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A tetrodotoxin-resistant sodium current mediates inflammatory pain in the rat

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Abstract

We report evidence for a contribution of tetrodotoxin-resistant sodium current (TTX-R I_{Na}) to prostaglandin E_2 (PGE_2)-induced hyperalgesia. Behavioral experiments were performed in rats chronically implanted with spinal cannulae. The study employed intrathecal administration of oligodeoxynucleotide (ODN) antisense to the recently cloned channel underlying TTX-R I_{Na} (PN3/SNS). The nociceptive flexion reflex was employed to determine changes in mechanical stimulus-induced paw-withdrawal threshold. Administration of antisense but not of sense or mismatch ODN, led to a decrease in PGE_2-induced hyperalgesia. PGE_2-induced hyperalgesia returned to normal 7 days after the last injection of antisense ODN. Antisense ODN selectively and significantly reduced TTX-R I_{Na} current density in cultured sensory neurons. Our observations support the hypothesis that modulation of TTX-R I_{Na}, present in peripheral terminals of primary afferent nociceptors, contributes, at least in part, to inflammatory hyperalgesia. Since TTX-R I_{Na} is found only in primary afferent nociceptors, our findings suggest TTX-R I_{Na} as a promising target for novel therapeutic interventions for the treatment of inflammatory pain.

Keywords: Tetrodotoxin-resistant sodium current; Pain; Hyperalgesia; Primary afferent nociceptor; Sensitization

The pain associated with inflammation results, in part, from the sensitization of primary afferent nociceptors innervating inflamed tissue. Since a tetrodotoxin-resistant voltage-gated sodium current (TTX-R I_{Na}), restricted to nociceptors, is modulated by hyperalgesic inflammatory mediators in a manner that is likely to enhance nociceptor excitability [4,7], such modulation may underlie nociceptor sensitization.

The involvement of TTX-R I_{Na} as an underlying mechanism of nociceptor sensitization has yet to be determined in vivo. The lack of selective agonists and antagonists for TTX-R I_{Na} preclude the use of traditional pharmacological methodology to address this question. However, through the use of antisense oligodeoxynucleotides (ODNs) to selectively knock-down expression of protein encoded by targeted mRNA [3], it has become possible to study the function of specific proteins (e.g. [8]). We have previously demonstrated that intrathecal administration of antisense ODNs can be used to specifically evaluate the function of proteins present in the peripheral terminals of primary afferent nociceptors [10]. The recent cloning from rat DRG of a TTX-R I_{Na}, referred to as PN3 [17] or SNS [1] now permits the use of this approach to evaluate, in vivo, the role of TTX-R I_{Na} in inflammatory hyperalgesia.

The experiments were performed on 250–350 g male Sprague–Dawley rats (Bantin and Kingman, Fremont, CA). Rats were chronically implanted with spinal cannulae into the lumbar intrathecal (i.t.) space [16] (the number of observations are given in the legend to the Figs. 1 and 2).

The antisense, sense and mismatch phosphodiester oligodeoxynucleotides (ODNs) used in this study were purchased from GIBCO BRL (Grand Island, NY). The antisense ODN, 5′-GGG GAG CTC CAT CTT CTC-3′, was directed against a unique sequence of the PN3/SNS clones spanning the initiating ATG. The mismatch ODN, 5′-GGG G**T**C **T**TC

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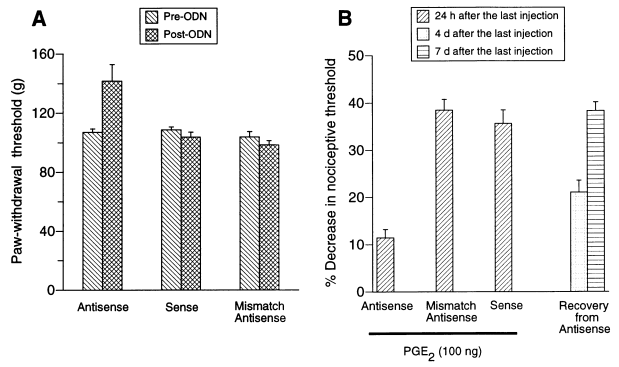

