A mixed Ca\(^{2+}\) channel blocker, A-1264087, utilizes peripheral and spinal mechanisms to inhibit spinal nociceptive transmission in a rat model of neuropathic pain

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Xu J, Chu KL, Zhu CZ, Niforatos W, Swensen A, Searle X, Lee L, Jarvis MF, McGaraughty S. A mixed Ca\(^{2+}\) channel blocker, A-1264087, utilizes peripheral and spinal mechanisms to inhibit spinal nociceptive transmission in a rat model of neuropathic pain. J Neurophysiol 111: 394–404, 2014. First published October 23, 2013; doi:10.1152/jn.00463.2013.—N-, T- and P/Q-type voltage-gated Ca\(^{2+}\) channels are critical for regulating neurotransmitter release and cellular excitability and have been implicated in mediating pathological nociception. A-1264087 is a novel state-dependent blocker of N-, T- and P/Q-type channels. In the present studies, A-1264087 blocked (IC\(_{50}\) = 1.6 \(\mu\)M) rat dorsal root ganglia N-type Ca\(^{2+}\) in a state-dependent fashion. A-1264087 (1, 3 and 10 mg/kg po) dose-dependently reduced mechanical allodynia in rats with a spinal nerve ligation (SNL) injury. A-1264087 (4 mg/kg iv) inhibited both spontaneous and mechanically evoked activity of spinal wide dynamic range (WDR) neurons in SNL rats but had no effect in uninjured rats. The inhibitory effect on WDR neurons remained in spinally transected SNL rats. Injection of A-1264087 (10 nmol/0.5 \(\mu\)l) into the spinal cord reduced both spontaneous and evoked WDR activity in SNL rats. Application of A-1264087 (300 nmol/20 \(\mu\)l) into the receptive field on the hindpaw attenuated evoked but not spontaneous firing of WDR neurons. Using electrical stimulation, A-1264087 (4 mg/kg iv) inhibited A\(\delta\)- and C-fiber evoked responses and after-discharge of WDR neurons in SNL rats. These effects by A-1264087 were not present in uninjured rats. A-1264087 moderately attenuated WDR neuron windup in both uninjured and SNL rats. In summary, these results indicate that A-1264087 selectively inhibited spinal nociceptive transmission in sensitized states through both peripheral and central mechanisms.

N-type Ca\(^{2+}\) channels; T-type Ca\(^{2+}\) channels; P/Q-type Ca\(^{2+}\) channels; neuropathic pain; spinal cord

VOLTAGE-GATED Ca\(^{2+}\) CHANNELS mediate Ca\(^{2+}\) influx in response to changes in membrane potential and trigger subsequent cellular signaling (Catterall 2011). Based on electrophysiological and pharmacological properties, these channels are classified into low-voltage activated T-type and high-voltage activated L-, P/Q-, N- and R-type Ca\(^{2+}\) channels (Catterall 2000, 2010). Among these subtypes, there is extensive evidence supporting N- and T-type Ca\(^{2+}\) channels as attractive molecular targets for the treatment of chronic pain (Santicioli et al. 1992; Zamponi et al. 2009). A role for P/Q-type Ca\(^{2+}\) channels in nociceptive signal transmission has also emerged, and there is some genetic evidence supporting a role for R-type channels as well (Saegusa et al. 2001; Yaksh 2006).

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N-type Ca\(^{2+}\) channels are expressed throughout the central nervous system, but are highly concentrated in dorsal root ganglia (DRG) and the synaptic terminals in laminae I and II of the spinal cord (Dubel et al. 1992; Gohil et al. 1994; Kerr et al. 1988). N-type channels mediate the synaptic transmission of somatosensory signals by converting neuronal action potentials into the release of neurotransmitters such as glutamate, CGRP, and substance P (Evans et al. 1996; Maggi et al. 1990; Santicioli et al. 1992). Following nerve injury, the expression of N-type Ca\(^{2+}\) channels is upregulated in the superficial layers of spinal dorsal horn (Cizkova et al. 2002). Deletion of N-type gene in mice inhibits the development of allodynia in models of pathological pain (Saegusa et al. 2001). Spinal administration of a selective toxin-derived N-type peptide antagonist, ziconotide, reduces pain in patients and allodynia/hyperalgesia in preclinical rodent studies (McGivern 2007; Miljanich 2004; Scott et al. 2002; Wang et al. 2000).

