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Diabetic-induced increased sodium channel activity attenuated by tetracaine in sensory neurons *in vitro*

Rathej Meerupally [1], Jitendra Narain Singh [*,1], Shyam S. Sharma [*]

Electrophysiology Laboratory, Department of Pharmacology and Toxicology, National Institute of Pharmaceutical Education and Research (NIPER), S.A.S. Nagar (Mohali) 160 062, Punjab, India

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ABSTRACT

The present study was aimed to explore correlation between the altered pain perception and Na^+ channel activity in diabetic animals as well as the effect of tetracaine on sensory neurons of diabetic rat. In streptozotocin-induced diabetic rats behavioral nociceptive parameters were assessed. The Na^+ current (I_{Na}) was obtained using whole-cell voltage-clamp configuration in dorsal root ganglion (DRG) neurons isolated from diabetic rat (*in vitro*). In addition, the effects of tetracaine on altered Na^+ channel activity associated with diabetes in small DRG neurons were evaluated. After induction of diabetes mechanical allodynia, thermal hyperalgesia and Na^+ channel activity were altered significantly in 4th and 6th week in relation to the control. Altered pain parameters were in correlation with increased I_{Na} in time-dependent manner. In comparison to age-matched control (-1.10 ± 0.20 nA) the I_{Na} was found to be -2.49 ± 0.21 nA at 4th week and -3.71 ± 0.28 nA at 6th week. The increased activity of Na^+ channels was blocked by tetracaine even in diabetic condition. The depression of the I_{Na} on tetracaine exposure was not sensitive to the voltage or time. The conductance curve shifted towards right around -8.0 mV. The alterations in neuropathic pain associated with diabetes and Na^+ channel activity has been clearly correlated in time-dependent manner. The I_{Na} density was increased significantly with the progression of neuropathic pain. Local anesthetic, tetracaine potentially blocked the Na^+ channel activity in diabetic sensory neurons.

1. Introduction

Diabetes is a serious health problem in developing as well as developed countries. Persistent hyperglycemia in diabetic patients leads to several complications like neuropathy, cardiomyopathy, nephropathy and retinopathy [1]. Diabetic neuropathy is one of the most frequent complications of diabetes associated with anomaly in pain perception and hyperalgesia [2,3]. Pathophysiology of diabetic neuropathy involve many mechanisms like hyperglycemia-induced oxidative stress, increased activity of polyol pathway and advanced glycation end products, deficiency of γ-linolenic acid and dysfunction of dorsal root ganglion (DRG) neurons [4–6], but exact mechanism for development of sensory disturbance in diabetic neuropathy is still unclear.

Streptozotocin (STZ)-induced diabetes is the most widely used experimental model in simulating the pathology of diabetes and its complications [7]. The altered pain behavior in STZ-induced rat model has clinical correlation with peripheral diabetic neuropathy [8]. The spontaneous generation of neural activity in Aδ and C fibers has been reported in STZ-induced model. These Aδ and C fibers correspond to small DRG neurons. The sensory neurons in DRG are not protected from the blood–brain barrier, therefore these neurons are vulnerable to hyperglycemia, triggering many changes in cellular functions of DRG neurons like altered expression of voltage-gated Na^+ channels [9,10]. Thus leading to hyperalgesia and allodynia characterized by spontaneous and prolonged episodes of pain [11].

Voltage-gated Na^+ channels (VGSCs) are important in excitability of neurons and play a vital role in the initiation of action potential generation. Due to hyperglycemia the expression of tetrodotoxin-sensitive (TTX-S) and tetrodotoxin-resistant (TTX-R) VGSCs were altered [12,13]. Reports suggests that expression of Na_v 1.3, Na_v 1.6, Na_v 1.7, Na_v 1.8, Na_v 1.9 were increased in diabetic neuropathy [10,14]. The altered activity of VGSCs in pain associated with diabetic neuropathy has supported the evidence for the functional involvement of these channels [15], where both TTX-S and TTX-R VGSCs play a critical role by altering the electrical

* Corresponding authors. Fax: +91 172 2214692.
E-mail addresses: jnsingh@niper.ac.in (J.N. Singh), sssharma@niper.ac.in (S.S. Sharma).
[1] RM, JNS have equally contributed in this work.

