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

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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

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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.