T-type Ca\(^{2+}\) channels have a unique role in regulating neuronal excitability as they can be activated by a small depolarization of the cell membrane (Todorovic and Jevtovic-Todorovic 2011). These channels contribute to action potential initiation, burst firing generation, and intrinsic neuronal membrane oscillations (Cain and Snutch 2010; Todorovic and Jevtovic-Todorovic 2011). Ca\(_{3.2}\), a specific T-type channel subtype, is highly expressed on DRG cell bodies (Scroggs and Fox 1992; Shin et al. 2003). T-type channels are also present in the central nervous system, including second-order projection neurons in the spinal cord and thalamus (Huguenard and Prince 1992; Ikeda et al. 2003). Nerve injuries can enhance both current densities and expression levels of T-type Ca\(^{2+}\) channels in DRG (Jagodic et al. 2007, 2008; Latham et al. 2009; Takahashi et al. 2010). Knockdown of T-type channels in the DRG with antisense oligonucleotides or pharmacological blockade of the channels with a selective antagonist, 3,5-dichloro-N-[(2,2-dimethyl-tetrahydropyran-4-ylmethyl)-4-fluoro-piperidin-4-ylmethyl]-benzamide (TTA-P2), attenuates allodynia in animal models of neuropathic pain (Bourinet et al. 2005; Choe et al. 2011; Messinger et al. 2009).

P/Q-type Ca\(^{2+}\) channels are present in DRG neurons, laminae II-VI of the spinal cord and various brain sites (D’Ascenzo et al. 2004; Kim et al. 2001; Westenbroek et al. 1998). This type of channel may be involved in the release of different transmitters, including glutamate (Araque et al. 1994; Tomiawa et al. 2002). Electrophysiological studies using a selective peptide antagonist, \(\omega\)-agatoxin, revealed a role of P/Q-type Ca\(^{2+}\) channels in polysynaptic nociceptive transmission (Hei-
nke et al. 2004). Deleting the P/Q-type gene in mice resulted in reduced allodynia in neuropathic and inflammatory pain models (Luvisetto et al. 2006).

Injury to a peripheral nerve can induce neuropathic pain. Aberrant afferent activity as well as the consequential spinal hyperexcitability underlie both the development and maintenance of this pathological state (Costigan et al. 2009). Calcium channels in these more active neurons would presumably spend more time in the open/inactivated states. Thus it has been postulated that a state-dependent Ca\(^{2+}\) channel blocker that preferentially binds to and stabilizes the inactivated state of these channels may beneficially inhibit hyperactive neurons while sparing Ca\(^{2+}\) channels in less active neurons underlying normal function (Snutch 2005; Winquist et al. 2005). In the present study, we demonstrated that a novel state-dependent mixed N-, T- and P/Q-type Ca\(^{2+}\) channel blocker, A-1264087, was anti-allodynic in neuropathic rats, and that this compound differentially affected inputs to spinal nociceptive neurons between naive and nerve-injured rats.

MATERIALS AND METHODS

All animal handling and experimental protocols were reviewed and approved by AbbVie's Institutional Animal Care and Use Committee. Animals were treated in accordance with the ethical principles for pain-related animal research of the American Pain Society. Adult male Sprague-Dawley rats (250–420 g, Charles River, Wilmington, MA) were used in the present study. Animals were housed in a temperature-controlled room on a 12:12-h light-dark cycle, and food and water were available ad libitum.

To induce neuropathic injury, a unilateral tight ligation of the L\(_5\) and L\(_6\) spinal nerves (SNL) was performed 2 wk before experiments on rats under anesthesia (Kim and Chung 1992).

A-1264087 (N\(^{2}\)-methyl-N-(3aR,4S,6aS)-2-[4 (trifluoromethyl)phenyl]-octahydrocyclopenta[\(\Phi\)]pyrrol-4-yl)-L-leucinamide, weight 397.48) was synthesized at AbbVie (North Chicago, IL) (Beebe et al. 2013). In recombinant cell lines, A-1264087 has an IC\(_{50}\) of 1.0 \(\mu\)M (inactivated state) and 4.4 \(\mu\)M (closed state) for N-type, 1.1 \(\mu\)M (inactivated state) and 5.3 \(\mu\)M (closed state) for T-type, and 1.9 \(\mu\)M (inactivated state) for P/Q-type Ca\(^{2+}\) channels. A-1264087 is inactive at L-type calcium channels (Zhu et al., unpublished observations). A-1264087 has a half-life of 15.4 h and a plasma clearance rate of 1.4 \(\text{hl}^{-1}\text{kg}^{-1}\) following intravenous administration at 1.2 mg/kg. The pharmacological selectivity of A-1264087 (10 \(\mu\)M) has been evaluated by radioligand binding for more than 90 G protein-coupled receptors, ion channels, transporters and enzymes and revealed little or no activity in all assays, except for the sigma and 5-HT\(_{2A}\) receptors (68 and 71% inhibition, respectively). A-1264087 had modest activity at Na\(_\text{v}1.7\) channels with an IC\(_{50}\) of 5.2 \(\mu\)M. We also assessed the activity of A-1264087 in the rat aorta to detect functional block of L-type calcium channels. A-1264087 was inactive in this assay (EC\(_{50}\) > 30 \(\mu\)M) (Zhu et al., unpublished observations). In addition, administration of A-1264087 (10, 30 and 100 mg/kg po) did not affect locomotor activity or motor coordination in naive rats (Zhu et al., unpublished observations).

