all of our experiments were conducted in the presence of TTX. This finding suggests that $\text{Na}_v1.8$ channels have different properties in different populations of small diameter DRG neurons. This variation in the sensitivity to a blocking agent might be related in part to the recent finding of Choi et al. (2007) suggesting that $\text{Na}_v1.8$ channels entered inactivation faster and recovered from slow inactivation more slowly in IB_4^+ DRG neurons than in IB_4^- neurons. These differences resulted in more rapid adaptation of firing to prolonged depolarizing pulses. Thus the ability of ralfinamide to selectively block repetitive firing in one population of neurons could be explained by a more rapid onset of inactivation and slower recovery from inactivation of $\text{Na}_v1.8$ channels in those CAPS-responsive neurons.

Another question relates to the greater sensitivity to the drug of tonic firing versus phasic firing. The first spike in a train of APs induced by a long depolarizing current pulse was very resistant to the depressant effect of ralfinamide; whereas later occurring APs were suppressed whether they occurred naturally or were unmasked by treatment with substance P or 4-aminopyridine. The frequency- and depolarization-dependent block of TTX-resistant Na^+ currents by the drug most likely reflects a delaying effect on the recovery of Na^+ channels from inactivation following an AP or increased stability of the channels to inactivating states at depolarized membrane potentials. Thus it is tempting to speculate that in the presence of ralfinamide the sustained depolarization necessary to induce tonic firing leads to inactivation of Na^+ channels and failure of firing after the first AP. The greater effect of ralfinamide on tonic CAPS-responsive versus CAPS-unresponsive neurons could be related to the observation of Choi et al. (2007) mentioned previously, that the recovery from inactivation of TTX-resistant $\text{Na}_v1.8$ channels is different in different subpopulations of DRG neurons. The prominent differences between these two populations of neurons was also evident in their responses to substance P which only converted phasic to tonic firing in CAPS-responsive neurons (Sculptoreanu and de Groat, 2007).

The suppression of the Na^+ currents by ralfinamide (25 μM) was relatively small (7% reduction) after a prepulse to -90 mV in comparison to the 58% suppression elicited after a depolarizing prepulse to -40 mV. Thus partially inactivated Na^+ channels induced by persistent depolarization seem to be more sensitive to the effects of ralfinamide. This is consistent with the greater inhibitory effect of ralfinamide on trains of APs elicited by prolonged depolarizing pulses. Local anesthetics, such as lidocaine and bupivacaine have also been reported to suppress TTX-resistant tonic firing in rat DRG neurons (Scholz et al., 1998; Scholz and Voge, 2000). The suppression of tonic firing by lidocaine, bupivacaine and ralfinamide is very similar in that the first spike is drug-resistant and the later spikes are very sensitive to blockade (Scholz et al., 1998).

The conversion of phasic to tonic firing in DRG neurons in vitro seems to be correlated with the hypersensitivity of afferent neurons in vivo. For example, chronic pathological conditions inducing hyperactivity of the urinary bladder and gastrointestinal tract, induced tonic firing in phasic afferent neurons innervating those organs (Hillsley et al., 2006; Moore et al., 2002; Yoshimura and de Groat, 1999). The emergence of tonic firing was associated with a decrease in A-type K^+ currents (Yoshimura and de Groat, 1999; Yoshimura et al., 2006). Continuous intrathecal administration of nerve growth factor (NGF), which is upregulated in the bladder

of animals with spinal cord injury and chronic cystitis (Vizzard, 2000), induces bladder hyperactivity, increases the number of spikes in response to long duration current injections and decrease A-type K^+ currents in phasic bladder DRG neurons (Yoshimura and de Groat, 1999; Yoshimura et al., 2006). In models of neuropathic pain such as sciatic nerve ligation or axotomy, the expression of mRNA for $K_v1.2$, 1.4 and 4.2 genes was reduced in L4-6 DRG neurons (Park et al., 2003; Yang et al., 2004). Thus, suppression of K^+ currents seems to be an important factor involved in the generating afferent hyperexcitability in models of sub-chronic or chronic nociceptive sensitization.

