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Hypoxia and high glucose activate tetrodotoxin-resistant Na^+ currents through PKA and PKC

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Voltage-gated sodium channels are critical for the initiation and propagation of action potentials and for the regulation of neuronal excitability. Hyperglycemia and hypoxia are two main changes in diabetes frequently associated with several complications. Although many studies on streptozotocin-induced diabetic rats indicate that early diabetic neuropathy is associated with increased amplitude and faster kinetics of sodium channels, the distinctive roles of high glucose and hypoxia have not been completely clarified. Here we show that hypoxic and high glucose conditions (overnight exposure) increase activation and inactivation of TTX-R I_{Na} in DRG neurons without affecting the level of expression. Hypoxia and high glucose alone were potent enough to induce similar or even greater sensitization than when both conditions were present, without any of them having a predominant effect. PKA is mainly responsible of the one condition effect, while under both hypoxia and high glucose PKC was also contributing to alter the kinetics, although not in a cumulative manner. These data indicate that TTX-R I_{Na} is significantly modulated under short-time exposure to hypoxia and high glucose, a mechanism which might be relevant for diabetes-related complications or other diseases associated with acute hypoxia.

Key words: high glucose, hypoxia, phosphorylation, PKC, PKA, TTX-R sodium channels

INTRODUCTION

High glucose is considered the leading causative factor of diabetes and of the pain associated with diabetic neuropathy (Dobretsov et al. 2001, Obrosova 2009). However, hypoxia might also have a significant earlier contribution as it is suggested by the lowered oxygen tension in diabetic arterial wall and sciatic nerve that precedes the formation of specific lesions (Zochodne et al. 1994) and the reduced transcutaneous oxygen pressure in diabetic patients before any symptoms of macro-vascular disease or diabetic neuropathy (Iwase et al. 2007). Similar redox, metabolic and pathophysiological changes evoked by either condition raise the possibility that high glucose and hypoxia may interact *via* common metabolic pathways to initiate and/or exacerbate complications of diabetes. Additive effects of hypoxia and high glucose were reported to be important for promoting the onset and progression of diabetic retinopathy (Nyengaard et al. 2004) and a cross-coupling between the two processes has been suggested as a new mechanism for the development of chronic complications of diabetes (Catrina et al. 2004). In a previous study, we showed that hypoxia rather than high glucose has the critical role in the sensitization of TRPV1 channels expressed in cultured DRG (Ristoiu et al. 2009), a new mechanism that could be of interest for other channels that are involved in the excitability of the peripheral neurons.

One possible target are the voltage-gated sodium channels, large trans-membrane proteins that contribute to the hyperexcitability of dorsal root ganglia (DRG) neurons associated with pain. Based on differential sensitivity to tetrodotoxin (TTX), sodium currents in the DRG neurons are classified into TTX-sensitive (TTX-S) and TTX-resistant (TTX-R). Small neurons, which are likely to be nociceptive in nature and are associated with C-fibers or thinly myelinated A-δ fibers, express primarily TTX-R channels $Na_v1.8$ and $Na_v1.9$, neurons of intermediate size exhibit both

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TTX-S and TTX-R Na^+ currents, whereas large DRG neurons typically associated with myelinated A-δ fibers have been shown to predominantly express TTX-S channels such as $Na_v1.1$, $Na_v1.2$ and $Na_v1.6$ with some TTX-R $Na_v1.8$ expression (Rush et al. 1998, Ekberg and Adams 2006). Alterations in sodium channels composition and expression in DRG sensory neurons are frequently associated with inflammatory and neuropathic pain states, including diabetic neuropathy. An increased amplitude and negative shift of voltage-dependent activation and steady-state inactivation curves of TTX-S and TTX-R sodium currents were observed in both small (Hong et al. 2004) and large (Hong and Wiley 2006) DRG neurons from diabetic rats, suggesting an implication in the pain associated with diabetic neuropathy. Studies on streptozotocin-induced diabetic rats showed that the abnormal functioning of sodium channels starts to develop early and persists during the whole period of diabetes (Hirade et al. 1999), being associated with a significant up-regulation of mRNA for the $Na_v1.3$, $Na_v1.6$ and $Na_v1.9$ and a down-regulation of $Na_v1.8$ mRNA, from 1 to 8 weeks after the onset of allodynia (Craner et al. 2002).

Based on these observations we examined the hypothesis that TTX-R I_{Na+} could be sensitized by exposure, to hypoxia and high glucose, in combination or alone. Our study shows that upon *in vitro* overnight exposure, TTX-R sodium channels are faster activating and inactivating in a PKA and PKC-dependent manner.

