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Changes in osmolality modulate voltage-gated sodium channels in trigeminal ganglion neurons

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Abstract

Voltage-gated sodium channels (VGSCs) are important channels which participate in many physiological functions. Whether VGSCs can be modulated by changes in osmolality in trigeminal ganglion (TG) neurons remains unknown. In this study, by using whole-cell patch clamp techniques, we tested the effects of hypo- and hypertonicity on VGSCs in cultured TG neurons. Our data show that tetrodotoxin-resistant sodium current (TTX-R current) was inhibited in the presence of hypo- and hypertonic solutions. In hypertonic solutions both voltage-dependent activation and inactivation curves shifted to the hyperpolarizing direction, while in hypotonic solutions only inactivation curve shifted to the hyperpolarizing direction. Transient Receptor Potential Vanilloid 4 receptor (TRPV4) activator mimicked the inhibition of TTX-R current by hypotonicity and the inhibition by hypotonicity was markedly attenuated by TRPV4 receptor blocker and in TRPV4^{-/-} mice TG neurons. We also demonstrate that inhibition of PKA selectively attenuated hypotonicity-induced inhibition, whereas antagonism of PLC and PI3K selectively attenuated hypertonicity-induced inhibition. We conclude that although hypo- and hypertonicity have similar effect on VGSCs, receptor and intracellular signaling pathways are different for hypo- versus hypertonicity-induced inhibition of TTX-R current.

Keywords

osmolality; voltage-gated sodium channels; TRPV4 receptor; intracellular signaling pathway; nociception

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Introduction

Osmolality may play an important role in regulating neuron excitability in the nervous system (Schwartzkroin et al., 1998). It has been reported that hippocampal excitability can be modulated by the complex actions exerted by changes in extracellular osmolality (Baraban and Schwartzkroin, 1998). Recently, in nociceptors throughout the body there is evidence that both hypo- and hypertonic stimuli can induce nociceptor hyperexcitability, increase its activity or sensitize nociceptors, indicating that changes in osmolality could be involved in the nociception by modulating neurons excitability in the peripheral nervous system (Alessandri-Haber et al., 2003; Alessandri-Haber et al., 2004; Alessandri-Haber et al., 2005). Voltage-gated sodium channels (VGSCs), which underlie the depolarization of action potential (AP) and determine the threshold and amplitude of AP, are important ion channels regulating the biophysical properties of nociceptive neurons (Amir et al., 2006; Waxman et al., 1999). Studies have demonstrated that VGSCs participate in nociceptive signal transduction in primary sensory neurons (Chahine et al., 2005; Wood et al., 2004). *In vivo*, hyperosmolar solutions selectively block APs in rat sensory myelinated fibers (Matsuka and Spigelman, 2004), implying that VGSCs may be involved in anisotonicity-induced nociception. However, whether VGSCs can be modulated by anisotonicity in sensory neurons is still unknown.

Transient Receptor Potential Vanilloid 4 (TRPV4) receptor is an important osmotic cellular sensor (Liedtke and Kim, 2005; Ramsey et al., 2006) and expressed in brain as well as in dorsal root ganglion (DRG) and trigeminal ganglion (TG) (Alessandri-Haber et al., 2003; Suzuki et al., 2003). Besides hypotonic stimulus, TRPV4 can also be activated by heat, phorbol ester compounds (4 α -PDD), and the metabolite of arachidonic acid (Liedtke and Kim, 2005; Voets et al., 2002; Vriens et al., 2004). Both *in vivo* and *in vitro* studies have shown that activation of TRPV4 participates in nociceptive behavior evoked by anisotonic stimuli. For example, TRPV4 has been reported to be necessary for nociceptive behavior elicited by subcutaneous injection of mild hypertonic and hypotonic saline solutions (Alessandri-Haber et al., 2005; Alessandri-Haber et al., 2004; Alessandri-Haber et al., 2003).

