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COMPARATIVE EVALUATION OF *IN VITRO* AND *IN VIVO* HIGH GLUCOSE-INDUCED ALTERATIONS IN VOLTAGE-GATED TETRODOTOXIN-RESISTANT SODIUM CHANNEL: EFFECTS ATTENUATED BY SODIUM CHANNEL BLOCKERS

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Abstract—Glucose uptake in neurons depends on their cellular/physiological activity and the extracellular concentration of glucose around the cell. High concentration of extra-cellular glucose, as under hyperglycemic conditions or pathological condition in diabetes, may persist for extended periods of time in neurons and trigger cellular damage by altering voltage-gated sodium channels (VGSCs), the exact mechanism of which remains unclear. Therefore, we hypothesized that high glucose may directly affect kinetics of the VGSCs in the dorsal root ganglion (DRG) neurons. DRG neurons were exposed to normal glucose (NG: 5.5 mM) and high glucose (HG: 30 mM) for 24 h. In another set of experiments, diabetic DRG neurons were also isolated from streptozotocin-induced diabetic rats. Effects of sodium channel blockers on nociceptive parameters and tetrodotoxin-resistant (TTX-R) Na^+ channel kinetics were elucidated by whole-cell patch-clamp in HG exposure and diabetes-induced rat DRG neurons. HG exposure and diabetes-induced DRG neurons demonstrated significant increase in TTX-R Na^+ current (I_{Na}) densities in comparison to the control. Both HG-exposed and diabetic DRG neurons demonstrated similar biophysical characteristics of I_{Na}. Lidocaine and tetracaine significantly decreased TTX-R I_{Na} density in a concentration- and voltage-dependent manner. Steady-state fast inactivation of I_{Na} was shifted in the hyperpolarizing direction whereas voltage-dependent activation was shifted in the rightward direction. Diabetic rats treated with lidocaine and tetracaine (3 mg/kg, i.p.) significantly improved thermal hyperalgesia, mechanical allodynia and motor nerve conduction velocity with a significant inhibition of TTX-R I_{Na} density as compared to the diabetic control. These results suggest that HG exposure increases the TTX-R Na^+ channel activity sensitive to Na^+ channel blockers, lidocaine and tetracaine.

Key words: diabetic neuropathy, dorsal root ganglion, high glucose, tetrodotoxin-resistant sodium channels, whole-cell patch-clamp.

INTRODUCTION

Voltage-gated sodium channels (VGSCs) generate and propagate action potential in dorsal root ganglion (DRG) neurons (Waxman, 2007). VGSCs are made up of a large pore-forming α-subunit composed of four repeated domains, each containing six transmembrane segments and the associated auxiliary β-subunits (Catterall and Yu, 2006; Patino et al., 2011). On the basis of tetrodotoxin (TTX) sensitivity; they have been classified as tetrodotoxin-sensitive (TTX-S: Na_v 1.1, 1.2, 1.3, 1.4, 1.6 and 1.7) and resistant (TTX-R: Na_v 1.5, 1.8 and 1.9). $Na_v1.5$ channel, a voltage-dependent cardiac sodium channel, is responsible for the depolarization of the cardiac cells (Abriel, 2007; Rook et al., 2012) whereas $Na_v1.8$ and $Na_v1.9$ are preferentially expressed in small diameter nociceptors that play a crucial role in the neuropathic and inflammatory pain conditions (Arbuckle and Docherty, 1995; Akopian et al., 1996; Sangameswaran et al., 1996; Porreca et al., 1999; Goldin et al., 2000). TTX-R $Na_v1.8$ channel produces slowly inactivating sodium current (I_{Na}) with relatively depolarized voltage-dependence of activation and inactivation, whereas $Na_v1.9$ channel elicits a persistent I_{Na} with a large overlap between activation and steady-state inactivation and a relatively hyperpolarized voltage-dependence (Cummins et al., 1999; Dib-Hajj et al., 1999a,b). $Na_v1.8$ channels play an important role in both inflammatory and neuropathic pain (Akopian et al., 1999; Lai et al., 2002; Thakor et al., 2009) and its selective blockade by A-803467 has been shown to attenuate allodynia and hyperalgesia in various animal models (Veneroni et al., 2003; Jarvis et al., 2007). $Na_v1.8$ mRNA and the protein levels are down regulated in the DRG neurons after the transaction of sciatic nerve and streptozotocin (STZ)-induced model of diabetic neuropathy with significant increase in the TTX-R I_{Na} density. The reason for such discrepancy is still unclear (Okuse et al., 1997; Dib-Hajj et al., 1998;

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Abbreviations: DRG, dorsal root ganglion; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; HG, high glucose; I_{Na}, sodium current; NG, normal glucose; STZ, streptozotocin; TTX, tetrodotoxin; VGSC, voltage-gated sodium channel.

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Novakovic et al., 1998; Lai et al., 2002; Hong et al., 2004). Therefore, we hypothesized that some structural and functional alterations may exist to correlate the TTX-R Na^+ channel kinetics and its expression in the extra-cellular HG-induced and the STZ-induced diabetic rat DRG neurons.