METHODS

DRG neurons culture

Adult male Wistar rats (150-200 g) were anesthetized by inhalation of 100% CO_2 (2 min exposure) followed by decapitation. The procedures were conducted in accordance with the Guidelines of University of Bucharest regarding the care and use of animals for experimentation. DRGs from the L1-L5 lumbar region were bilaterally removed as previously described (Ristoiu et al. 2002). Briefly, they were incubated for 1h at 37ºC in IncMix solution containing (in mM): NaCl 155; KH_2PO_4 1.5; HEPES 5.6; NaHEPES 4.8; glucose 5 and 50 µg/ml gentamicin, to which 1 mg/ml collagenase (type XI, Sigma) and 1 mg/ml dispase (nonspecific protease, Gibco) were added. Neurons were dissociated by trituration and plated for 1h on 35 mm culture Petri dishes (Corning) treated with poly-D-Lysine (0.1 mg/ml, 30 min) and were cultured at 37ºC in a NGF-free 1:1 mixture of DMEM and Hams's F10 medium with 10% horse serum and 50 µg/ml gentamicin. For the experiments, the cells were exposed overnight (18-20 hours) to hypoxia [4% O_2 (Nyengaard et al. 2004) and 5% CO_2 in nitrogen] and high glucose (25 mM glucose; Hong et al. 2004). For normal conditions, the cells were maintained in normoxia (5% CO_2 and 7% O_2) and normal glucose (7.4 mM glucose; Scott et al. 1999). In some experiments, cells were exposed to 7% P_{O2} and 25 mM glucose conditions, or 4% P_{O2} and 7.4 mM glucose conditions. Both normal and hypoxic conditions were generated using a two-gas incubator (MCO-5AC, Sanyo, Japan).

Solutions and drugs

The extracellular solution contained (in mM): NaCl 35, $MgCl_2$ 5, $CaCl_2$ 0.1, CholineCl 65, TEA 30, HEPES 10 (pH 7.4 with NaOH at 25ºC) to which glucose was added on the day of the experiment. Reduced extracellular Na^+ was required to reduce the magnitude of sodium currents to improve the fidelity of the voltage clamp (Hong et al. 2004). The intracellular solution contained (in mM): CsCl 80, $MgCl_2$ 2, $CaCl_2$ 1, TEA 30, EGTA/NaOH 3/6, HEPES 10, Mg_2ATP 2 mM, Li_2GTP 1 mM (pH 7.2 at 25ºC with CsOH) and filtered at 0.2 µm immediately before use. When PKC, PKA and p38 MAPK involvement was tested, 1 µM of specific inhibitors Chelerythrine chloride (1 mM stock in water), H-89 (1 mM stock in water) or SB203580 (1 mM stock in DMSO) was added into medium and incubated overnight with the cells. If not otherwise mentioned, all the reagents were purchased from Sigma.

Electrophysiological recordings

Sodium currents were recorded in the whole-cell patch-clamp configuration using a WPC-100 amplifier (ESF Electronic Göttingen, Germany) at 25ºC. Electrodes (2-5 MΩ when filled with intracellular solution) were pulled from borosilicate glass pipettes (GC150T, Harvard Apparatus) using a vertical puller (WPI, Germany). After formation of a tight seal (>3 GΩ), membrane resistance, series resistance and capacitance (C_m) were determined, and the series resistance was compensated 75-80% with the amplifier circuitry as necessary. The recordings were conducted

only when the access resistance was lower than 10 MΩ.

To explore the voltage dependence of sodium currents we took advantage of the previously described activation and inactivation properties of peripheral nerve Na^+ currents (Flake et al. 2004, Lopez-Santiago et al. 2006). Thus, a protocol with 500 ms pre-pulse to -110 or -40 mV, followed by an 80 ms test pulse from -65 to +45 mV in 5 mV increments was applied to generate current-voltage (*I-V*) curves. Current evoked from -40 mV was considered to be TTX-R I_{Na} while the difference between the current evoked from -110 and -40 mV was considered to be TTX-S I_{Na} (Gold et al. 1996, Flake et al. 2004, Lopez-Santiago et al. 2006). Because of the steady-state availability properties of the two currents, the voltage step to -40 mV completely inactivates TTX-S I_{Na}, while TTX-R I_{Na} is still fully available for activation. This protocol has the advantage of obtaining separate *I-V* relationships for both TTX-R and TTX-S sodium currents in the absence of TTX, thus saving time and reducing cell deterioration (Lopez-Santiago et al. 2006).

For current density measurements, the currents were divided by the cell capacitance (C_m) as read from the amplifier. 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 that $G=I/(V_m-V_{rev})$, 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 (Flake et al. 2004). The activation curve was fitted with a Boltzmann equation of the following form: $G_{Na} = G_{max}/(1+\exp[(V_{1/2}-V_m)/k]$, where G_{max} is the maximum G_{Na}, $V_{1/2}$ is the potential at which half of the sodium channels are activated and k is the slope factor.

Steady-state inactivation was measured by applying a double-pulse protocol, consisting of a 1000 ms pre-pulse ranging from -110 mV to 0 mV (in 10 mV increments), followed by a test pulse to 0 mV to test the fraction of the maximum Na^+ current remaining after each pre-pulse. A mean of all data sets (plots of peak I_{Na} during the 0 mV test pulse *vs* pre-pulse voltage) was fit with a Boltzmann equation of the following form: $I_{Na} = I_{max}/(1+\exp[(V_{1/2}-V_m)/k]$ where I_{max} is the maximal value for I_{Na}, V_m is the membrane potential achieved using a pre-pulse potential, $V_{1/2}$ is the potential at which I_{Na} is half inactivated and k is the slope factor. Data were filtered at 3 kHz (low-pass Bessel 3-pole filter with WPC-100 amplifier) and sampled at 10 kHz with pClamp 8.1 software (Axon Instruments, USA).