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properties of the membrane, and contributing to the genesis of ectopic discharges. Local anesthetics, tricyclic anti-depressants and anti-convulsants are well known Na^+ channel blockers; these have been evaluated for therapeutic efficacy in the treatment of neuropathic pain. Considering these in the present study, nociceptive parameters like mechanical allodynia (Von Frey, Randall Selitto tests), thermal hyperalgesia (Hargreave's Plantar test) and nerve conduction velocity were assessed in STZ-induced diabetic rats and the effects of tetracaine on altered Na^+ channel activity due to diabetes-induced hyperglycemia in the sensory neurons were evaluated.

2. Materials and methods

2.1. Induction of diabetes

Experiments were carried out in accordance with Committee for the Purpose of Control and Supervision on Experiments on Animals, Government of India; guidelines and approval of Institutional Animal Ethics Committee of National Institute of Pharmaceutical Education and Research, SAS Nagar. All experiments were performed using adult male Sprague–Dawley rats (250–260 g). The animals were housed in room maintained at approximately 24 ± 1 °C temperature and humidity of 55 ± 5% with 12-h light/dark cycle. Free access to food and water were allowed. Details experimental procedures for the behavioral parameters recording is mentioned in Supplementary material (Appendix A).

Diabetes was induced using a single dose of streptozotocin (STZ; 50 mg/kg, i.p.) which was dissolved in citrate buffer (pH = 4.4). The age matched control rats were given an equal volume of vehicle (citrate buffer). Diabetes was confirmed after 48 h of STZ injection by estimating plasma glucose levels using GOD/POD kit (Accurex®, India). The animals before induction of diabetes were considered as zero week for diabetic group.

2.2. Electrophysiological recordings

DRG neurons (L4–L6) were cultured as described previously with slight modifications from adult rat [16–18]. Whole-cell patch-clamp recordings were performed using Axopatch-200B amplifier (Axon Instruments, USA). Pipettes were fabricated from borosilicate glass and pipettes were polished by using microforge (Narishige, Japan) to give resistances of 1–2 MΩ. Data acquisition and pulse protocols were controlled with the pCLAMP-software (Axon Instruments, Foster City, USA) and digitized using analog/digital converter (Axon Instruments, USA). Experiments were performed at temperature (20 ± 2 °C) using a bipolar temperature controller (Harvard Apparatus, USA). Currents were filtered at 5 kHz and sampled at 20 kHz.

Na^+ currents were isolated using, the extra-cellular solution containing in (mM): NaCl, 65; choline chloride, 50; tetraethylammonium chloride, 20; KCl, 5; $CaCl_2$, 0.01; $MgCl_2$, 5; glucose, 5 and HEPES, 10 and the pH was adjusted to 7.4 by the NaOH and intracellular (pipette) solution contained in (mM): CsF, 110; $MgCl_2$, 5; EGTA, 11; NaCl, 10; HEPES, 10 and pH was adjusted to 7.2 by the CsOH. The osmolarity of these solutions was kept in the range of 310–325 mOsm/kg. The holding potential was maintained at −67 mV and currents were recorded at voltages between −57 and 63 mV with an increment of 5 mV steps [18–20]. A P/4 protocol was used for leak subtraction. Series resistance and whole-cell capacitance were read from the dials of patch clamp amplifier after cancelation of capacitive transient currents obtained during a small depolarizing test pulse and continuously monitored in all recordings.

2.3. Statistical analysis

All results are expressed as mean ± SEM. Statistical comparisons between two different treatment groups were performed by Paired/Unpaired Student's *t*-test and ANOVA. $P \leqslant 0.05$ was considered as statistically significant. Since neurons are varied in size, the values of current density were normalized by dividing the current amplitude (pA) by the whole cell capacitance. The normalized current was calculated by dividing the peak current. Analysis of digitized data traces was done using Clampfit 9.0 (Molecular Devices, USA).