Sodium channel blockers like lidocaine and tetracaine generally blocks the impulse conduction and transmission in peripheral nerves by inhibiting the sodium channels in their inactivated state. Their putative binding site is supposed to be localized to the inner pore of the channel (mainly phenylalanine) in the S6 subunit of domain IV (Ragsdale et al., 1994). These therapeutics are useful in local anesthesia, analgesia (Vu, 2004), treatment of epilepsy and cardiac arrhythmia (Liu and Wood, 2011). They influence the transmission of sensory impulses by altering the resting membrane potential, timing of repolarization after an action potential, neuronal excitability and neuronal firing pattern (McCleskey, 2007; Park et al., 2009). An exact role of lidocaine and tetracaine on the modulation of TTX-R sodium channel in diabetic neuropathy is still unclear. Therefore, in this study we have compared the effects of hyperglycemia in the absence of insulin on the TTX-R sodium channel kinetics in the DRG neurons from *in vitro* [normal glucose (NG; 5.5 mM) and high glucose (HG; 30 mM)] and *in vivo* conditions [neurons isolated from non-diabetic (ND) and STZ-induced diabetic rats]. Additionally, we have also evaluated the effects of systemic treatment with lidocaine and tetracaine on nociceptive parameters in rats.

EXPERIMENTAL PROCEDURES

Animals

Adult Sprague–Dawley male rats (12–15 weeks; 250–275 g) were procured from the Central Animal Facility, National Institute of Pharmaceutical Education and Research, S.A.S. Nagar and the experiments were approved by the Institutional Animal Ethics Committee. Rats were maintained in a climate-controlled and sound isolated room (21 °C) under a 12:12-h light:dark cycle, with 40–60% relative humidity and were fed on *ad libitum* provision of feed (pellets) and water. Care was taken to restrict the number of animals to the minimum possible.

STZ-induced model of diabetic neuropathy

Diabetes was induced by STZ (50 mg/kg; ip) and the confirmation of diabetes was made by collecting blood samples from the tail vein after 48 h. Rats with a plasma glucose level more than 250 mg/dl (> 15 mM) were considered diabetic. The plasma glucose measurements were carried out after the induction of diabetes at specific intervals i.e., 0th week; before start of sixth week and end of eighth week using GOD/POD kit as described elsewhere (Kharatmal et al., 2015a). We assessed the functional (motor nerve conduction velocity and nerve blood flow) and the behavioral (thermal hyperalgesia and mechanical allodynia) parameters for different experimental groups as per our laboratory protocols described elsewhere (Negi et al., 2009, 2011a,b; Negi and Sharma, 2015). For *in vivo* experiments, 3 mg/kg doses of each drug were administered intraperitoneally for two weeks after the sixth week of the development of diabetic neuropathy in rats.

DRG neuron culture

DRG neurons were isolated as per our laboratory protocol described elsewhere (Singh et al., 2013; Meerupally et al., 2014). Briefly, L_4–L_6 DRG were aseptically removed from the spinal column of the anesthetized rats and were dissociated in the collagenase–trypsin solution (1 mg/ml each) for 20 min. The tissues were gently triturated with fire-polished Pasteur pipettes and then the cells were plated onto the poly-D-lysine (0.1 mg/ml) coated 35-mm culture plates and were incubated at 37 °C with 95% relative humidity and 5% CO_2. The cell diameter was visually determined using a calibrated reticule in the light path of the microscope during recording, using an approximate average of the longest and the shortest dimensions of the cells that deviated slightly from spherical shape. The small-diameter DRG neurons (20–25 μm) were selected for the electrophysiological recordings. The DRG neurons were exposed to normal glucose (NG; 5.5 mM) and high glucose (HG; 30 mM) for short-term duration of 24 h (Vincent et al., 2005; Singh et al., 2013).

Electrophysiological recordings

All the recordings were conducted at room temperature (~21 °C) within 24 h on the small-diameter (⩽25 μm) DRG neurons using Axopatch 200B amplifier (Molecular Devices, CA, USA) as per the laboratory protocol described elsewhere (Singh et al., 2009, 2013; Meerupally et al., 2014; Kharatmal et al., 2015a,b). The extra-cellular solution contained (in mM): NaCl, 65; choline chloride, 50; tetraethylammonium chloride, 20; KCl, 5; $CaCl_2$, 0.01; $MgCl_2$, 5; glucose, 5; HEPES, 10 and TTX, 0.0003 and the pH was adjusted to 7.4 by 5 N Tris base. The pipette solution contained (in mM): CsF, 110; $MgCl_2$, 5; EGTA, 11; NaCl, 10 and HEPES, 10 and the pH was adjusted to 7.2 by the addition of 5 N CsOH. Osmolarity of the solutions remained in the range of 300–320 mOsm/L. After the addition of extra-cellular glucose, the osmolarity of the medium was not altered. The pipette resistance was approximately 1 MΩ. All the recordings were conducted only when seal resistance > 1 GΩ and series resistance < 10 MΩ.

In activation protocol, currents were elicited by each step of depolarization from −57 to +63 mV in 5 mV increments for 15 ms from a holding potential of −70 mV. A pre-pulse protocol comprised of each step of depolarization from −50 to +60 mV in 5 mV increments for 30 ms from a holding potential of −70 mV with pre-pulse of −120 mV for 10 ms. To measure the steady-state fast inactivation of the TTX-R Na^+ channel, the cells were held at −80 mV, stepped to the inactivating pre-pulse potentials from −110 to −10 mV (in 5 mV increments) for 250 ms, before stepping the cells to 0 mV for 10 ms to measure the available current after the inactivation. In the DRG neurons exposed to the NG