Analysis was performed using pClamp 8.1, Origin 6.0 (Microcal Software Inc., USA) and Prism 3.02 (GraphPad Software Inc., USA) software. All data were expressed as means ± SEM. Statistical analyses were performed using one way ANOVA and two-tailed Student's *t*-test, the significance being assessed at $p<0.05$.

Real-time transcription-PCR

In order to quantify the expression levels of different voltage activated Na^+ channels in rat, the total RNA was extracted using the GenElute Mammaliam Total RNA MiniPrep Kit (RTN70, Sigma) according to the manufacturer's instructions. RNA concentrations were determined by spectrophotometry. Reverse transcription was performed using the High-Capacity cDNA Archieve Kit (Applied Biosystems). The relative abundance of Na_v isoform transcripts was assessed by quantitative real-time reverse transcription-polymerase chain reaction (real-time RT-PCR) using TaqMan methodology and the ABI Prism 7300 Sequence Detection System (Applied Biosystems). Primer and probe sets (Applied Biosystems) for rat genes of interest (Rn00578439_m1 for Nav 1.1, Rn00565438_m1 for Nav 1.3, Rn00570506_m1 for Nav 1.6, Rn00591020_m1 for Nav 1.7, Rn00568393_m1 for Nav 1.8, Rn00570487_m1 for Nav 1.9) and the housekeeping gene GAPDH (4352338E) were used in accordance to the manufacturer's guidelines. The mRNA quantity of the investigated genes was analyzed in triplicate, normalized against GAPDH, and expressed in relation to a calibrator sample (control cell cultures) using the comparative Ct method (Livak and Schmittgen 2001).

RESULTS

Overnight exposure to hypoxia and high glucose is associated with altered kinetics of TTX-R sodium current

The cells expressing TTX-R Na^+ channels represented 69% (20 of 29) in control conditions and 82% (27 of 33) under hypoxia and high glucose conditions. Using the cell capacitance (C_m) as read from the amplifier, assuming a specific $C_{m(s)}$ of 1 μF/cm² and that the

cells have a spherical shape, the cell-surface area and diameter was estimated from the formula $C_m = C_{m(s)}4\pi r^2$. Based on these criteria, DRG neurons expressing TTX-R currents were assigned as small, with a mean diameter of 30.42±1.37 µm (n=20) in normal conditions and 29.32±1.10 µm (n=27) in hypoxia/high glucose conditions (Petruska et al. 2000). Total I_{Na}, the sum of TTX-S and TTX-R I_{Na} (Rush et al. 1998) was recorded using a series of depolarizing voltage commands with a pre-pulse to -110 mV for 500 ms from a holding potential of -80 mV. The maximum current density of total I_{Na} was -48.00±3.85 pA/pF at -5 mV in normal cells and -52.36±5.82 pA/pF at -10 mV in neurons under hypoxia and high glucose (p>0.05). When the *I-V* protocol with a pre-pulse to -40 mV was applied, the TTX-R current was obtained (Fig. 1A). The peak current density of TTX-R I_{Na} was -38.81±3.50 pA/pF (n=20) in normal conditions and -39.40±5.47 pA/pF (n = 27) in hypoxia and high glucose conditions at the same voltage steps as for the total I_{Na}, without any statistical difference (p>0.05), (Fig. 1B). Since the current-voltage relationship showed a shift towards the