Based on the sensitivity to tetrodotoxin (TTX), VGSCs are divided into TTX-resistant (TTX-R) and TTX-sensitive channels (TTX-S). TTX-R channels, including Na v 1.8, and Na v 1.9 isoforms, are preferentially expressed in small- and medium-sized TG and DRG neurons and involved in pain transduction (Amaya et al., 2000; Kim and Chung, 1999; Novakovic et al., 1998). VGSCs are modulated by several intracellular signaling pathways (Chahine et al., 2005). In primary sensory neurons, PKA and PKC systems are two pathways which have been extensively studied. The two pathways are activated by many biological compounds, such as prostaglandin E $_2$, bradykinin and serotonin, and then enhance the function of TTX-R channels inducing inflammatory hyperalgesia (Cardenas et al., 1997; Gold et al., 1998; Ikeda et al., 2005; Smith et al., 2000). Other pathways such as PKG (Renganathan et al., 2002) and lipid cascade (Hourez et al., 2005) have also been reported to participate in modulating the function of VGSCs. Our previous study shows that anisotonicity can sensitize capsaicin evoked current (I_{caps}) via different intracellular pathways (Liu et al., 2007). In the present study, we tested the effect of anisotonicity on TTX-R current in TG neurons and then explored whether TRPV4 receptor and specific intracellular pathways were involved in the effect of hypo- and hypertonicity.

Materials and Methods

Cell culture

Trigeminal ganglion (TG) neurons from male Sprague–Dawley rats (180–200 g) and mice (C57BL/6 wild type and TRPV4 knockout) were cultured as described previously (Liu et al., 2006). Briefly, trigeminal ganglia were dissected aseptically and collected in modified Hank's

Balanced Salt solution (mHBSS). After being washed in mHBSS, the ganglia were diced into small pieces and incubated in mHBSS for 20–40 min at 37 °C with 0.1% collagenase (Type XI-S). Individual cells were dissociated by triturating them through a fire-polished glass pipette, followed by a 10 min incubation at 37 °C with 10 µg/ml DNase I (Type IV) in F-12 medium (Life Technologies, Gaithersburg, MD) and centrifuged for 5 min at 1500 rpm/min. After centrifuging three times, the cells were cultured in F-12 supplemented with 10% fetal bovine serum. The cells were plated on poly-D-lysine coated glass coverslips (15 mm diameter) and cultured for 24 h at 37 °C in a water saturated atmosphere with 5% CO₂. The cell diameter (µm) was measured with a calibrated eyepiece under phase contrast illumination.

Care of animals conformed to standards established by the National Institutes of Health. All animal protocols were approved by the Duke University Institutional Animal Care and Use Committee. All efforts were made to minimize animal suffering and to reduce the number of animals used.

Patch clamp recordings

All experiments were carried out at room temperature (22–23 °C). Whole-cell patch clamp recordings were obtained using an Axopatch-200B patch clamp amplifier (Axon Instruments, Foster City, CA) and the output was digitized with a Digidata 1322A converter (Axon Instruments). The sampling rate was 10 kHz and filtered at 5 kHz. The capacitance and series resistance were compensated $\geq 90\%$. Data obtained from neurons in which uncompensated series resistance resulted in voltage-clamp errors > 5 mV were not taken in further analysis. Liquid junction potentials were compensated before patching. When the osmolality of external solutions was changed from 300mOsm to hypotonicity or to hypertonicity, measurements of the changes in liquid junction potentials were less than 2 mV and were not corrected.

The voltage-dependent activation curve (G–V curve) was measured by 20 ms depolarizing pulses from -80 to $+40$ mV stepping by 5 mV with interval of 2s. The voltage-dependent inactivation curve (inactivation–voltage curve) was obtained by double pulses: precondition pulses (20ms) were from -100 to $+20$ mV in 5 mV steps and following 0 mV test pulse (20 ms) with interval of 4s. For recording 4 α -PDD-induced current, the holding potential was -60 mV. Other experiments, unless stated, the holding potential was -80 mV.