In Vitro Patch-Clamp Electrophysiology

DRG neurons were isolated from naive adult Sprague-Dawley rats that were CO\(_2\)-euthanized, cervically dislocated and decapitated, and their vertebral columns removed. DRG neurons were dissected from the lumbar level of the vertebral column and placed in DMEM containing 0.3% collagenase B/collagenase-dispase for approximately 90 min at 37°C and 5% CO\(_2\). The collagenase solution was removed and washed in fresh DMEM. Ganglia were dissociated by trituration using increasingly smaller bore-sized sterile fire-polished Pasteur pipettes to obtain a single-cell suspension. DRG neurons were then plated on poly-\(\alpha\)-lysine-coated 12-mm glass coverslips at a density of approximately one-half DRG per coverslip in 1 ml DMEM supplemented with 10% FBS, NGF (50 ng/ml) and 100 IU/ml penicillin-streptomycin (Invitrogen, Carlsbad, CA). Neurons were used for electrophysiological recording within 2–24 h after plating.

For whole-cell voltage-clamp experiments, neurons were continuously superfused by a room temperature extracellular recording solution consisting of the following (mM): 5 BaCl\(_2\), 87.5 CsCl, 40 TEA-Cl, 1 MgCl\(_2\), 10 HEPES, and 10 glucose. The pH was adjusted to 7.2 with CsOH, and the osmolarity was adjusted to ~310 mosM with sucrose. Intracellular solution consisted of the following (mM): 101 CsCl, 24 CsF, 1.8 NaCl, 7.4 EGTA, 9 HEPES, 4 Mg\(_2\)ATP, 14 creatine phosphate, and 0.3 GTP. The pH was adjusted to 7.3 with CsOH with an osmolarity of ~290 mosM.

To assess inhibition of N-type current under depolarized conditions, DRG neurons were voltage-clamped at approximately −60 to −70 mV, to achieve ~40% inactivation of the channels, and stepped to 0 mV for 60 ms at a frequency of 0.2 Hz. Inactivation levels were determined by comparing the current elicited after 90 s to the current elicited initially from a hyperpolarized holding potential of −105 mV. To assess inhibition of N-type current under hyperpolarized conditions, DRG neurons were voltage-clamped at −105 mV and stepped to 0 mV for 50 ms to elicit current. Initial experiments from the hyperpolarized potential demonstrated that, when neurons were stepped to 0 mV once every 20 s, 10 \(\mu\)M A-1264087 inhibited 88 ± 7% of the calcium current. However, when neurons were instead stepped to 0 mV only once every 60 s, A-1264087 produced less inhibition (60 ± 6%, \(P < 0.05\)). These data suggest that, even from a −105 mV holding potential, there can be a significant use-dependent component to the inhibition. To minimize this use-dependent component during the hyperpolarized protocol, neurons were stepped to 0 mV once per 60 s, and block was assessed after 3 min in compound. For both the depolarized and the hyperpolarized protocols, the step to 0 mV was preceded by a 200-ms step to −30 mV to inactivate T-type calcium channels. To minimize the contribution from non-N-type high-threshold calcium channels, 1 \(\mu\)M nifedipine, 150 nM SNX-482 (Peptides International, Louisville, KY), and 300 nM \(\omega\)-Aga-IVA (Sigma-Aldrich, St. Louis, MO) were included in the external solution to block L-type (Mery et al. 1996), R-type (Newcomb et al. 1998), and P/Q-type (Mintz et al. 1992) currents, respectively. Under similar conditions, ~85% of the remaining DRG calcium current were sensitive to the N-type selective peptide inhibitor \(\omega\)-conotoxin GVIA (Abbade et al. 2010). Data were collected from rat DRG neurons, which ranged between 25 and 35 \(\mu\)M in diameter.

For determining compound IC\(_{50}\) values for manual electrophysiology, averaged percent control values were plotted as a function of compound concentration and fitted using a four-parameter logistic equation with the minimum fixed at zero and maximum fixed to 100% of control.

Behavioral Studies

To assess the effect of A-1264087 on behavioral mechanical hypersensitivity, rats were placed into inverted individual plastic containers (20 × 12.5 × 20 cm) on top of suspended wire mesh with a 1-cm\(^2\) grid. After acclimation to the testing area (20 min), mechanical thresholds were assessed by applying von Frey monofilaments (Stoelting, Wood Dale, IL) on the hindpaw ipsilateral to the nerve ligation. The von Frey filaments were presented perpendicularly to the plantar surface of rat hindpaw and then held in this position for ~8 s with enough force to cause a slight bend in the filament. Positive responses included an abrupt withdrawal of the hindpaw from the stimulus, or flinching behavior immediately following removal of the stimulus. A 50% withdrawal threshold was determined using an up-down procedure (Chaplan et al. 1994a; Dixon 1980). The strength
of the maximum filament used for von Frey testing was 15.0 g. A percent maximal possible effect of testing compound was calculated following the formula: \[ \log(\text{compound-treated threshold}) - \log(\text{vehicle-treated threshold})/\log(\text{maximum threshold}) - \log(\text{vehicle-treated threshold}) \times 100\% \], where the maximum threshold was taken as 15.0 g.