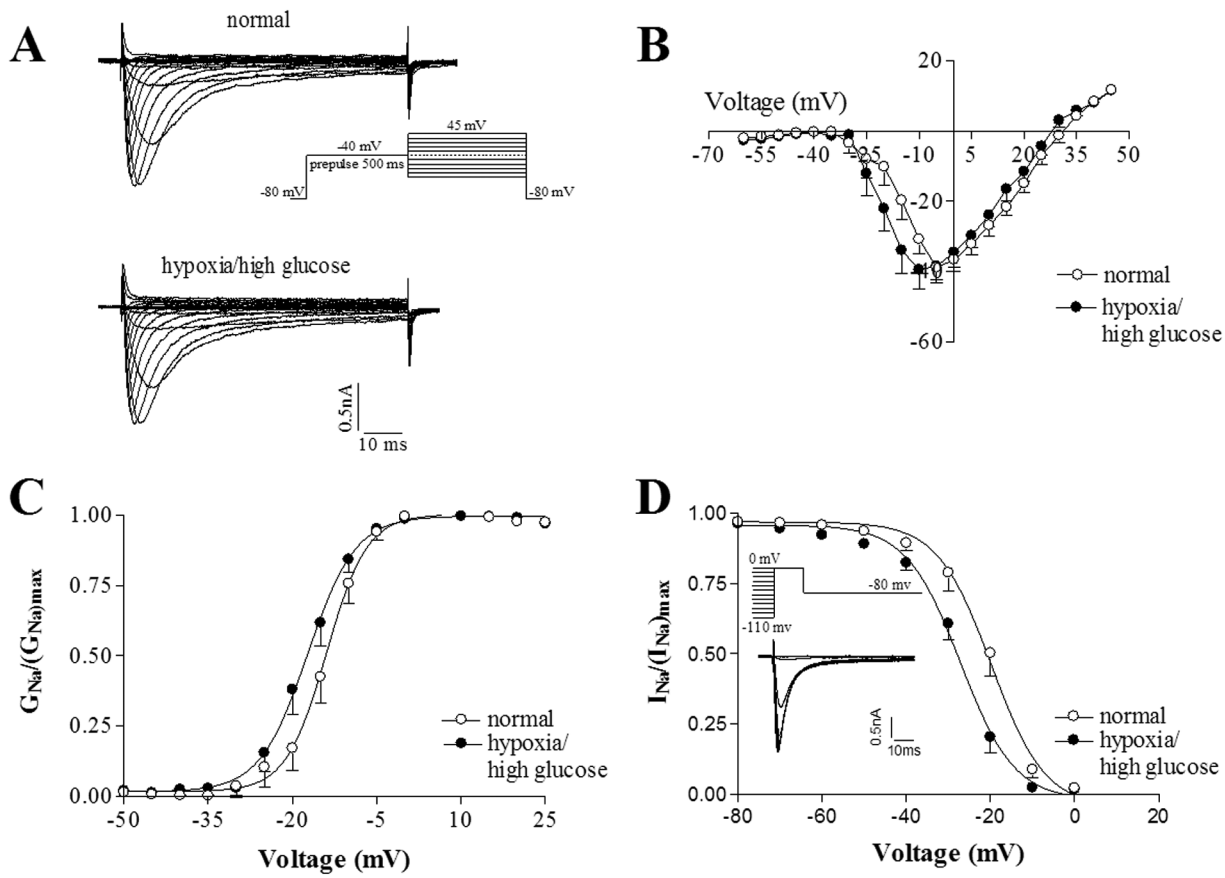

Fig. 1. Changes of activation and inactivation properties of TTX-R I_{Na} in DRG neurons under hypoxia and high glucose. (A) Typical traces of TTX-R I_{Na} generated in voltage-clamp mode under normal (*top*) or hypoxia and high glucose (*bottom*) conditions. Cells were voltage-clamped at -80 mV and the currents were elicited by 80 milliseconds voltage steps ranging between -65 and +45 mV following a 500 ms conditioning step to -40 mV. (B) Average peak I_{Na} density-voltage relationships for TTX-R current shifted leftward in neurons exposed to hypoxia and high glucose compared to normal neurons, but there is no difference in the amplitude. (C) Normalized to G_{Max} TTX-R I_{Na} conductance-voltage curve fitted with a single Boltzmann equation shows a significant shift of ~3 mV in the hyperpolarizing direction under hypoxia and high glucose condition compared to normal. (D) Steady-state inactivation obtained with a voltage step at 0 mV after 1000 ms pre-pulse ranging from -110 mV to 0 mV (*inlet*) has a significant negative shift in neurons under hypoxia and high glucose (p<0.001, student t-test). Error bars indicate SEM.

hyperpolarizing direction under hypoxia and high glucose, we determined the kinetics of activation by fitting the rising phase of the conductance curve using a single exponential Boltzmann equation as shown in Fig. 1C. The midpoint of the voltage-dependence of activation was -17.28±0.20 mV in neurons under hypoxia and high glucose conditions, significantly more negative than the value obtained for the control neurons (-13.94±0.21 mV) (p<0.001).

For the steady-state inactivation we used a double-pulse protocol and took advantage of the differences in the voltage-dependence of inactivation of TTX-S channels compared to TTX-R channels. Since TTX-S I_{Na} (fast) is inactivated at more negative voltages than TTX-R I_{Na} (slow) (Lopez-Santiago et al. 2006), we measured the TTX-R I_{Na} inactivation curve at the peak of the I_{Na} evoked following a -40 mV pre-pulse. Each data set was normalized to the maximum current evoked by the first pulse preceded by the initial -40 mV pre-pulse and the mean of individual curves was fit with a single Boltzmann equation as shown in the method section. The midpoint of steady-state inactivation was -27.13±0.8 mV (n=16) in neurons under hypoxia and high glucose conditions, and significantly more depolarized in normal conditions -19.79±0.99 mV (n=13, p<0.001, Fig. 1D). These results suggest that overnight exposure to hypoxia and high glucose is a potent condition to induce a significant TTX-R Na^+ channels sensitization.

To examine whether the sensitization of Na^+ current under hypoxic/high glucose condition was due to an increase of the level of expression, and if yes, to which TTX-R Na^+ channel isoform, we performed a real-time PCR analysis. The fold change of mRNA coding for the TTX-S ($Na_v1.1$, $Na_v1.3$, $Na_v1.6$, $Na_v1.7$) and TTX-R ($Na_v1.8$ and $Na_v1.9$) channels which are known to be present in the DRG neurons (Cummins et al. 2007), indicated no difference between the two experimental conditions (Fig. 2A). The predominant Na^+ channels were TTX-R $Na_v1.8$ (Fig. 2B). This observation, together with the fact that the voltage-dependence of steady-state inactivation of the TTX-R Na^+ current is closer to the one of $Na_v1.8$ TTX-R current ($V_{1/2}$ = -30 mV) than to that of $Na_v1.9$ ($V_{1/2}$ = -45 mV) or of the fast-inactivating TTX-sensitive currents ($V_{1/2}$ = -70 mV) (Dib-Hajj et al. 2002, Patrick-Harty and Waxman 2007), made us conclude that most of the TTX-R Na^+ current in our experiments was carried by the $Na_v1.8$ isoform. The lack of change of the mRNA expression level between the two experimental conditions combined with the same current densities values, suggest that the sensitization of the TTX-R Na^+ channels was not dependent on changes in the expression levels.