For voltage-clamp experiments, the resistance of the glass pipettes (No. 64-0817(G85150T-3), Warner Instruments Inc., Hamden, CT, USA) was 1–2 M Ω when filled with pipette solution composed of (in mM): CsF 135, NaCl 10, CaCl₂ 1, MgCl₂ 2, EGTA 10, HEPES 10, Tris-ATP 5 at pH 7.3 and osmolality 300mOsm. The external solution was composed of (in mM): NaCl 30, KCl 5, MgCl₂ 3, TEA-Cl 20, Choline-Cl 35, 4-AP 3, D-Mannitol 106, HEPES 10, TTX 0.0002 at pH 7.4 and osmolality 300mOsm. The use of this low Na solution was to reduce the magnitude of the rapidly activating sodium currents to overcome possible voltage errors that may arise due to poor space clamp during the voltage command. Hypo- and hypertonic external solutions were obtained by adjusting the concentration of D-Mannitol. When recording 4 α -PDD-induced current the external solution was “standard external solution” composed of (in mM): NaCl 147, KCl 5, MgCl₂ 1, CaCl₂ 2, D-glucose 10, HEPES 10 at pH 7.4 and osmolality 300mOsm. The osmolality was measured using a vapor pressure osmometer (Model 3300, Advanced Instruments, Norwood, MA).

Data analysis

The amplitude of TTX-R current was calculated as peak current. Data were analyzed using pClamp (Axon Instruments) and SigmaPlot (SPSS Inc., Chicago, IL, USA) software. All of data were presented as mean $\pm$ S.E.M. and the significance was indicated as $P < 0.05$ (*) and $P < 0.01$ (**) tested by paired or unpaired Student's *t*-tests. G–V curve and inactivation–voltage

curve were fitted by Boltzmann functions, which $G/G_{\max} = 1/(1 + \exp((V_{0.5} - V_m)/k))$ or $I/I_{\max} = 1/(1 + \exp((V_{0.5} - V_m)/k))$, with $V_{0.5}$ being membrane potential (V_m) at which 50% of activation or inactivation was observed and k being the slope of the function. The dose-response curve was fitted by Hill equation, in which $I_{\text{peak}} = I_{\text{peakmax}}/[1 + (IC_{50}/C)^n]$, with n as the Hill coefficient, and IC_{50} as the concentration producing 50% inhibition.

Chemicals

Cell culture materials were purchased from GIBCO (Life Technologies, Rockville, MD, USA). KT-5823, U73122 (1-[6-((17 β -3-Methoxyestra-1,3,5(10)-trien-17-yl) amino) hexyl]-1H-pyrrole-2,5-dione), D(-)Mannitol and 4 α -PDD (4 α -phorbol-12,13-didecanoate) were obtained from CALBIOCHEM (San Diego, CA, USA) and others, unless stated, all came from Sigma Chemical Company.

4 α -PDD, KT-5823, U73122, Wortmannin, pCPT-cAMP (8-(4-Chlorophenylthio)-adenosine 3',5'-cyclic monophosphate sodium salt), pCPT-cGMP (8-(4-Chlorophenylthio)-guanosine 3',5'-cyclic monophosphate sodium salt), BIM (Bisindolylmaleimide II), PMA (Phorbol 12-myristate 13-acetate), LY294002 (2-(4-Morpholinyl)-8-phenyl-1(4H)-benzopyran-4-one hydrochloride), H-89 (N-[2-(p-Bromocinnamylamino)ethyl]-5-isoquinolinesulfonamide dihydrochloride) and KT5720 ((9S,10S,12R)-2,3,9,10,11,12-Hexahydro-10-hydroxy-9-methyl-1-oxo-9,12-epoxy-1H-di indolo[1,2,3-fg:3',2',1'-kl]pyrrolo[3,4-i][1,6] benzodiazocine-10-carboxylic acid hexyl ester) were prepared as stock solutions in DMSO. The final concentration of DMSO in the bath chamber or pipette solution was <0.1%.

KT5823, H-89, U73122, Wortmannin, LY294002 were present in the pipette solution, while 4 α -PDD, pCPT-cAMP, pCPT-cGMP, BIM, PMA, KT5720 were applied in the bath solution.

Results

Effect of anisotonic stimuli on TTX-R current in rat TG neurons

In this study, rat TG neurons having projected soma diameters ranging between 15 and 30 μm were used to explore the effect of anisotonic stimuli on TTX-R current.