In Vivo Spinal Cord Electrophysiology

Mechanical hypersensitivity was also assessed on the SNL rats used for the in vivo electrophysiological experiments to confirm the development of allodynia. On the day of recording, animals were assessed for allodynia by applying a 6-g von Frey hair stimulus to the midplantar surface of the rat right hindpaw for a maximum of 5 s. This was repeated at least 3 times, and, if the animal did not respond, it was excluded from the study and was not used in electrophysiological experiments.

The experiment procedures for in vivo extracellular recordings of spinal dorsal horn neurons were similar to the methods described previously (McGaraughty et al. 2009). Briefly, the rat was initially anesthetized with pentobarbital (50 mg/kg ip). The left and right external jugular veins were cannulated for propofol infusion and drug delivery, respectively. A laminectomy was then performed to expose the dorsal spinal cord at the lumbar enlargement between 12th thoracic and 3rd lumbar vertebra. In a subgroup of animals, the spinal cord was transected between the 9th and 10th thoracic vertebrae. The animal body temperature was kept at ~37°C during surgeries and subsequent neuronal recording with an underbody circulating water blanket. Throughout the recording period, anesthesia was maintained by continuous infusion of propofol at a rate of 8–12 mg·kg⁻¹·h⁻¹ (iv).

A platinum-iridium microelectrode (impedance of ~1 MΩ at 1 kHz, Frederick Huer, Brunswick, ME) was slowly advanced into the spinal cord with a hydraulic micromanipulator (David Kopf Instruments, Tujunga, CA). After being amplified and filtered (Neurolog, Welwyn Garden City, UK), neuronal activities were continuously monitored on an oscilloscope, digitized, and stored in a computer for off-line analysis (SciWorks, Datawave Technologies, Longmont, CO). Single-unit activity was unambiguously discriminated based on an analysis of multiple waveform parameters. Except for one experiment in which two neurons with distinct waveforms were recorded simultaneously, only one neuron was studied per rat.

Recording protocol. Mechanical stimulation (tapping and pinching) was applied to the ipsilateral hindpaw, while the microelectrode was slowly advanced into spinal tissue to search for relevant dorsal horn neurons. Neurons were kept for further study if their mechanical receptive fields (RF) were located on the plantar or digit region, had well isolated and clearly distinguishable spike waveforms, and were characterized as wide dynamic range (WDR) neurons. WDR neurons were those cells that responded to both low- and high-intensity stimulation, but responded with greater firing frequency to high-intensity stimulation. After a neuron was identified, the baseline level of spontaneous activity was recorded for 5 min. The neuron was then characterized based on its response properties to various stimuli applied to the RF for 2–3 s, including low-intensity (brush, tap, air puff, and 10 g von Frey filament) and high-intensity (pinch with a 28-mm curved Bulldog Serrefine clamp, Fine Science Tools, Foster City, CA) mechanical stimulation. Predrug (baseline) evoked activity was then measured by applying a mechanical or electrical stimulus three times with a 5- or 10-min interstimulus interval, respectively. Baseline evoked activity was represented by the mean firing rate from the three stimulation applications. Following the measurement of baseline response, vehicle or drug was administered systemically or locally (see below). For most experiments, evoked and spontaneous firing was then measured at 5, 15, 25 s, and 35 min after vehicle or drug administration. For experiments with intra-RF injections, postdrug measurements started 15 min after administration due to extraneous activity related to needle penetration into the hindpaw. Postdrug spontaneous activity was measured during the 5-min period preceding each stimulus.

For mechanical stimulation, a 10-g von Frey hair filament was used and applied to the neuronal RF for 15 s (action potentials during the 15-s period were counted as the neuronal response). Transcutaneous electrical stimulation was delivered through a pair of needle electrodes. The needle electrodes were placed subcutaneously at the proximal and distal part of rat plantar hindpaw (ipsilateral to the injury) at the beginning of each experiment. The electrical stimulus was composed of a train of 16 electrical pulses (2-ms duration, 1 Hz). The intensity of the electrical stimulus delivered was three times the threshold current for C-fiber activation. The threshold current to activated C fiber was approximately between 0.5 to 3.0 mA, Aβ-, Aδ-, and C-fiber evoked WDR responses were separated based on response latencies using a poststimulus histogram (Aδ fiber: 0–20 ms; Aδ fiber: 20–400 ms; C fiber: 90–350 ms) (Chapman and Dickenson 1997; Rahman et al. 2006). Neuronal responses occurring 350–800 ms poststimulation were considered as C fiber related after-discharges. “Wind-up” of WDR neurons was also measured following the electrical stimulation and was quantified by subtracting nonpotentiated neuronal responses from the total number of action potentials in the C-fiber range (90–800 ms). The nonpotentiated neuronal response was calculated by multiplying the number of action potentials in C-fiber range following the first pulse of the train by 16 (the total number of pulses in a train).