Hypoxia or high glucose alone is sufficient to sensitize TTX-R Na^+ channels

To investigate the mechanisms underlying the TTX-R Na^+ current sensitization, we tested the effect of hypoxia or high glucose alone on the kinetics of the channel and compared to the responses in normal conditions as shown in Fig. 1. The midpoint of voltage-dependent activation was -16.62±0.20 mV (n=5) for cells cultured under hypoxia (4% O_2) with normal (7.4 mM) glucose condition, and -16.74±0.82 mV (n=5) for cells cultured under high glucose (25 mM) with normal oxygen (7%) (Fig. 3A). The ~3 mV shift towards negative values was significantly different compared to normal (p<0.001) and very similar to the condition

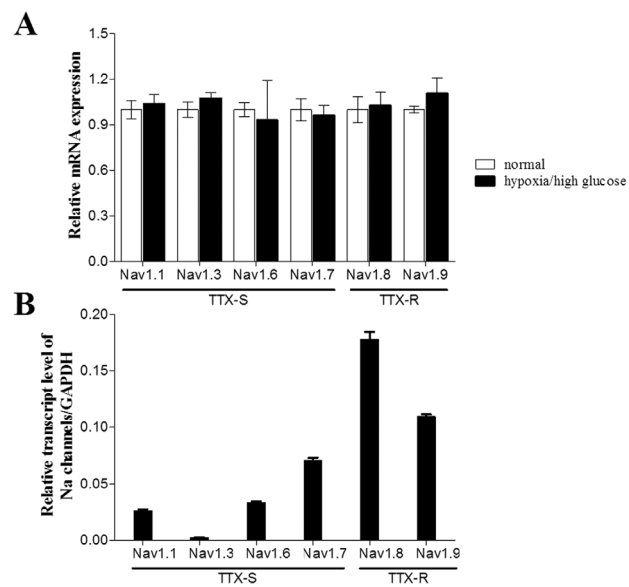

Fig. 2. Effect of overnight exposure to hypoxia and high glucose on Na_v mRNA levels. Level of sodium channels mRNA was determined by quantitative RT-PCR and normalized using GAPDH as a reference gene. Data are mean ± SEM of 3 samples per experimental condition (1 animal/sample), containing mRNA derived from pooled L1-L5 DRGs in one animal and analyzed separately in triplicate. (A) Transcriptional profile after overnight exposure to hypoxia and high glucose showed no significant difference for all the Na_v isoforms. (B) The predominant Na_v isoform was $Na_v1.8$.

when both hypoxia and high glucose were present (Fig. 1C), suggesting that only hypoxia or high glucose alone are sufficient to induce a change of TTX-R activation kinetics. The midpoints of steady-state inactivation were -32.37±1.69 mV (n=5) for cells cultured under only hypoxia condition, and -28.37±1.52 mV (n=5) for cells cultured under only high glucose condition (Fig. 3B). The values are significantly different compared to normal (p<0.001) and indicate a stronger effect on TTX-R Na^+ inactivation than when both conditions (hypoxia and high glucose) are present (Fig. 1D). These results suggest that hypoxia and high glucose are both important for TTX-R Na^+ current sensitization which can be induced by modulating either activating or inactivating mechanisms of the channel.

PKC and PKA are involved in the TTX-R Na^+ sensitization under hypoxia and/or high glucose

To further investigate through which mechanism hypoxia and high glucose, alone or in combination, might modulate the TTX-R Na^+ channel kinetics, we tested the effects of the kinases known to be involved in the sensitization of the channel. PKC and PKA modulate the peripheral nerve sodium channels (Gold et al. 1998, Vijayaragavan et al. 2004), and are activated under diabetic conditions (Hayase et al. 2007) or by oxygen deprivation (O'Reilly et al. 1997). Mitogen activated protein kinase (MAPK) p38 has an important role in the nervous system modulating $Na_v1.8$ and $Na_v1.6$ channels (Hudmon et al. 2008, Wittmack et al. 2005). It is activated by hypoxia (Maulik et al. 2008) and has a role in the etiology of diabetic neuropathy (Purves et al. 2001). Previously we showed that hypoxic/high glucose conditions promote significantly membrane translocation of PKCε which is inhibited by overnight incubation with 1 μM chelerythrine chloride (Ristoiu et al., unpublished data). Similarly, in these experiments we tested the effect of overnight incubation with 1 μM of Chelerythrine chloride which inhibits the translocation of all PKC isoforms, 1 μM H-89 to inhibit PKA and 1 μM SB203580 to inhibit p38 MAPK.