A similar shift in firing pattern can be induced acutely by suppressing K^+ channels with substance P or 4-aminopyridine as shown in the present and prior studies (Sculptoreanu et al., 2004; Sculptoreanu and de Groat, 2007). Substance P, which is released at afferent terminals of a subgroup of DRG neurons enhances afferent firing in the urinary bladder (Morrison et al., 1999) and facilitates firing of CAPS-responsive DRG neurons by activating NK_2 receptors, an effect which is presumably due in part to an inhibition of K^+ channels (Sculptoreanu et al., 2004; Sculptoreanu and de Groat, 2007). 4-Aminopyridine, an A-type K^+ channel inhibitor (Sculptoreanu et al., 2004; Yoshimura and de Groat, 1999), and heteropodatoxin, a selective blocker of K_v4 K^+ channels (Zarayskiy et al., 2005), mimics the effect of substance P to increase firing in rat DRG neurons (Sculptoreanu and de Groat, 2007). The effects of substance P on firing were reversed by a novel A-type K^+ channel opener, KW-7158 (Sculptoreanu et al. 2004). KW-7158 (Sculptoreanu et al., 2004) also suppressed bladder hyperactivity induced by chemical cystitis raising the possibility that K^+ channel openers may be useful analgesic agents. The present results indicate that a similar inhibition of tonic activity in the afferent neurons sensitized by substance P or 4-aminopyridine can be elicited by suppressing TTX-resistant Na^+ channels with ralfinamide.

In summary, ralfinamide which has been demonstrated to have analgesic effects in animal models of somatic pain (formalin injection, inflammation induced by complete Freund's adjuvant and sciatic nerve ligation, Veneroni et al., 2003) as well as visceral pain (CYP- and acetic acid-induced bladder hyperactivity, Geisselman et al., 2005; Artim et al., 2005) has prominent suppressant effects on the tonic firing and hyperexcitability in CAPS-responsive neurons induced by substance P and 4-aminopyridine. It seems likely that these effects contribute to the analgesic actions of ralfinamide.

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Fig. 1.

Voltage- and frequency-dependent inhibition of TTX-resistant Na^+ currents by ralfinamide (NW). (A) TTX-resistant Na^+ currents were elicited by step depolarization to -10 mV with duration of 20 ms from 2 s prepulses of -90 mV, -70 mV and -40 mV, respectively, in presence of TTX (0.5 μ M). Ralfinamide inhibited peak Na^+ currents by 7%, 21% and 58% in -90 mV, -70 mV and -40 mV, respectively. (B) TTX-resistant Na^+ currents (in presence of 0.5 μ M TTX) were evoked by 40 consecutive 5 ms pulses to -10 mV from a holding potential of -90 mV were applied at frequencies of 5 Hz and 14 Hz. Current amplitudes were normalized to the amplitude of the first response in the sequence. At the 40th pulse ralfinamide inhibited 2% and 17% of peak currents at frequencies of 5 Hz and 14 Hz, respectively, compared with first response in the sequence. TTX (0.5 μ M) was present throughout the experiments. Data are means $\pm$ S.E.M. from 3 neurons.

Fig. 2.

The action of ralfinamide (NW) on firing was dose-dependent and occurred in a reversible manner. Firing of TTX-resistant APs was evoked in a CAPS-responsive tonic neuron by prolonged current injection 600 ms duration with 500 pA of intensity in presence of TTX (0.5 μ M). Resting membrane potential was adjusted to -58 mV prior to recording action potentials. The steady-state effects of ralfinamide were confirmed by repeated recordings every 10 s for 3–5 min. TTX (0.5 μ M) was present throughout the experiments.

Fig. 3.

Ralfinamide (NW) suppressed tonic firing in CAPS-responsive neurons. Upper panels are typical traces from a CAPS-responsive (A) and a CAPS-unresponsive (C) tonic neuron in response to a current pulse 600 ms duration with 500 pA of intensity before and after ralfinamide (25 and 50 μ M) treatment. Lower graphs show the average numbers of overshoot of CAPS-responsive (B) and CAPS-unresponsive (D) tonic neurons for experiment as shown in A and C from 12 and 8 neurons, respectively. Current pulses 600 ms duration were injected in 50 pA increments every 10 sec. Ralfinamide dramatically suppressed repetitive firing in a CAPS-responsive neuron, but had little effects on firing in a CAPS-unresponsive neuron. TTX (0.5 μ M) was present throughout the experiments. Data is means $\pm$ S.E.M.