Fig. 1. Treatment of rats with oligodeoxynucleotides (ODNs) antisense to PN3/SNS increases mechanical nociceptive threshold and blocks PGE_2-induced hyperalgesia. Rats received a 2-μg intrathecal injection of 18-mer ODNs in 10 μl, once daily for 3 days, via an indwelling catheter. ODNs consisted of a unique sequence of PN3/SNS (sense), a sequence antisense to the sense sequence, or a mismatch (three base-pairs were switched) of the antisense sequence. Twenty-four hours following the last ODN injection, mechanical nociceptive threshold was measured and then PGE_2 was injected intradermally (100 ng/2.5 μl) into the dorsum of the hindpaw. Changes in mechanical paw-withdrawal threshold were assessed with an Ugo Basile analgesiometer©. (A) Mechanical nociceptive threshold was significantly increased in rats treated with ODN antisense to TTX-R I_{Na} compared with the rats treated with sense and mismatch ODNs ($P < 0.001$, one-way ANOVA, Fisher post hoc) (antisense $n = 20$, sense $n = 14$ and mismatch $n = 10$). (B) PGE_2-induced decrease in paw-withdrawal threshold (expressed as a percentage decrease from baseline threshold) was significantly attenuated (antisense $n = 14$, sense $n = 10$ and mismatch $n = 10$) in rats treated with ODN antisense to TTX-R I_{Na}, compared with the rats treated with sense and mismatch ODNs ($P < 0.05$, one-way ANOVA, Fisher post hoc). PGE_2-induced hyperalgesia was partially recovered 4 days after the last antisense injection (4 day recovery, $n = 8$), and was fully recovered in 7 days (7 day recovery, $n = 8$).

CA**A GC**T CTC-3′, corresponds to the antisense sequence except that three pairs of bases have been switched (bold face indicates mismatched base sequences). Prior to being used, ODNs were lyophilized and resuspended in 0.9% NaCl to a concentration of 2 μg/10 μl. A search of EMBL and GeneBank™ data bases identified no sequences homologous to those used in this experiment.

The nociceptive flexion reflex of awake, lightly restrained rats, was quantified by a Ugo Basile Analgesymeter© (Stoelting, Chicago, IL), which applies a linearly increasing force to the dorsum of the rat's hindpaw. Beginning at 2 days following implantation of i.t. cannulae, rats were trained in the paw-withdrawal reflex test at 5 min intervals for 1 h each day for 1 week [18]. The baseline paw-withdrawal threshold was defined as the mean of the last six measurements, before the injection of ODN. ODNs (2 μg) were injected daily in a volume of 10 μl followed by 10 μl of saline, for 3 days. Twenty-four hours after the last i.t. injection of ODN, paw-withdrawal thresholds were determined again, at 5 min intervals for 1 h. The mean of the last six measurements was defined as the threshold after i.t. injection of ODN. This paw-withdrawal threshold (after ODN treatment) was used to calculate the percentage change in nociceptive threshold after intradermal injection of prostaglandin E_2 (PGE_2) into the paw.

PGE_2 was injected into the dorsum of each hindpaw, in a volume of 2.5 μl. Thresholds for paw-withdrawal were then re-determined 10, 15 and 20 min post-PGE_2 injection. The mean of these three consecutive readings was defined as the threshold in the presence of PGE_2. The effect of PGE_2 was calculated as follows: [(paw threshold in the presence of PGE_2 − paw threshold after ODN treatment)/paw threshold after ODN treatment] × 100.