Drug Administration and Site of Action

For systemic administration to rats in both electrophysiological (4 mg/kg iv over a 6- to 7-min infusion period) and behavioral (1, 3, and 10 mg/kg po) tests, A-1264087 was dissolved in 10% dimethyl sulfoxide and polyethylene glycol 400 (Sigma-Aldrich, St. Louis, MO). For behavioral tests, testing started 60 min following compound administration. For electrophysiological experiments, in addition to examining effects in intact rats, spinally transected SNL rats also received systemic injection of A-1264087 to delineate the potential role of supraspinal Ca²⁺ channels in modulation of WDR neuronal activity. For intraspinal injection (McGaraughty et al. 2006), vehicle (60% N-methyl-2-pyrrolidinone in H₂O) or A-1264087 (10 nmol/0.5 μl) was delivered through an angled glass infusion pipette (outer diameter 75–80 μm) attached to the recording electrode. The tip of glass pipette and that of the recording electrode were separated by ~300 μm laterally and by 30–100 μm dorsoventrally. Vehicle or drug was applied over a 2-min period with a 1-μl Hamilton syringe. Finally, for intra-RF administration, vehicle (5% dimethyl sulfoxide in polyethylene glycol 400) or A-1264087 (300 nmol/20 μl) was injected into the neuronal RF on the ipsilateral hindpaw or the corresponding area on the contralateral hindpaw. The injection was made with a 31-gauge needle over a 2-min period.

Data Analysis

One-way analysis of variance (ANOVA) was performed for behavioral data. Bonferroni multiple-comparison test was used for post hoc analysis. For the in vivo electrophysiological data, postdrug firing levels were calculated as a mean percentage of baseline values ± standard error of the mean (SEM) for both evoked and spontaneous activity. The recording depth and baseline evoked responses of WDR neurons between uninjured and SNL rats were analyzed with t-test. To compare baseline and postdrug activity, statistical significance was examined by using a repeated-measures ANOVA followed by Fisher’s least significant difference test. A two-way ANOVA followed by Fisher’s least
significant difference test was used to compare the vehicle and drug groups. Data were presented as means ± SE, and significance was set at \( P < 0.05 \).

RESULTS

A-1264087 Inhibited N-type Currents in a Voltage-dependent Manner

The activity of A-1264087 was assessed on native N-type \( \text{Ca}^{2+} \) channels from acutely dissociated naive rat DRG neurons. Under depolarized conditions, where N-type channels are biased toward the inactivated state, A-1264087 inhibited N-type currents with an IC\(_{50}\) of 1.6 \( \mu M \) (Fig. 1). Under more hyperpolarized conditions, where N-type channels are largely in the closed state, A-1264087 inhibited current less potently with an IC\(_{50}\) of 8.4 \( \mu M \). These results are consistent with a state-dependent blocking mechanism for A-1264087 and a >5-fold preference for open/inactivated channels over closed channels.

Anti-allodynic Activity of Systemic A-1264087 in SNL Rats

Rats were allodynic 2 wk following the SNL surgery as the mean paw withdrawal threshold to von Frey hairs was 2.7 ± 0.5 g. A-1264087 at 1, 3 and 10 mg/kg (po) elevated mechanical thresholds in a dose-dependent manner, with a maximal decrease of 38.2% at 10 mg/kg po (Fig. 2, \( P < 0.01 \)). Plasma levels was 190 ng/ml 70 min after the 10 mg/kg dose.

Baseline Activity of WDR Neurons

Twenty-seven and eighty-two spinal WDR neurons were recorded from uninjured and SNL rats, respectively (Table 1). The mean baseline spontaneous activity of WDR neurons in SNL rats was significantly higher (\( P < 0.01 \)) than that in uninjured rats and is similar to levels reported previously (Chu et al. 2004; McGaraughty et al. 2012). C-fiber evoked responses were significantly elevated in SNL rats compared with the uninjured rats (\( P < 0.05 \)); however, no other measure of evoked firing was different between the two groups.

Effect of Systemic A-1264087 on Electrically Evoked Activity of WDR Neurons in SNL and Uninjured Rats

In SNL rats, systemic administration of A-1264087 (4 mg/kg iv) significantly reduced A\( \delta \)- and C-fiber evoked responses as well as the after-discharges of WDR neurons with maximal decreases of 38.2 ± 4%, 40.3 ± 10.4% and 25.1 ± 8% from baseline levels, respectively (Fig. 3, C, D, and E). In contrast, these parameters were not affected by A-1264087 in uninjured rats (Fig. 3). A-1264087 did not significantly alter A\( \delta \)-fiber-mediated input to WDR neurons in either SNL or uninjured rats (Fig. 3B).