Fig. 4.

Ralfinamide (NW) suppressed substance P-facilitated firing in CAPS-responsive phasic neurons. Upper panels are typical responses in a CAPS-responsive neuron (A) and a CAPS-unresponsive (C) phasic neuron in response to a current pulse 600 ms duration with 500 pA of intensity. Substance P (0.5 μ M) increased firing numbers in CAPS-responsive neurons, which was inhibited by ralfinamide (25 and 50 μ M). Substance P and ralfinamide had no effects on CAPS-unresponsive phasic neurons. Lower graphs show the average numbers of overshoot of CAPS-responsive (B) and CAPS-unresponsive (D) phasic neurons for experiment as shown in A and B in 6 and 5 neurons, respectively. Current pulses 600 ms duration were injected in 50 pA increments every 10 sec. TTX (0.5 μ M) was present throughout the experiments. Data is means $\pm$ S.E.M.

Fig. 5.

4-Aminopyridine mimicked the effects of substance P in CAPS-responsive phasic neurons. Representative traces evoked by current pulse 600 ms duration with 600 pA of intensity are shown. 4-aminopyridine (50 μ M) increased the number of firing to 6 spikes. Ralfinamide (NW, 25 μ M) reversed the effect of 4-AP. TTX (0.5 μ M) was present throughout the experiments.

Fig. 6. Effects of ralfinamide (NW) on action potentials in CAPS-responsive tonic (A), phasic (B), CAPS-unresponsive tonic (C) and phasic (D) neurons. Single action potentials were elicited by step current pulse injection 5 ms duration with 50 pA increment every 10 sec. Each parameter of the action potentials is summarized in Table 1. For clarity, traces evoked by current pulses with 0, 200, 400, 600 pA of intensities are shown. TTX (0.5 μ M) was present throughout the experiments.

Table 1

Effects of ralfinamide (NW) on parameters of TTX-resistant action potentials.

	Threshold (mV)	Threshold Δ %	dV/dt _{max} (V/s)	dV/dt _{max} Δ %	Amplitude (mV)	Amplitude Δ %
Tonic CAPS-responsive cells (N=13)						
Control	-25.6±1.7		34.8±1.6		27.9±2.3	
NW 25 μ M	-19.5±1.7**	-24	24.4±4.2**	-30	21.0±3.9	-25
NW 50 μ M	-16.4±1.3	-36	19.4±3.1	-44	17.6±3.2	-37
Phasic CAPS-responsive cells (N=8)						
Control	-25.8±1.7		30.5±2.6		26.1±3.2	
SP 0.5 μ M	-29.3±2.3	+14	29.7±3.4	-3	25.2±3.6	-3
SP + NW 25 μ M	-23.9±1.9	-18 [†]	20.6±6.4	-31 [†]	18.6±6.0	-26 [†]
SP + NW 50 μ M	-18.0±1.8	-39 [†]	15.1±6.0	-49 [†]	14.5±6.4	-42 [†]
Tonic CAPS-unresponsive cells (N=6)						
Control	-24.0±2.0		34.8±2.8		31.6±2.1	
NW 25 μ M	-23.9±2.0	0	32.2±3.6	-7	28.0±3.2	-9
NW 50 μ M	-20.4±3.1	-15	26.2±3.9*	-25	24.7±3.3	-22
Phasic CAPS-unresponsive cells (N=5)						
Control	-28.1±1.6		34.1±3.0		27.0±3.3	
NW 25 μ M	-23.7±2.9	-16	29.3±9.4	-14	22.5±7.8	-17
NW 50 μ M	-21.4±2.1	-24	21.9±7.7	-36	15.7±7.7	-42

NW, ralfinamide; SP, substance P; CAPS, capsaicin, dV/dt_{max}, maximum rate of rise; %, percent changes. Action potential parameters were determined for single spike responses, just above threshold, to current injection 5 ms in duration of 50 pA increment every 10. TTX (0.5 μ M) was present throughout the experiments. Data are means $\pm$ S.E.M.

* p<0.05,

** p<0.01 significantly different compared with control,

† p<0.05,

†† p<0.01 compared with substance P. Percent changes were calculated by dividing each value by control value or substance P value[†].