Hypotonic stimuli—Fig. 1A shows when the external solution was changed from isotonicity (300mOsm) to hypotonicity (260mOsm) TTX-R current was inhibited by $31.7 \pm 7.2\%$ from -137.4 ± 20.0 pA/pF to -92.9 ± 17.6 pA/pF ($n=17$, paired t -test, $P<0.05$). The inhibition by hypotonicity was largely reversible and recovered to -131.5 ± 22.3 pA/pF after hypotonicity was washed out for 3 min. We also found that G–V curve did not markedly shift before and during hypotonic treatment (paired t -test, $P>0.05$) (Fig. 1C).

Fig. 1D shows the effect on inactivation–voltage curve when the extracellular solution was changed from 300mOsm to 260mOsm. In the presence of hypotonicity, the inactivation–voltage curve markedly shifted to the hyperpolarizing direction (paired t -test, $P<0.05$).

Hypertonic stimuli—As seen in Fig. 2A, TTX-R current was also inhibited when the external solution was changed from isotonicity (300mOsm) to hypertonicity (330mOsm). On the average, TTX-R current was inhibited by $38.8 \pm 8.5\%$ from -140.1 ± 20.7 pA/pF to -83.1 ± 10.7 pA/pF ($n=16$, paired t -test, $P<0.01$). Different from the inhibition by hypotonicity, the inhibition of TTX-R current by hypertonicity was irreversible and the amplitude was -90.4 ± 9.2 pA/pF after a 3 min washout. Also different from the inhibition by hypotonicity, both G–V curve (Fig. 2C) and inactivation–voltage curve (Fig. 2D) shifted to the hyperpolarizing direction during hypertonic treatment (paired t -test, $P<0.05$).

The dose–response curve of the inhibition by anisotonic stimuli was presented in Fig. 2E. Since 260mOsm and 330mOsm exhibited maximal or near maximal inhibition of TTX-R current and since these changes in tonicity were modest, these concentrations were used in all subsequent experiments.

Effect of TRPV4 agonist, 4 α -PDD, on TTX-R current

TRPV4 receptors are osmosensitive receptors and have been shown to be activated in anisotonic media (Liedtke et al., 2000; Liedtke, 2005). To test the hypothesis that TRPV4 receptors may be involved in the modulation of TTX-R current by anisotonicity, we tested whether 4 α -PDD, the agonist of TRPV4 receptor (Watanabe et al., 2003), could mimic the inhibition by hypotonicity or hypertonicity. As shown in Fig. 3A, after exposed to 4 α -PDD (3 μ M) for 3 min, TTX-R current was reduced by $50.3 \pm 7.2\%$ from -138.3 ± 24.7 pA/pF to -65.0 ± 9.5 pA/pF ($n=11$, paired t -test, $P<0.01$). Moreover, the response was recoverable after washout. In the presence of 3 μ M 4 α -PDD, there was no shift in G–V curve (paired t -test, $P>0.05$) (Fig. 3C). However, 4 α -PDD caused a hyperpolarizing shift in the inactivation–voltage curve (paired t -test, $P<0.05$) (Fig. 3D). The concentration–dependent inhibition of TTX-R current by 4 α -PDD is shown in Fig. 3E. TTX-R current was inhibited $6.1 \pm 1.9\%$ (from -140.1 ± 11.7 pA/pF to -131.7 ± 10.5 pA/pF, $n=6$, paired t -test, $P>0.05$) and $69.2 \pm 6.8\%$ (from -139.9 ± 13.3 pA/pF to -43.9 ± 7.1 pA/pF, $n=6$, paired t -test, $P<0.01$) by 0.01 and 100 μ M 4 α -PDD, respectively. The dose–response curve was fitted by Hill equation with IC_{50} being 2.4 μ M.