Lumbar dorsal root ganglion (DRG) neurons were obtained from adult male Sprague–Dawley rats. Ganglia were enzymatically treated and mechanically dispersed by methods described previously [6]. DRG neurons were plated on cover slips coated with laminin and ornithine and incubated for 30 min at 37°C, 90% humidity and 3% CO_2 in minimal essential medium (MEM) containing fetal bovine serum (10% final concentration), MEM vitamins, penicillin/streptomycin and 10–20 ng/ml mouse nerve growth factor (NGF) (2.5S). Cover slips were then washed with serum-free MEM, and MEM was replaced with MEM containing N2 supplement, MEM vitamins, penicillin/streptomycin, 10–20 ng/ml NGF and 20 μM of one of the three different ODNs used in the in vivo study. Neurons were studied 22 to 26 h after the addition of ODNs.

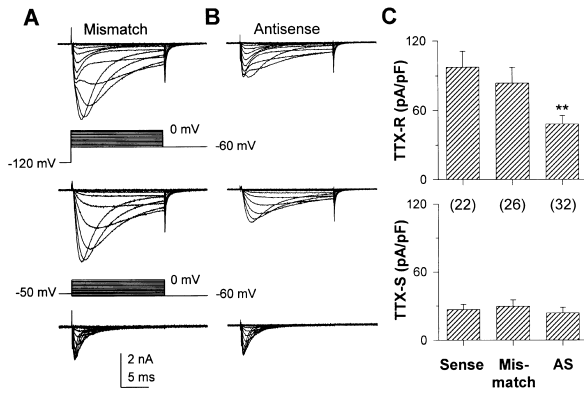

Fig. 2. Oligodeoxynucleotides (ODNs) antisense to PN3/SNS selectively reduce TTX-R I_{Na}. (A) Voltage-gated sodium currents recorded from a 33.5 pF DRG neuron incubated in mismatch ODN. Top panel: voltage-gated sodium current evoked during 15 ms command potentials ranging between –60 and 0 mV, following a 500 ms pre-pulse to –120 mV. The voltage-clamp protocol used to evoke the currents is shown beneath the current traces. Middle panel: TTX-R I_{Na} was isolated by changing the pre-pulse amplitude to –50 mV, a potential at which TTX-S I_{Na} is inactivated. Bottom panel: TTX-S I_{Na} was isolated as the difference between the current evoked from –140 mV and the current evoked from –50 mV. (B) Voltage-gated sodium currents recorded in a 25.5 pF DRG neuron incubated in antisense ODN. Current traces in the top, middle and bottom panels were obtained as described in (A). (C) The density of TTX-R I_{Na} (Top panel) was significantly reduced in neurons pre-incubated with antisense (AS) ODN compared with the neurons pre-incubated with sense or mismatch ODNs ($P < 0.01$, one-way ANOVA with Fisher post-hoc). There was no difference between groups with respect to the density of TTX-S I_{Na} (bottom panel). Numbers between the two panels represent the number of neurons studied in each group.

The experiments were carried out on small-diameter lumbar DRG neurons. Conventional whole-cell voltage clamp electrophysiological techniques were used. To study Na^+ currents in isolation, bath and electrode solutions were composed to minimize K^+ and Ca^{2+} currents, as well as to maintain clamp control over voltage-gated Na^+ currents. The bath solution contained (in mM): 50 NaCl; 50 choline-Cl; 30 TEA-Cl; 0.1 $CaCl_2$; 5 $MgCl_2$; 10 HEPES; 10 glucose. pH was adjusted to 7.4 with Tris-base and osmolarity was adjusted with sucrose to 325 mOsm. The electrode solution contained (in mM): 90 CsCl, 10 NaCl; 40 TEA-Cl; 0.1 $CaCl_2$, 2 $MgCl_2$, 11 EGTA, 10 HEPES, 2 Mg-ATP, 1 Li-GTP. pH was adjusted to 7.2 with Tris-base and osmolarity was adjusted with sucrose to 310 mOsm.