Conductance–voltage (G–V) curves were constructed from I–V curves by dividing the peak evoked current by the driving force of the current, such *i.e.* $\{G = I/(V_m - V_{rev})\}$; where, V_m is the potential at which current was evoked and V_{rev} is the reversal potential for the current determined by extrapolating the linear portion of the I–V curve through 0 current. The test potential at which G is half of its maximal value (G_{max}) is termed $V_{0.5}$, and the slope factor of normalized conductance–voltage relationship is termed k. $V_{0.5}$ and k were determined from least squares fit to the data of a rising sigmoidal relationship *i.e.* $\{G/G_{max} = 1/1 + \exp(V_{0.5} - V)/k\}$; where, G/G_{max} is the normalized peak G and V is the test potential. The activation curve was fitted with a Boltzmann equation *i.e.* $\{G_{Na} = G_{max}/(1 + \exp[(V_{0.5} - V_m)/k]\}$; where, G_{max} is the maximum G_{Na}, $V_{0.5}$ is the potential, at which half of the Na^+ channels are activated and k is the slope factor.

2.4. Drugs and solution

Tetracaine was dissolved in the extra-cellular solution to prepare 10 mM stock solution. Stock solutions were stored in a freezer and thawed just before use. To obtain the desired concentration, drug dilutions were freshly prepared in extra-cellular solution from stock. Drug was applied near to patch cell after control Na^+ currents were recorded. All the chemicals used in this study were obtained from Sigma–Aldrich, USA unless mentioned otherwise.

3. Results

3.1. Plasma glucose levels

Two days after administration of STZ; 80% of the rats developed high blood glucose levels of 578 ± 53 mg/dl (32.1 ± 2.9 mmol/L), whereas control rats had normal glucose levels of 130.4 ± 2.2 mg/dl (7.2 ± 0.1 mmol/L) (Fig. 1A). The elevated glucose levels were consistent throughout the experimental period and significantly different from age matched control rats (Fig. 1A, $n = 5$–9, $P < 0.001$, Unpaired Student's *t*-test) during 2, 4, 6 weeks.

3.2. Altered nociceptive parameters in diabetic rats

3.2.1. Thermal hyperalgesia

After induction of diabetes the paw withdrawal latency of diabetic animal were assessed during 0, 2, 4, 6 week time points. At 2nd and 4th week diabetic rats, paw withdrawal latencies were around 13.8 ± 0.3, 14.1 ± 0.3 s respectively, as compared to age matched control rats, (14.2 ± 0.3, 14.3 ± 0.2 s respectively). At 6th week diabetic rats showed significant decrease in paw withdrawal latency 10.9 ± 0.6 s (Fig. 1B, $n = 5$–9; Unpaired Student's *t*-test, $P < 0.05$, *t*-test.) as compared to age matched control rats (13.8 ± 0.4 s), which showed that thermal hyperalgesia was induced.