Effect of TRPV4 antagonist, ruthenium red, on TTX-R current in anisotonic conditions

To further determine whether TRPV4 receptor was involved in the effects of hypo and hypertonicity, ruthenium red (RR), a non-specific TRPV4 receptor antagonist (Voets et al., 2002) was used to determine how it would affect TTX-R current under isotonic (300mOsm), hypo- (260mOsm) and hypertonic conditions (330mOsm). In isotonic condition, after exposure to 10 μ M RR for 3 min, TTX-R current was reduced by $12.1 \pm 3.7\%$ from -136.9 ± 16.1 pA/pF to -120.9 ± 15.0 pA/pF ($n=8$, paired t -test, $P<0.05$). The inhibition of TTX-R by hypotonicity was reduced from $31.7 \pm 7.2\%$ ($n=17$) to $9.0 \pm 2.1\%$ ($n=13$) upon pre-incubation with 10 μ M RR (unpaired t -test, $P<0.05$). In contrast, the inhibition by hypertonicity was not affected in the presence of RR (330mOsm: $38.8 \pm 8.5\%$, $n=16$; 330+RR: $40.3 \pm 7.1\%$, $n=13$) (unpaired t -test, $P>0.05$) (Fig. 4A).

Here, it was noted that after pre-application of RR for 3 min, TTX-R current was not significantly changed before and during 3 μ M 4 α -PDD treatment (RR: -121.1 ± 11.9 pA/pF, RR+4 α -PDD: -123.7 ± 12.4 pA/pF; $n=10$, paired t -test, $P>0.05$).

Effect of anisotonicity on TTX-R current in TG neurons from TRPV4 wild type (TRPV4^{+/+}) and TRPV4 knockout (TRPV4^{-/-}) mice

We further explored the possible role of TRPV4 receptor by measuring the effect of anisotonicity on TTX-R current in TRPV4^{+/+} and TRPV4^{-/-} mice TG neurons with soma diameters ranging between 10 and 25 μ m.

Hypotonic stimuli—Effects of hypotonicity (260mOsm) on TTX-R current obtained from TRPV4^{+/+} and TRPV4^{-/-} mice are shown in Fig. 4B. Similar to the results found in rat TG neurons, TTX-R current was reversibly reduced by $32.1 \pm 8.4\%$ from -147.1 ± 36.3 pA/pF to -99.8 ± 14.9 pA/pF ($n=13$, paired t -test, $P<0.01$) during hypotonic treatment in TRPV4^{+/+} mice TG neurons. In contrast, TTX-R current was only reduced by $14.1 \pm 6.7\%$ from -144.7 ± 24.0 pA/pF to -128.3 ± 12.1 pA/pF ($n=13$, paired t -test, $P<0.05$) in TG neurons from TRPV4^{-/-} mice when exposed to hypotonicity. There was marked difference between the

inhibition of TTX-R current by hypotonicity in TRPV4^{+/+} and in TRPV4^{-/-} mice (unpaired *t*-test, $P < 0.05$).

Here, TTX-R current was not markedly affected by 3 μ M 4 α -PDD in TRPV4^{-/-} mice TG neurons (300mOsm: -142.1 ± 13.4 pA/pF, 4 α -PDD: -143.1 ± 22.7 pA/pF; $n=8$, paired *t*-test, $P > 0.05$).

Hypertonic stimuli—TTX-R current was inhibited irreversibly when the extracellular osmolality was changed from 300mOsm to 330mOsm. In the presence of hypertonicity, TTX-R current was reduced by $38.1 \pm 9.1\%$ from -150.2 ± 11.4 pA/pF to -92.3 ± 19.6 pA/pF ($n=14$, paired *t*-test, $P < 0.01$) in TG neurons from TRPV4^{+/+} mice. After exposure to hypertonicity, TTX-R current was decreased by $41.5 \pm 9.7\%$ from -152.4 ± 16.3 pA/pF to -90.9 ± 23.6 pA/pF ($n=15$, paired *t*-test, $P < 0.01$) in TG neurons from TRPV4^{-/-} mice. No statistical difference was found in the reduction of TTX-R current by hypertonicity between TRPV4^{+/+} and TRPV4^{-/-} mice (unpaired *t*-test t , $P > 0.05$) (Fig. 4B).

Taken together, these data demonstrated that anisotonicity had similar effect on TTX-R current in TG neurons from rats and TRPV4^{+/+} mice. However, it is noticeable that the inhibition by hypotonicity seen in TRPV4^{+/+} mice was markedly attenuated in TRPV4^{-/-} mice, suggesting the involvement of TRPV4 receptor in hypotonic-induced inhibition.