Whole-cell impedance and capacitance were determined for each cell by stepping the membrane potential to −100 mV 4 times (10 ms pulses) (capacitance was estimated from the area of the transient associated with the voltage step). The current–voltage (I–V) relationship for voltage-gated Na^+ currents was determined with 15-ms depolarizing voltage steps to potentials ranging between −50 and +50 mV evoked at 5-mV intervals every 2 s (evoked current was sampled at 50 kHz, filtered at 5 kHz and a P/4 protocol was utilized to eliminate leak current and capacitative transients). Data for current–voltage (I–V) curves were collected at holding potentials of −100 mV and −50 mV, and/or before and after the application of 500 nM TTX. TTX-sensitive sodium currents (TTX-S I_{Na}) were eliminated by both a holding potential of −50 mV and the addition of TTX. I–V curves for TTX-S I_{Na} were constructed by plotting the difference between the current evoked from −100 mV and mnius50 mV, or from −100 mV before and after application of TTX (500 nM). The current density for TTX-S and -R I_{Na} was determined by dividing the peak evoked current by the cell capacitance.

Data are presented as mean ± SEM and were analyzed using the Student's *t*-test or one-factor analysis of variance (ANOVA), where applicable. Where ANOVA showed significant group effects, Fisher post-hoc test was used to determine which groups were significantly different.

Antisense but not sense or mismatch ODNs administration increased baseline threshold (Fig. 1). The PGE_2-induced decrease in paw-withdrawal threshold in rats treated with sense and mismatch ODNs was similar to the PGE_2-induced decrease in paw-withdrawal threshold consistently observed in untreated control rats [11]. However, the PGE_2-induced decrease in paw-withdrawal threshold was significantly reduced in rats treated with antisense ODN ($P < 0.05$) (Fig. 1). PGE_2-induced hyperalgesia was partially recovered 4 days after the last antisense injection, and was fully recovered within 7 days (Fig. 1).

To determine the relative selectivity of the ODN sequences used in vivo on the expression of TTX-R I_{Na}, in DRG neurons we assayed the effects of the ODNs on TTX-R I_{Na} current density (pA/pF) in the cell body of cultured DRG neurons. The addition of ODNs to the culture medium resulted in no gross abnormalities in DRG neuron morphology, survival, or size distribution. We studied a population of DRG neurons with cell body diameters <35 μm, since TTX-R I_{Na} is expressed in this population of neurons [7,13]. Neurons treated with sense and mismatch ODNs were from three different rats while the neurons incubated with antisense ODNs were from four different rats; data obtained with each ODN treatment were pooled. There were no statistical differences in the mean cell body capacitance (a measure for cell body size) between neurons treated with sense (35.4 ± 2.1, $n = 22$), mismatch (35.2 ± 2.4, $n = 26$) and antisense (35.4 ± 2.2, $n = 32$) ODN ($P > 0.05$). In addition, incubating DRG neurons with ODNs had no significant effect on the density of TTX-S I_{Na} (26.9 ± 4.5 pA/pF, 29.6 ± 5.6 pA/pF and 23.7 ± 5.0 pA/pF, for sense, mismatch and antisense, respectively; Fig. 2). Also, there was no difference in the mean TTX-R I_{Na} density between neurons incubated with sense (97.4 ± 13.8 pA/pF) and mismatch ODNs (83.6 ± 13.7 pA/pF (Fig. 2). However, TTX-R I_{Na} density was significantly reduced in neurons incubated with antisense ODNs (48.3 ± 7.2 pA/pF) (Fig. 2).

We have presented evidence which suggests that TTX-R I_{Na} contributes to mechanical nociceptive threshold and inflammatory hyperalgesia in the intact animal. This is achieved, presumably by the ability of the antisense ODN to decrease the expression of TTX-R I_{Na} in the DRG, consequent to decreasing its transport to the peripheral terminal. Recovery of PGE_2-induced hyperalgesia 7 days following cessation of antisense treatment suggests that the inhibitory effect of antisense observed was not due to its toxicity. This time course for recovery is similar to the time course of recovery observed for other proteins present in nociceptor peripheral terminals [2].