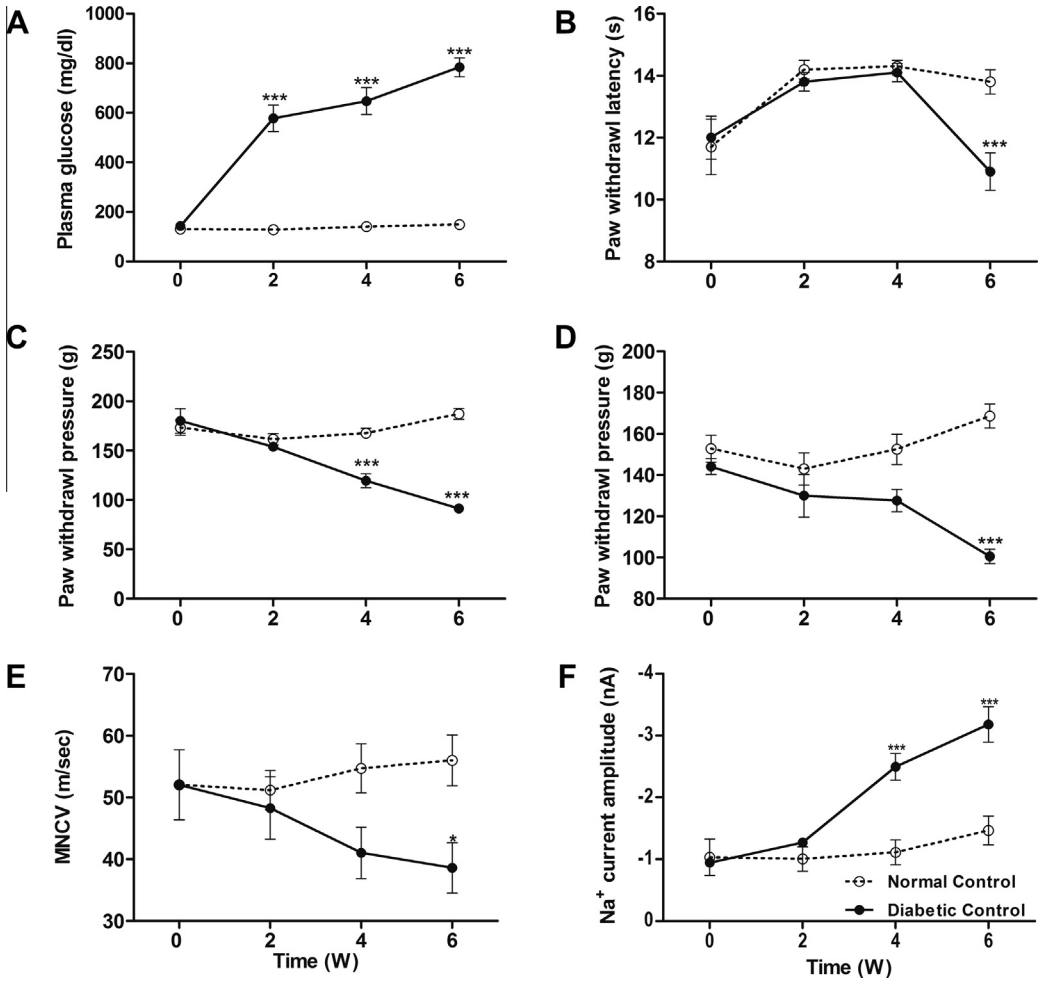

Fig. 1. A correlation between pain parameters and Na^+ current in time-dependent manner (W = week). (A) Plasma glucose levels in rats were increased after administration of streptozotocin (STZ) till the end of 6th week compared to age matched control. (B) Thermal hyperalgesia (Hargreaves Plantar test) of diabetic rats after administration of STZ was increased at the end of 6th week compared to age matched control. (C) Mechanical allodynia (Von Frey test) of normal control and diabetic control after administration of STZ were increased gradually till the end of 6th week compared to age matched control. (D) Mechanical allodynia (Randall Selitto test) of diabetic rats after administration of STZ was increased gradually till the end of 6th week compared to age matched control. (E) Deficit in motor nerve conduction velocities of rats were observed after administration of STZ gradually as compared to age matched control. (F) Na^+ current amplitude was increased after administration of STZ gradually as compared to age matched control. ***Significantly different from the age matched normal control rats ($P < 0.001$). *Significantly different from the age matched normal control rats ($P < 0.05$).