Systemic administration of A-1264087 (4 mg/kg iv) inhibited wind-up in both SNL rats (\( P < 0.05 \), maximal inhibition of 33.4 ± 9%; Fig. 4B) and uninjured (\( P < 0.05 \), maximal inhibition of 21.8 ± 7.1%; Fig. 4C).

The plasma level 15 min after injection of 4 mg/kg (iv) of A-1264087 was 366 ng/ml.
A-1264087 INHIBITS SPINAL NOCICEPTIVE SIGNALS

Fig. 2. Systemic A-1264087 was anti-allodynic in spinal nerve ligation (SNL) rats. A-1264087 (1, 3 and 10 mg/kg p.o.) dose-dependently reduced mechanical allodynia 60 min after administration. n = 6 rats per group. Data are expressed as the percentage of maximal possible effect (%MPE) ± SE. *P < 0.05, **P < 0.01 vs. vehicle treated rats.

A-1264087 (mg/kg, p.o.)

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<th>Vehicle</th>
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<td>%MPE</td>
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group (Fig. 5, E and F). The inhibitory effect of A-1264087 on the mechanical responses of WDR neurons was evident 5 min postinjection and had a maximal decrease of 52.2 ± 12.8% from baseline levels at 35 min postinjection (Fig. 5B). The effect of A-1264087 on spontaneous activity was also observed 5 min postinjection and lasted for the remainder of the recording period with a maximum reduction of 44.5 ± 7.8% occurring 15 min postinjection (Fig. 5C). In uninjured animals, systemic injection of A-1264087 (4 mg/kg iv) did not alter either the mechanical responses or the spontaneous activity of WDR neurons (Fig. 5, E and F).

Sites of A-1264087 Action on WDR Neuronal Activity in SNL Rats

To address the possible role of descending modulation in the systemic actions of A-1264087, the spinal cord was transected at the level of T9 (rostral to the WDR recording site) in SNL rats. Systemic A-1264087 (4 mg/kg iv) attenuated mechanically evoked and spontaneous activity of WDR neurons compared with both baseline and vehicle group (P < 0.05, Fig. 6, A and B). These effects by A-1264087 in spinally transected rats were comparable to those in intact rats.

Injection of A-1264087 (300 nmol/0.5 μl) into the RF on the ipsilateral hindpaw inhibited the responses of WDR neurons to the 10-g von Frey hair stimulation at each time point examined (15–35 min, P < 0.05, Fig. 6C) in SNL rats. The mechanical response was maximally reduced by 31.5 ± 4.7% from baseline levels, occurring 35 min postinjection. Intra-RF injection of A-1264087 did not decrease the spontaneous activity of WDR neurons at any time point examined (Fig. 6D), but produced transient increase in ongoing activity 15 min postinjection. Injection of A-1264087 (300 nmol/0.5 μl) into the contralateral hindpaw did not alter either the evoked or spontaneous firing of WDR neurons in SNL rats.

Intraspinal injection of A-1264087 (10 nmol/0.5 μl) to SNL rats significantly reduced both mechanically evoked and spontaneous activity of WDR neurons by 5 and 15 min postinjection, respectively, and the effect lasted for the remainder of the recordings (Fig. 6, E and F). The greatest reduction in WDR responses to mechanical stimulation (35.5 ± 4.6%) and spontaneous activity (41.2 ± 6.5%) was observed 15 min postinjection of A-1264087.

Table 1. Recording depth and baseline evoked responses of wide dynamic range neurons in SNL and uninjured rats

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<th>SNL</th>
<th>Uninjured</th>
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<tr>
<td>Recording depth, μm</td>
<td>634 ± 23</td>
<td>591 ± 20</td>
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<tr>
<td>Spontaneous activity, Hz</td>
<td>4.35 ± 0.49**</td>
<td>1.39 ± 0.34</td>
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<td>von Frey 10 g, AP</td>
<td>225 ± 34</td>
<td>197 ± 24</td>
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<tr>
<td>Aβ-fiber response, AP</td>
<td>12 ± 3</td>
<td>16 ± 3</td>
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<tr>
<td>Aδ-fiber response, AP</td>
<td>20 ± 5</td>
<td>32 ± 7</td>
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<tr>
<td>C-fiber response, AP</td>
<td>94 ± 12*</td>
<td>149 ± 17</td>
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<tr>
<td>After-discharge, AP</td>
<td>231 ± 35</td>
<td>159 ± 25</td>
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<tr>
<td>Wind-up, AP</td>
<td>150 ± 25</td>
<td>123 ± 20</td>
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Values are means ± SE. SNL, spinal nerve ligation; AP, mean number of action potentials evoked by each stimulus during the stimulation periods. *P < 0.05, **P < 0.01 vs. uninjured group.