Under hypoxia and high glucose condition, the $V_{1/2}$ potential for activation was rightward shifted up to -13.64±0.99 mV (n=6) and -13.82±0.25 mV (n=5) due to Chel C and H-89 treatment (p<0.001, ANOVA test) (Fig. 4A) and had no effect under SB203580 p38 inhibitor (-15.78±0.8552, n=5) (data not shown) compared to the initial hypoxia and high glucose condition (Fig. 1C). Under hypoxia or high glucose alone ChelC had no effect on the midpoint of voltage-dependent activation, while H-89 significantly reduced it up to -13.06±0.77 mV, (n=10, p<0.001, ANOVA test) under hypoxia and up to -14.26±0.44 mV (n=8, p<0.05, ANOVA test) under high glucose (Fig. 4A).

For the inactivation, under both hypoxia and high glucose ChelC and H-89 elicited a $V_{1/2}$ rightward shift up to –21.24±1.55 mV, (n=6) and -19.94±0.64 mV, (n=5) (Fig. 4B), significantly different compared to the initial

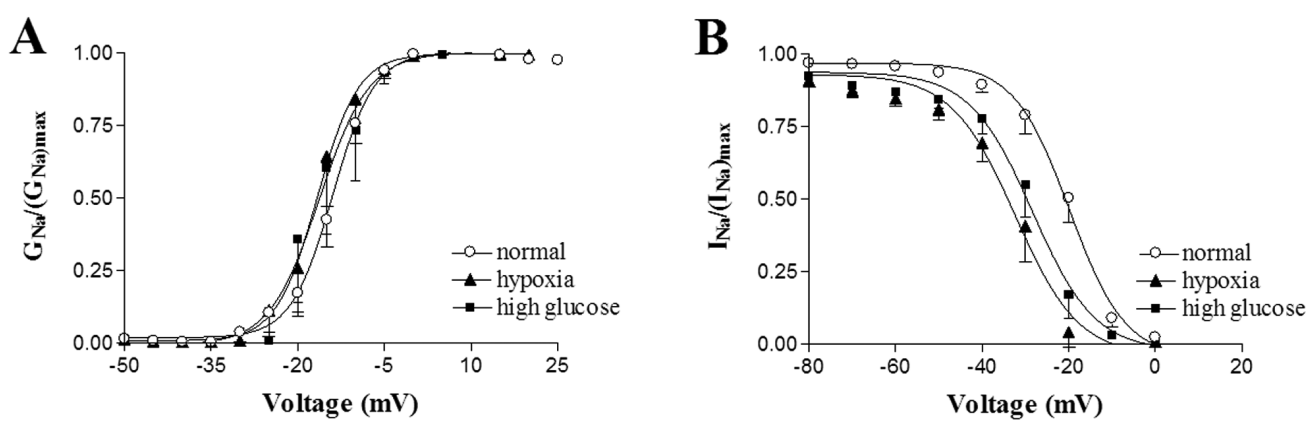

Fig. 3. Hypoxia and high glucose alone sensitize TTX-R Na^+ channels. (A) Normalized conductance-voltage relationship of TTX-R I_{Na} shows a hyperpolarizing shift under hypoxia or high glucose condition alone of the same amplitude (~3 mV) as under both hypoxia and high glucose (p<0.001, student t-test). (B) The voltage-dependence of TTX-R I_{Na} inactivation significantly shifted in the negative potential direction by either hypoxia or high glucose compared to the normal cells (p<0.001, student t-test). Error bars indicate SEM.

hypoxia and high glucose condition (Fig. 1D), (p<0.001, ANOVA test). ChelC didn't have any effect on the mid-point of the steady-state inactivation under hypoxia but significantly blocked the PKC activation under high glucose (-22.86±0.25 mV, n=7, p<0.01, ANOVA test), while H-89 significantly reduced it up to -24.49±0.25 mV (n=9) under hypoxia and up to -21.19±0.30 mV (n=8) under high glucose (Fig. 4B) (p<0.001, ANOVA test). These results suggest that hypoxia and high glucose alone sensitize TTX-R channels mainly through PKA, while a combination of hypoxia and high glucose modulates activation and inactivation kinetics of Na^+ channels through both PKA and PKC, without having cumulative effects.

DISCUSSION

Voltage-gated sodium channels are critical for the initiation and propagation of action potentials and for the regulation of neuronal excitability (Chahine et al. 2005). In the present study, using an experimental model previously developed by us (Ristoiu et al. 2006), we investigated if hypoxic and high glucose cellular environment affects sodium channels expression and function, which might therefore contribute to increased excitability in the early stages of diabetes. After overnight exposure of rat DRG neurons to a combination of hypoxia (4% O_2) and high glucose (25 mM) conditions, we found significant changes of TTX-R sodium channels kinetics, which were not associated with altered mRNA level of expression. In small DRG neurons, TTX-R I_{Na} isolated with a pre-pulse to -40 mV showed a significant faster activation and inactivation under hypoxia and high glucose condition compared to normal (7% O_2 and 7.4 mM glucose) (Fig. 1C, D), even though the current amplitude was not increased. To induce these kinetic changes, the combination of hypoxia and high glucose was not absolutely required since the exposure to hypoxia and high glucose alone was enough to activate the TTX-R I_{Na} in the same proportion as when both conditions were present (Fig. 3A), while the effect on the inactivation was even stronger than the exposure to both conditions (Fig. 3B). These data suggest a different effect on the activation and inactivation gate of the channel, which raise the possibility of very subtle modulations of sodium channels activity depending on the metabolic change. The sensitization of these channels through a hyperpolarized shift of activation and inactivation is predicted to enhance the neurons excitability by decreasing the