3.2.2. Mechanical allodynia

After induction of diabetes the paw withdrawal pressure of diabetic animal were assessed during 0, 2, 4, 6 week time points. At 4th week diabetic rats the Randall Selitto test has shown decrease in paw withdrawal pressure 127.7 ± 5.4 g as compared to control 152.5 ± 7.4 g, whereas for Von Frey test diabetic rats showed significant decrease in paw withdrawal up to 119.4 ± 7 g (Fig. 1C and D, $n = 5–9$ $P < 0.05$, Unpaired Student's t-test.) as compared to age matched control 167.7 ± 4.8 g. At 6th week time point the diabetic rats showed significant decrease in paw withdrawal up to 91.3 ± 3.8 g, 100.6 ± 3.5 g (Fig. 1C and D; $n = 5–9$; Unpaired Student's t-test, $P < 0.05$, t-test.) respectively for Von Frey test, Randall Selitto test as compared to age matched control rats 187.1 ± 5.6 g, 168.7 ± 5.9 g respectively.

3.3. Deficit in motor nerve conduction velocity of diabetic rats

The motor nerve conduction velocity was decreased up to 41.05 ± 4.17 m/s, as compared to age matched control rats 54.72 ± 3.9 m/s in 4th week diabetic rat. However at 6th week diabetic rat the conduction velocity had significantly decreased up to 38.6 ± 4.09 m/s; (Fig. 1E, $P < 0.05$, Unpaired Student's t-test) as compared to age matched control rats 56.0 ± 4.10 m/s.

3.4. Alteration of sodium current in diabetic rats

The Na^+ current were recorded from the diabetic rat small DRG neurons (< 25 μm) with whole cell capacitance 12.22 ± 1.5 pF and access resistance 0.89 ± 0.05 MΩ included in this study. The peak I_{Na} has been increased in a time-dependent manner (Fig. 1F, $n = 6$, Fig. 2A and B) from control to 2–6 weeks after induction of diabetes. 2nd week diabetic rat showed peak current amplitude of −1.26 ± 0.06 nA, 4th week diabetic rat showed significant increased peak current amplitude up to −2.49 ± 0.21 nA as compared to age matched control −1.10 ± 0.20 nA, in 6th week, diabetic rat showed significant increase in peak current amplitude up to −3.71 ± 0.28 nA from age matched control −1.46 ± 0.23 nA, (Fig. 1F, $n = 6$, Unpaired Student's t-test, $P < 0.05$). The current density was increased up to −127.6 ± 26.80 pA/pF in 4th week diabetic rat and −236.23 ± 12.82 pA/pF in 6th week diabetic rat as compared to age matched control −51.8 ± 8.1 pA/pF (Fig. 2C).