DISCUSSION

In SNL rats, systemic administration of A-1264087 dose-dependently alleviated mechanical allodynia. This anti-allodynic activity of A-1264087 is consistent with the effects of other Ca2+ channel blockers in neuropathic rats, including A-1048400 (Scott et al. 2012), TROX-1 (Abbadie et al. 2010), and NP118809 (Pajouhesh et al. 2010), a T-type preferring channel blocker, TTA-P2 (Choe et al. 2011), and a peptidyl N-type blocker (Bowersox et al. 1996; Wallace et al. 2006). A-1264087 also attenuated the transmission of low-intensity mechanical stimulation to WDR neurons in SNL animals. This action of A-1264087 on WDR neurons likely underlies its anti-allodynic activity in vivo.

WDR neurons receive sensory signals from the periphery through small-, medium-, and large-diameter primary afferent fibers. Their activity is further modulated by intraspinal excitatory and inhibitory interneurons as well as supraspinal descending control (both facilitatory and inhibitory) (Baron 2006). It has been suggested that N- and P/Q-type Ca2+ channels in the rostral ventromedial medulla contribute to neuropathic allodynia through modulation of descending facilitatory pathways (Urban et al. 2005). In the present study, the inhibitory effect of systemic A-1264087 on mechanically evoked WDR firing remained unaltered following spinal transection, indicating that descending modulation had a minor role, if any, in this effect. Although this result does not rule out a modulatory role for Ca2+ channels in the supraspinal pain-processing areas, it does suggest that blocking peripheral and/or spinal Ca2+ channels is sufficient for a mixed Ca2+ channel blocker to inhibit spinal mechanotransmission in neuropathic rats.

Confirming a role for peripheral and/or spinal Ca2+ channels, local injection of A-1264087 into the hindpaw or spinal cord decreased the responses of WDR neurons to mechanical stimulation in SNL rats. In agreement, blockade of spinal N-, P-, or T-type Ca2+ channels with locally applied ω-conotoxin-GVIA, ω-agatoxin-IVA, or ethosuximide, respectively, inhibited the processing of innocuous mechanical signals by WDR neurons in SNL rats (Matthews and Dickenson 2001a, 2001b). Contribution of spinal N- and T-type Ca2+ channels to the development of neuropathic allodynia has also been suggested by behavioral studies (Bowersox et al. 1996; Chaplan et al. 1994b; Miljanich 2004; Wen et al. 2010). The peripheral
effects of A-1264087 may be mediated by inhibition of T-type channels (Todorovic and Jevtovic-Todorovic 2011), although N-type inhibition may also contribute since intraplantar administration of the selective N-type blocker, SNX-111, dose-dependently attenuated mechanical allodynia in neuropathic rats (Dogrul et al. 2003; White and Cousins 1998), but intraplantar injection of the P/Q-type blocker, SNX-230, was without effect (White and Cousins 1998).

WDR neurons spontaneously fired at a higher rate in neuropathic rats compared with the uninjured animals and is consistent with previous reports (Chu et al. 2004; McGarraughty et al. 2009, 2012; Suzuki and Dickenson 2006). An increased level of spontaneous discharge in WDR neurons following tissue or nerve injury indicates spinal hyperexcitability and may also suggest that there is stimulus-independent ongoing pain in these animals (Chu et al. 2004; Suzuki and Dickenson 2006). The inhibitory effect of systemic A-1264087 on WDR spontaneous activity indicates that blocking Ca\(^{2+}\) channels attenuates spinal hyperactivity and may result in reduced ongoing pain under neuropathic conditions. This effect of A-1264087 on neuronal spontaneous activity is likely mediated by spinal Ca\(^{2+}\) channels, since intraplantarly applied A-1264087 had no effect, and removal of descending modulation (by spinal transection) did not alter the inhibitory action of systemic A-1264087 on spontaneous activity. Consistent with our findings, Matthews and Dickenson (2001b) previously observed that spinally applied α-conotoxin-GVIA or ω-agatoxin-IVA inhibited spontaneous WDR neuronal activity in SNL animals. More recently, Jacus and colleagues (2012) found that TTA-P2 attenuated the elevated frequency of spontaneous excitatory synaptic activity in spinal cord slices from a rodent model of diabetic neuropathic pain. These data suggested that presynaptic T-type Ca\(^{2+}\) channels have a role in tuning excitability of spinal sensory neurons. Despite the lack of effect on spontaneous WDR activity by A-1264087 applied into the neuronal RF, peripheral Ca\(^{2+}\) channels at the site of injury may still influence ongoing firing. Topical application of nonselective Ca\(^{2+}\) channel blockers to the site of nerve injury suppressed ectopic discharge of primary afferents, which may then block the peripheral drive of spinal hyperactivity (Xie et al. 1993).