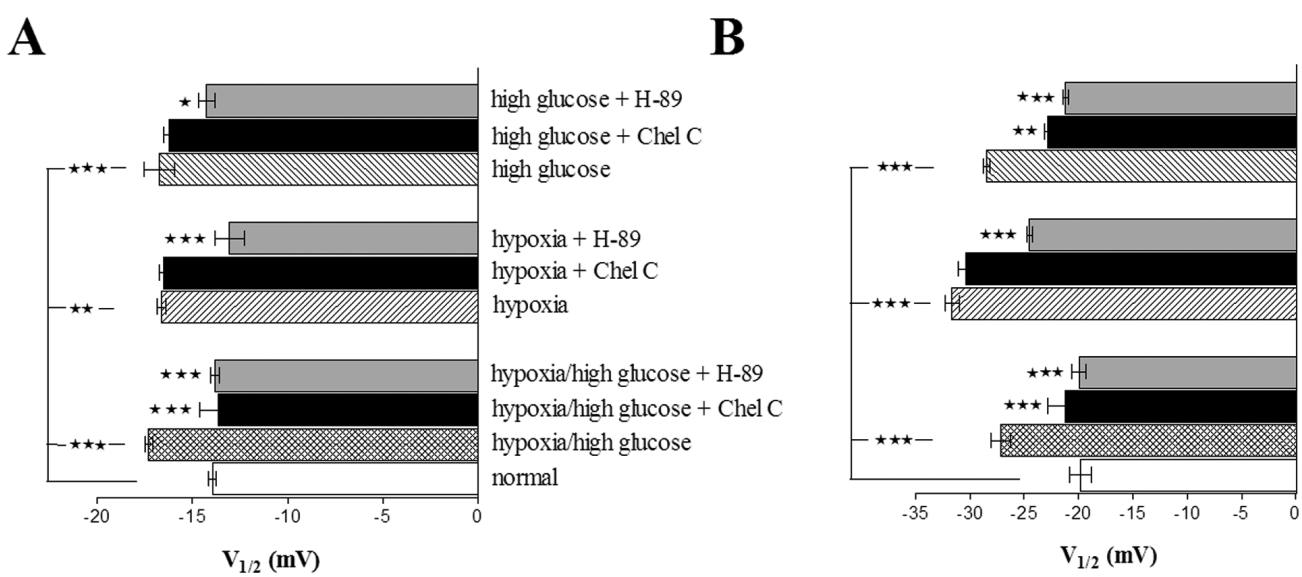

Fig. 4. Hypoxia and high glucose sensitize TTX-R Na^+ through PKA and PKC. Potential of half activation (A) and inactivation (B) of TTX-R I_{Na} in neurons exposed to hypoxia and high glucose, alone or in combination, in the absence or presence of ChelC (Chelerythrine chloride) and H-89 inhibitors of PKC and PKA respectively. Note that the legend for each row in the activation graph is also valid for the inactivation graph. ***p<0.001, **p<0.01 and *p<0.05 indicate the level of significance difference as calculated by ANOVA test between an experimental condition *vs* normal condition (see connecting lines) or between an experimental condition with inhibitors *vs* without inhibitors. Error bars are SEM.

threshold and increasing the likelihood of action potential firing, which eventually generates pain. The sensitization can occur rapidly (within minutes) by post-translational regulation *via* phosphorylation (Woolf and Costigan 1999) or slowly (from hours to days) via transcriptional regulation (Cheng and Ji 2008). In our study we showed that the sensitization was done in a phosphorylation-dependent manner, with PKA predominantly involved when only one condition was present and PKC contributing when both conditions were present (Fig. 4A, B).

$Na_v1.8$ and $Na_v1.9$ are the two TTX-R I_{Na} which are expressed primarily in the small DRG neurons. The TTX-R I_{Na} recorded in our study was predominantly $Na_v1.8$ as identified by the specific protocol (Flake et al. 2004, Lopez-Santiago et al. 2006) and the inactivation properties (Dib-Hajj et al. 2002, Patrick-Harty and Waxman 2007). No $Na_v1.9$ current was recorded in the present study because of the ultraslow inactivation these channels undergone during the -110 mV pre-pulse applied to remove the fast inactivation of TTX-S currents (Huang and Song 2008) which yielded a total TTX-R current minus the $Na_v1.9$ current. $Na_v1.8$ has been shown to contribute most of the sodium current underlying the upstroke of action potential (Renganathan et al. 2001) and to have a very important role in inflammatory and, although controversial, in neuropathic pain (Cummins et al. 2007). In contrast to the robust and almost universal up-regulation of $Na_v1.3$ in various neuropathy models, the $Na_v1.8$ mRNA and protein level are significantly reduced in most, but not all, *in vivo* models of neuropathic pain (Rogers et al. 2006). Under diabetic conditions, an increased TTX-R sodium current due to a higher degree of phosphorylation has been shown in DRG neurons (Hirade et al. 1999, Hong et al. 2004), while the level of mRNA and protein expression decreased by 25% in streptozotocin-induced diabetic rats (Craner et al. 2002). In contrast to these results, our data shows that under hypoxia and high glucose conditions the $Na_v1.8$ mRNA level (Fig. 2) or the TTX-R current density (Fig. 1B) does not change, while the voltage-dependence of activation and inactivation (Fig. 1C, D) was shifted towards hyperpolarizing values. These data suggest that short-term exposure to hypoxia and/or high glucose induce the sensitization of the TTX-R Na^+ channels not by changing the expression level, but by fast post-translational regulation.