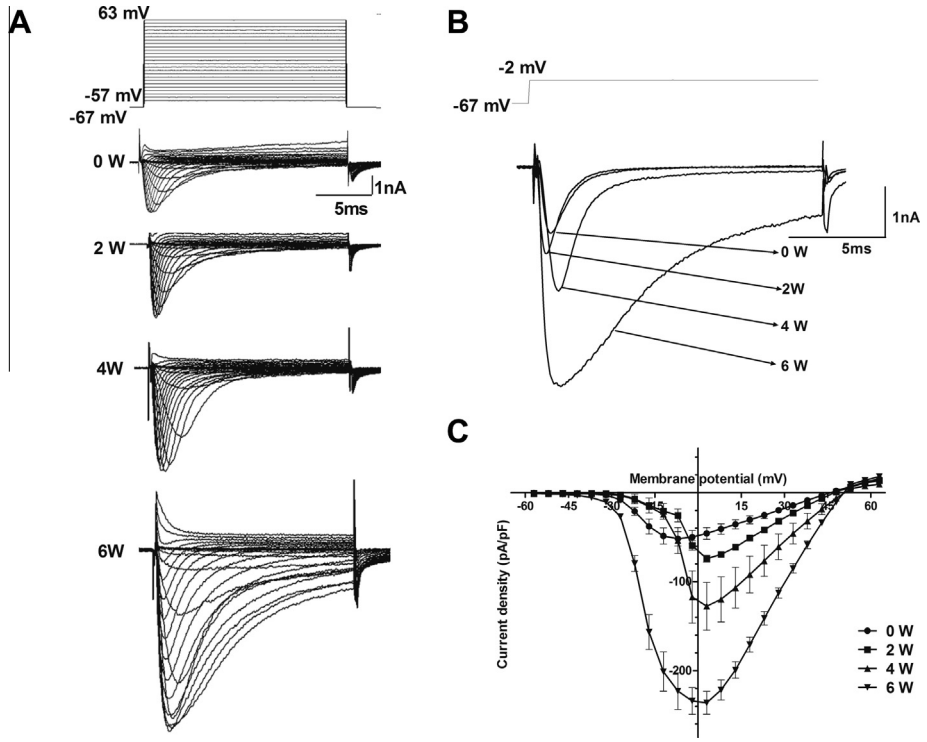

Fig. 2. Diabetic-induced alteration of Na^+ current from acutely dissociated DRG neurons. (A) Original tracings of the Na^+ current were evoked by voltage pulse from a holding potential of −67 mV and given a 15 ms test pulse to potentials between −57 and 63 mV with 5 mV increment; 0 W: (control rat); 2 W: (2nd week diabetic rat); 4 W: (4th week diabetic rat); 6 W: (6th week diabetic rat). Na^+ current was significantly increased as compared to 0 week (control) after administration of STZ. (B) Original tracings showing increased Na^+ current in time-dependent manner recorded from DRG neurons at −2 mV. (C) A time-dependent increase in Na^+ current density was observed after induction of diabetes.

3.5. Effects of tetracaine on sodium current

Exposure of tetracaine depressed Na^+ current of diabetic rat DRG neurons in a concentration-dependent manner (Fig. 3). The diabetic DRG neurons prior tetracaine exposure has shown peak current of −3.3 ± 0.41 nA. On exposure of 10 μM tetracaine the peak current was depressed to −2.6 ± 0.12 nA; whereas exposure of 30, 100 and 300 μM tetracaine the peak current has depressed significantly to −1.5 ± 0.16 nA, −0.8 ± 0.08 nA and −0.3 ± 0.05 nA ($P < 0.05$, $n = 6$, Paired Student's t-test).

The diabetic DRG neurons prior tetracaine exposure showed Na^+ current density of −215.1 ± 9.5 pA/pF at −2 mV. On exposure of 10 μM tetracaine the current density was depressed to −149.4 ± 7.2 pA/pF; whereas on exposure of 30, 100 and 300 μM tetracaine the current density has depressed significantly to −98.9 ± 14.8 pA/pF, 65.0 ± 8.1 pA/pF, −18.7 ± 6.3 pA/pF (Fig. 3, $P < 0.05$, One way ANOVA).

The sigmoid curve has shifted towards right and the G/G_{max} has depressed on exposure to tetracaine (Fig. 3C). The diabetic DRG neurons prior tetracaine exposure showed G/G_{max} of 0.80 ± 0.03. On exposure of 10 μM tetracaine the current density was depressed to 0.63 ± 0.02; whereas on exposure of 30, 100 and 300 μM tetracaine the current density has depressed to 0.36 ± 0.04, 0.16 ± 0.02, 0.06 ± 0.004 respectively. The biophysical parameters like $V_{0.5}$ (half maximal activation voltage) and K (slope factor) were reduced after exposure of tetracaine. After tetracaine exposure $V_{0.5}$ curve shifted −8 mV towards right (Supplementary Table 1).

The percent inhibition of Na^+ channel current on exposure of tetracaine shown to be increase with increasing concentrations of tetracaine. After fitting data to the Hill equation inhibitory curve was obtained (Supplementary Fig. 1C). At holding potential −57 mV the IC_{50} and hill coefficient were 31.91 ± 6.88 and 1.05 ± 0.188 μM, respectively and at −70 mV (with prepulse of −120 mV) IC_{50} and hill coefficient were 30.34 ± 6.88 and 0.9 ± 0.155 μM, respectively (Supplementary Fig. 1) .