A decrease in the expression of TTX-R I_{Na}, in vivo, presumably will result in a decrease in TTX-R I_{Na} density throughout the afferent neuron. TTX-R I_{Na} is present in the cell body in vivo [15] and there is evidence that it is present in the central terminals [6] and peripheral axons [14] of afferent neurons, as well. Due to the increasing evidence indicating that the excitability of the cell body [9] and the central terminal of primary afferents may contribute to pain, a decrease in the expression of TTX-R I_{Na} in these areas may contribute to the antinociceptive effects of the intrathecally administered antisense ODN. However, in the present study, PGE_2 was administered locally in a small volume to peripheral tissue, and such an administration protocol has been shown to sensitize the peripheral terminal of primary afferent nociceptors [12]. Therefore, we suggest that the results of the present study reflect the contribution of TTX-R I_{Na}, present in peripheral terminals, to PGE_2-induced hyperalgesia.

Numerous observations support the suggestion that the antisense ODN sequences used in the present experiment are specific for PN3/SNS TTX-R I_{Na}. First, a blast search of the GeneBank and EMBL data bases revealed no homo-

logous sequences. Second, neither the sense ODN nor a rearrangement of the antisense sequence (mismatch) had any detectable effect in vivo. Third, the effects of antisense treatment were fully reversible following cessation of antisense treatment. Fourth, pre-incubation of DRG neurons in vitro with antisense ODN selectively and significantly attenuated TTX-R I_{Na}; there was no significant effect on TTX-S I_{Na} current density. Thus, our observations are consistent with the hypothesis that inflammatory hyperalgesia results, in part, from the modulation of TTX-R I_{Na} in primary afferent nociceptors.

A pivotal role for TTX-R I_{Na} in inflammatory mediator-induced hyperalgesia suggests a direction for a novel pharmacological approach to treating inflammatory pain. In our experiments, the hyperalgesic response to PGE_2 after knock-down of TTX-R I_{Na} was decreased by two-thirds. Since PGE_2 contributes significantly to inflammatory pain and hyperalgesia [5,19], this attenuation of PGE_2 hyperalgesia would be expected to afford clinically significant relief from inflammatory pain. In addition, antagonism of TTX-R I_{Na} may be even more efficacious than non-steroidal anti-inflammatory analgesics currently used for the treatment of inflammatory pain, since other inflammatory mediators that are not products of the cyclo-oxygenase pathway of arachidonic acid metabolism also appear to sensitize nociceptors via modulation of TTX-R I_{Na} [7]. Furthermore, it is possible that following administration of a higher dose of antisense ODN, inflammatory hyperalgesia would be more markedly decreased. In addition, since the distribution of TTX-R I_{Na}, is restricted to a subpopulation of sensory neurons with properties of nociceptors [1,7,17], therapeutic interventions targeting TTX-R I_{Na} may enable development of therapies that provide profound pain relief with few side-effects.