TTX-R I_{Na} in DRG neurons are modulated by several kinds of intracellular signaling molecules, including PKA and PKC which were frequently associated with increased TTX-R I_{Na} kinetics accompanied or not by higher peak amplitude (Gold et al. 1996, 1998, Vijayaragavan et al. 2004, Ikeda et al. 2005). PKA and PKC can have convergent or divergent effects on sodium channels (Denac et al. 2000, Cantrell et al. 2002) and PKC might be necessary to enable the PKA-mediated effect (Gold et al. 1998). Analyses of $Na_v1.2$ brain channels have shown that the cytoplasmic loop between domains DI & DII and DIII & DIV possesses several shared PKA and PKC phosphorylation sites, while the inactivation gate has a unique site for PKC phosphorylation (Chahine et al. 2005). Differences in trafficking might explain why activation of PKA potentiates the currents of some Na^+ channels isoforms ($Na_v1.9$ and $Na_v1.8$ which are TTX-R) but not others ($Na_v1.2$ and $Na_v1.7$, which are TTX-S) (Chahine et al. 2005, Liu et al. 2009). For PKC, the effects could be more subtle, since more isoenzymes have been described. Among them, PKCβ isoform has potentiating effects on TTX-R I_{Na} under diabetic conditions (Hayase et al. 2007), while PKCε can reduce or potentiate $Na_v1.8$ in rat DRG neurons (Vijayaragavan et al. 2004, Cang et al. 2009) . Under our experimental conditions, when hypoxia or high glucose alone were present, only PKA was activated with significant effects on both activation and inactivation, while PKC was only slightly activated under high-glucose (Fig. 4). When both conditions were present, PKC becomes activated with no potentiating effect on activation (Fig. 1C, 3A), but with a limiting effect on inactivation (Fig. 1D, 3B). In contrast to previous studies where PKC activity is necessary to enable the expression of PKA-mediated effects (Gold et al. 1998), in our study PKC activation requires PKA activity but PKA-induced modulation of TTX-R I_{Na} does not require PKC activity. Given our duration of exposure (18-20 h) compared to other acute application studies (Gold et al. 1998, Ikeda et al. 2005, Cang et al. 2009), it is highly possible that a different degree of phosphorylation at the different sites involved in activation and inactivation is responsible for the specific effects we have noticed.

In a previous study we have investigated the effect of the same conditions on the TRPV1 channels expressed in native and heterologous systems and noticed that hypoxia is the critical factor of TRPV1 sensitization in a PKCε- and HiF-1α-dependent manner (Ristoiu et al., unpublished data). The PKCε translocation from cytosol to the membrane was prevented by chelerythrine, a

process coupled with Hif1-α activation possibly through a ROS producing mechanism. In the present study however, the situation is more complex, with both hypoxia and high glucose having an effect without any predominant role for any of them. PKCβ isoform that potentiate TTX-R I_{Na} under diabetic conditions (Hayase et al. 2007) can be activated by high glucose which elevate diacylglycerol (DAG) and consequently activate PKC (Inoguchi et al. 1992). There are no data about the effect of hypoxia on this particular isoform or about high glucose effect on the PKCε translocation which can also potentiate $Na_v1.8$ in rat DRG neurons (Cang et al. 2009). However, it is possible that under hypoxia and high glucose both PKCε and PKCβ isoformes translocate to the membrane, contributing to the TTX-R I_{Na} increased activation and inactivation, although not in a cumulative manner compared to PKA effect. High glucose stimulates increased PKA activity through increased cAMP (Zhang et al. 2000), which can increase $Na_v1.8$ trafficking (Liu et al. 2009) and thus potentiate the current. To our knowledge, there are no other studies on the effect of hypoxia on the TTX-R I_{Na} in the DRG neurons, although there are some data showing that hypoxia can directly activate PKA in cortical neurons or bone cells (Semenov et al. 2008, Chao and Xia 2010, Zhang et al. 2010). Our data so far, doesn't support the involvement of certain kinase isoformes to the observations we have made. More detailed analyses at molecular level will be required to confirm their contribution and identify the specific role of PKA and PKC subtypes in the modulation of sodium channels activity under short-term exposure to hypoxia and/or high glucose.

CONCLUSIONS

A short-term exposure to hypoxia and high glucose alone or in combination is sufficient to cause functional changes of the TTX-R sodium channels in DRG neurons. This happens mainly through a PKA-dependent mechanism under hypoxia or high glucose alone or a PKA and PKC combination effect when both conditions are present. The predominant effect of PKA compared to PKC is a new and interesting observation which could be relevant for DRG neurons activity in the first stages of diabetes. A further study on the effect of progressive longer exposure to these conditions will help to understand the interplay between these kinases and the possible functional consequences for neurons excitability.

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