4. Discussion

This study demonstrates a correlation between altered nociception behavior with increased Na^+ channel activity with progression of diabetes in STZ-induced diabetic rat model. Na^+ channel blocker (tetracaine) depressed the increased Na^+ channel activity due to hyperglycemia in a concentration-dependent manner. The elevated Na^+ current is the major cause of excessive firing DRG neurons and altered pain perception. To block the excessive firing of DRG neurons local anesthetics tetracaine can be employed which block the impulses by interfering with the function of Na^+ channels. Tetracaine activity was neither altered in a time-dependent manner nor in voltage-dependent manner under hyperglycemia.

Diabetic rats showed significant increase in thermal hyperalgesia and mechanical allodynia after six weeks of diabetic induction. Sciatic motor nerve conduction velocity was also significantly reduced in diabetic rats. In correlation to the pain parameters and nerve conduction velocity the Na^+ channel activity of DRG neurons has also altered. Na^+ current of 4th and 6th week diabetic rat were significantly increased as compared to normal control rats. The firing of DRG neurons is largely dependent on the density of Na^+ channels in the cell membrane [18,21]. It is evident that the expression and activity of voltage gated Na^+ channels is altered in diabetic sensory neurons [10,22]. At a molecular level, increase in Na^+ channel expression on sensory DRG neurons contribute to the decrease in pain threshold resulting in hyperalgesia [15]. We have shown direct evidence in a time-dependent manner for the