To further examine Ca\(^{2+}\) channel-mediated modulation ofafferent input to WDR neurons, electrical stimulation was applied to the hindpaw. Systemic A-1264087 reduced WDR responses to A\(\beta\)- and C-fiber inputs (including C-fiber-related

![Image](image_url)
after-discharges) in SNL, but not uninjured rats. Although there was a trend toward a reduction, A-1264087 did not significantly inhibit WDR responses related to Aβ-fiber inputs. Spinally applied ω-conotoxin-GVIA and ethosuximide moderately attenuated Aβ-fiber transmission to WDR neurons, but had a greater effect on Aδ- and C-fiber inputs in SNL animals (Matthews and Dickenson 2001a, 2001b). In the same study, spinal application of ω-agatoxin-IVA had a greater effect on Aδ-fiber mediated responses of WDR neurons compared with the effect on Aβ- and C-fiber responses. Thus, taken together, inputs from Aδ fibers were consistently inhibited by the different compounds: C-fiber inputs appear to be diminished, and Aβ-fiber inputs may be modulated but to a limited degree by blockade of Ca\(^{2+}\) channels. Blocking Ca\(^{2+}\) channels in neuropathic rats inhibits spinal processing of inputs from diverse somatosensory primary afferents, and this likely contributed to the anti-allodynic actions of A-1264087.

Wind-up (temporal summation) reflects a short-term sensitization of spinal neurons where nociceptive afferent inputs are amplified. The moderate inhibition of wind-up by A-1264087 in SNL rats suggests that blocking Ca\(^{2+}\) channels impairs the development of central sensitization. N- and P-type Ca\(^{2+}\) channels modulate the release of glutamate and substance P (Millan and Sanchez-Prieto 2002; Qian and Noebels 2001; Smith et al. 2002) which are key components to the generation of wind-up (Herrero and Laird 2000). Additionally, T-type channels have been reported to have a role in excitatory synaptic transmission and contribute to activity-dependent long-term potentiation of synaptic strength in the spinal cord (Ikeda et al. 2003, 2006). Thus it is not a surprise that A-1264087 affected wind-up. Spinally applied ethosuximide also reduced the wind-up of WDR neurons in SNL rats (Matthews and Dickenson 2001a). Interestingly, in the absence of a neuropathic injury, A-1264087 also had a moderate effect on wind-up. It suggests that the compound is primarily effective in states of facilitated spinal processing, which is induced either by a nerve injury or by repetitive electrical stimulation. Gabapentin, which likely modulates Ca\(^{2+}\) channels through binding at \(\alpha2\delta\) site (Gee et al. 1996), also inhibits wind-up-like pain in healthy subjects, but does not alter pain associated with an acute punctuate stimulus (Harding et al. 2005).

Except for the effect on wind-up, A-1264087 selectively inhibited WDR neuronal activity in SNL rats. This injury-specific effect of A-1264087 may partially arise from the intrinsic property of the compound. As a state-dependent...
blocker, A-1264087 is assumed to selectively bind to the inactivated state of Ca\(^{2+}\)/H11001 channels and stabilize channels into the inactivated conformation. Following a nerve injury or during the induction of wind-up, sensory neurons can become sensitized and actively fire at higher rates than normal. A-1264087 may preferentially bind to and modulate those hyperactive neurons without compromising the activity of nonhyperactive neurons performing housekeeping functions. Based on this rationale, several novel voltage-dependent Ca\(^{2+}\)/H11001 channel blockers have demonstrated analgesic activity while having minimal effects on other functions (Abbadie et al. 2010; Choe et al. 2011; Pajouhesh et al. 2010; Scott et al. 2012). The selective effect of A-1264087 in SNL animals may also reflect an enhanced role of Ca\(^{2+}\)/H11001 channels in nociceptive transmission after injury. Increased current density and/or expression levels of N- and T-type Ca\(^{2+}\)/H11001 channels may contribute to the shift of channel function under neuropathic conditions (Cizkova et al. 2002; Latham et al. 2009; Takahashi et al. 2010).

In this study, we focused on WDR neurons to investigate the underlying physiological mechanisms for the anti-allodynic
Fig. 6. Site(s) of action of A-1264087 on WDR neuronal activity in SNL rats. Systemic A-1264087 (4 mg/kg, i.v.) to spinally transected SNL rats decreased the mechanical responses (A) and the spontaneous activity (B) of WDR neurons comparable to the effects observed in intact SNL rats. Data from intact rats are shown for comparison. C: the responses of WDR neurons to 10 g von Frey hair stimulation was reduced by ipsilateral but not contralateral injection of A-1264087 (300 nmol/20 μl) into the neuronal receptive field (RF) on the rat hindpaw. D: spontaneous activity of WDR neurons was not affected by either ipsilateral or contralateral injection of A-1264087 into the RF. Direct injection of A-1264087 (10 nmol/0.5 μl) into the spinal cord reduced the mechanical evoked (E) and spontaneous activity (F) of WDR neurons in neuropathic rats. n = 5–8 neurons per group. Results are expressed as means ± SE. *P < 0.05, **P < 0.01, vs. baseline levels; *P < 0.05, **P < 0.01 vs. vehicle-treated group.
REFERENCES