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[1] Akopian, A.N., Sivilotti, L. and Wood, J.N., A tetrodotoxin-resistant voltage-gated sodium channel expressed by sensory neurons, Nature, 379 (1996) 257–262.
[2] Aley, K.O. and Levine, J.D., Different mechanisms mediate development and expression of tolerance and dependence for peripheral mu-opioid antinociception in rat, J. Neurosci., 17 (1997) 8018–8023.
[3] Crooke, S.T., Progress in antisense therapeutics discovery and development, Ciba Found. Symp., 209 (1997) 158–168.
[4] England, S., Bevan, S. and Docherty, R.J., PGE_2 modulates the tetrodotoxin-resistant sodium current in neonatal rat dorsal root ganglion neurones via the cyclic AMP-protein kinase A cascade, J. Physiol. (Lond.), 495 (1996) 429–440.
[5] Ferreira, S.H., Prostaglandins, aspirin-like drugs and analgesia, Natl. New Biol., 240 (1972) 200–203.
[6] Gold, M.S., Dastmalchi, S. and Levine, J.D., Co-expression of nociceptor properties in dorsal root ganglion neurons from the adult rat in vitro, Neuroscience, 71 (1996) 265–275.
[7] Gold, M.S., Reichling, D.B., Shuster, M.J. and Levine, J.D., Hyperalgesic agents increase a tetrodotoxin-resistant Na^+ current in nociceptors, Proc. Natl. Acad. Sci. USA, 93 (1996) 1108–1112.
[8] Hunter, J.C., Woodburn, V.L., Durieux, C., Pettersson, E.K., Poat, J.A. and Hughes, J., c-fos Antisense oligodeoxynucleotide increases formalin-induced nociception and regulates preprodynorphin expression, Neuroscience, 65 (1995) 485–492.
[9] Kajander, K.C., Wakisaka, S. and Bennett, G.J., Spontaneous discharge originates in the dorsal root ganglion at the onset of a painful peripheral neuropathy in the rat, Neurosci. Lett., 138 (1992) 225–228.
[10] Khasar, S.G., Gold, M.S., Dastmalchi, S. and Levine, J.D., Selective attenuation of mu-opioid receptor-mediated effects in rat sensory neurons by intrathecal administration of antisense oligodeoxynucleotides, Neurosci. Lett., 218 (1996) 17–20.
[11] Khasar, S.G., Ho, T., Green, P.G. and Levine, J.D., Comparison of prostaglandin E_1- and prostaglandin E_2-induced hyperalgesia in the rat, Neuroscience, 62 (1994) 345–350.
[12] Martin, H.A., Basbaum, A.I., Kwiat, G.C., Goetzl, E.J. and Levine, J.D., Leukotriene and prostaglandin sensitization of cutaneous high-threshold C- and A-delta mechanonociceptors in the hairy skin of rat hindlimbs, Neuroscience, 22 (1987) 651–659.
[13] Ogata, N. and Tatebayashi, H., Kinetic analysis of two types of Na^+ channels in rat dorsal root ganglia, J. Physiol. (Lond.), 466 (1993) 9–37.
[14] Quasthoff, S., Grosskreutz, J., Schroder, J.M., Schneider, U. and Grafe, P., Calcium potentials and tetrodotoxin-resistant sodium potentials in unmyelinated C fibres of biopsied human sural nerve, Neuroscience, 69 (1995) 955–965.
[15] Ritter, A.M. and Mendell, L.M., Somal membrane properties of physiologically identified sensory neurons in the rat: effects of nerve growth factor, J. Neurophysiol., 68 (1992) 2033–2041.
[16] Sakura, S., Hashimoto, K., Bollen, A.W., Ciriales, R. and Drasner, K., Intrathecal catheterization in the rat. Improved technique for morphologic analysis of drug-induced injury, Anesthesiology, 85 (1996) 1184–1189.
[17] Sangameswaran, L., Delgado, S.G., Fish, L.M., Koch, B.D., Jakeman, L.B., Stewart, G.R., Sze, P., Hunter, J.C., Eglen, R.M. and Herman, R.C., Structure and function of a novel voltage-gated, tetrodoxtoxin-resistant sodium channel specific to sensory neurons, J. Biol. Chem., 271 (1996) 5953–5956.
[18] Taiwo, Y.O., Coderre, T.J. and Levine, J.D., The contribution of training to sensitivity in the nociceptive paw-withdrawal test, Brain Res., 487 (1989) 148–151.
[19] Vane, J.R., Inhibition of prostaglandin synthesis as a mechanism of action for aspirin-like drugs, Natl. New Biol., 231 (1971) 232–235.