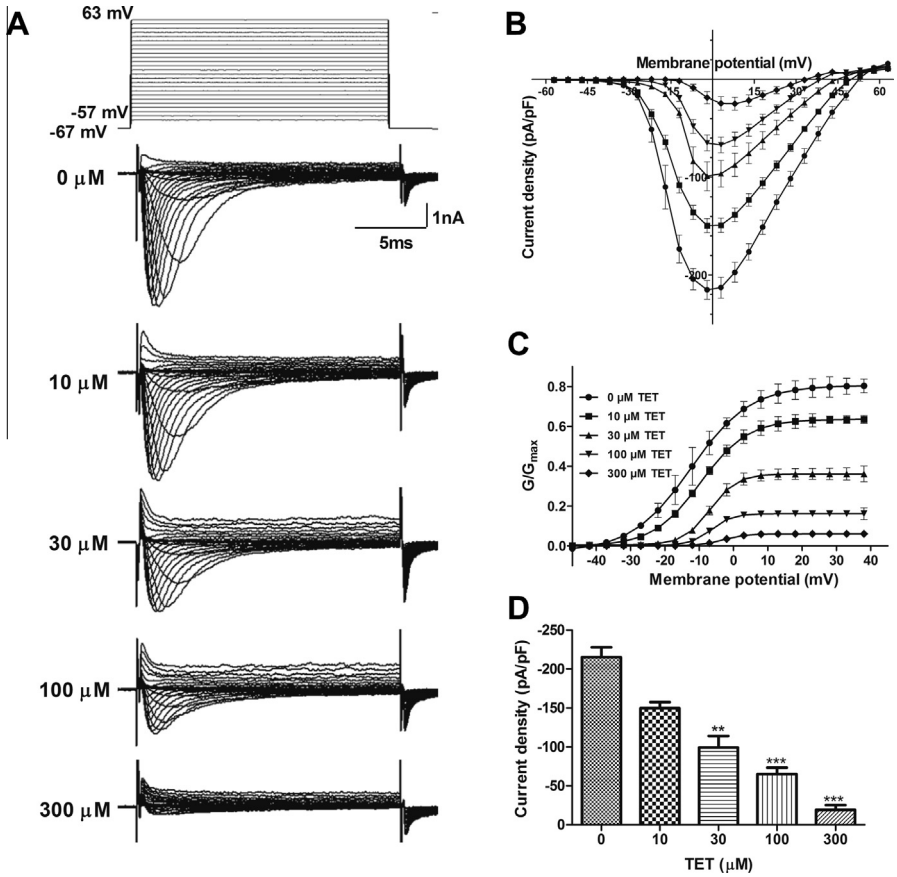

Fig. 3. Effects of the tetracaine on the Na^+ current from acutely dissociated DRG neurons of diabetic rat. (A) Original tracings of Na^+ current were evoked by voltage pulse from a holding potential of −67 mV and given a 15 ms test pulse to potentials between −57 and 63 mV with 5 mV increment; the concentration-dependant depression of Na^+ current were observed after exposure of tetracaine. (B) Graph was showing the relationship of current density and membrane potential after exposure of tetracaine. (C) A plot between G/G_{max} (control) and membrane potential the lines represent a fit to the Boltzmann equation showing rightward shift in conductance on tetracaine exposure. (D) Bar graphs were showing the significant depression of peak Na^+ current density in a concentration-dependent manner. ***Significantly different from the control ($P < 0.001$). **Significantly different from the control ($P < 0.01$).

progression of the diabetic neuropathy and the corresponding electrophysiological changes in Na^+ channel activity, strengthening that pain progression involves altered sodium channel activity.

About the site(s) or receptor(s) involved in Na^+ current suppression by tetracaine and, from our present knowledge of membrane composition and structure, the voltage-dependent gate of ion channels senses the voltage potential gradient within a membrane that is given by subtracting the outer surface potential from the transmembrane potential plus the inner surface potential. In presence of higher concentration of tetracaine (300 μM) Na^+ conductance activation curve was shifted right. In previous studies of local anesthetics benzocaine acting on voltage-dependent Na^+ channel in isolated squid giant axons, observed the opposite shifts in the voltage dependency of steady-state activation (right shift) and inactivation (left shift) [23]. In isolated single myelinated rat and frog nerve fibers, voltage shift were not observed by tocainide [24]. Lidocaine has been shown to produced 5 and 10 mV right voltage shift in *Xenopus oocytes* and rat isolated ventricular myocytes, respectively [25,26]. Another contribution to the membrane surface potential could be brought by calcium ions, entering the cell via Na^+/Ca^{2+} exchangers activated by the massive Na^+ influx in the absence of tetracaine. In the presence of tetracaine, a much lower Na^+ inflow would result in a lower subsequent Ca^{2+} inflow and a smaller positive surface charge density on the inner membrane leaflet, hence the requirement of a larger depolarization for half-activation and the resulting right voltage shift. A positive surface charge on the internal membrane leaflet would also explain the voltage shifts in opposite direction for voltage-dependent activation and steady-state inactivation, since larger hyperpolarization would be required to remove the positively charged gating particle from its binding site at the inner mouth of the permeation pathway. Tetracaine as a sodium channel blockers will block the motor nerve conduction velocity and as a local anesthetic, may lead to the some other complication; so the effects of tetracaine have not been recorded for the behavioral parameters. Local anesthetics mainly produce its effect by blocking peripheral nerve potential and impulse propagation in axons, which is sensitive to Na^+ channels [27]. Local anesthetics block impulses by interfering with the Na^+ channels function through selective inhibition of open and inactivated states of channel. Tetracaine inhibited I_{Na} in concentration-dependent manner. In the presence of tetracaine the I_{Na} has depressed and at sufficiently higher concentrations, enough Na^+ channels were impaired and fewer currents were produced. Our study provides, evidences for inhibition of I_{Na} in a concentration-dependent manner in same condition. In the presence of tetracaine, I_{Na} is decreased in hyperglycemic condition, and at a sufficiently high anesthetic concentration, enough Na^+ channels were impaired and fewer currents were produced. Moreover, tetracaine may not be useful in the neuropathic pain due to very short plasma half life, however, N-butyl tetracaine is reported to produce rapid onset and nerve impairment lasting in two weeks [28,29]. The prolonged functional

impairment by tetracaine analogy in the sensory nerves can last in two weeks or months but the sensory and motor functions may return when nerves fibers regenerate [28,29].

In conclusion, we clearly elucidated the role of Na^+ channel in neuropathic pain associated with diabetes in time-dependent manner. Tetracaine potentially blocked the Na^+ channel activity in diabetic sensory neurons by altering the excitability, delineating the most relevant mechanism for its efficacy as a local anesthetic.

Conflict of interest

None.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2014.09.035.

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