Intracellular pathways involve in the inhibition of TTX-R current by anisotonicity

In this study, we tested several intracellular pathways under isotonic (300mOsm), hypotonic (260mOsm) and hypertonic (330mOsm) conditions to determine which, if any, of them are involved in the effect of hypo- and hypertonicity on TTX-R current.

PKA system—PKA system is one of the important intracellular signal pathways modulating VGSCs. The effects of PKA are various depending on different types of cells, subtypes of VGSCs and phosphorylation sites (Chahine et al., 2005). In sensory neurons, activation of PKA by pro-inflammatory hyperalgesic agents such as serotonin and prostaglandin E₂ results in an increase of TTX-R currents (Gold et al., 1996; Gold et al., 1998). Here, under isotonic condition, TTX-R current was increased from -137.4 ± 7.9 pA/pF to -156.2 ± 22.1 pA/pF ($n=8$, paired *t*-test, $P < 0.05$) by application of 1 mM pCPT-cAMP (agonist of PKA). TTX-R current was decreased from -140.3 ± 12.8 pA/pF to -119.9 ± 9.6 pA/pF ($n=17$, paired *t*-test, $P < 0.05$) and from -138.8 ± 9.9 pA/pF to -120.3 ± 11.8 pA/pF, ($n=15$, paired *t*-test, $P < 0.05$) by pre-incubation of PKA antagonists, H-89 (10 μ M) or KT5720 (1 μ M) 10 min, respectively. Fig. 5A shows that following pre-incubation with H-89 or KT5720 for 10 min, the inhibition by hypotonicity was markedly attenuated (unpaired *t*-test, $P < 0.05$), whereas the inhibition by hypertonicity was unaffected (unpaired *t*-test, $P > 0.05$).

We further tested the effect of PKA antagonists on the inhibition of TTX-R current by 4 α -PDD. It was found that after pre-application of H-89 or KT5720, the inhibition by 3 μ M 4 α -PDD was reduced to $11.7 \pm 1.3\%$ ($n=11$) and $13.6 \pm 2.7\%$ ($n=11$) respectively, which was markedly different from that in normal pipette solution ($50.3 \pm 7.2\%$) (unpaired *t*-test, $P < 0.05$). These data indicated that PKA system selectively contributed to the inhibition of TTX-R current by hypotonicity.

PKC system—PKC system is another important intracellular messenger pathway modulating VGSCs. In DRG neurons, activation of PKC causes a dose-dependent increase in the amplitude of TTX-R current (Baker, 2005; Gold et al., 1996; Gold et al., 1998). In the present study, under isotonic condition, TTX-R current was enhanced from -137.5 ± 11.0 pA/pF to -153.8 ± 8.8 pA/pF ($n=10$, paired *t*-test, $P < 0.05$) in the presence of 1 μ M PMA (PKC agonist) and decreased from -136.5 ± 14.3 pA/pF to -106.6 ± 8.7 pA/pF ($n=11$, paired *t*-test, $P < 0.05$)

in the presence of 1 μ M BIM (PKC antagonist). As shown in Fig. 5B, TTX-R current by hypo- or hypertonicity was not altered by pre-application of BIM, implying that PKC system did not underlie the modulation of TTX-R current by anisotonicity.

PKG system—Consistent with previous study (Liu et al., 2004), it was found that activation of PKG reduced the amplitude of TTX-R current. In this study, TTX-R current was reduced from -139.5 ± 14.9 pA/pF to -111.9 ± 10.6 pA/pF ($n=11$, paired t -test, $P<0.05$) when extracellular application of 1 mM pCPT-cGMP (PKG activator) 3 min. TTX-R current was increased from -137.1 ± 13.8 pA/pF to -149.8 ± 15.2 pA/pF ($n=11$, paired t -test, $P<0.05$) when neurons were pre-incubated with 10 μ M KT5823 (PKG antagonist) 10 min. As shown in Fig. 5C, following pre-incubation with KT5823, the inhibition by neither hypotonicity nor hypertonicity was markedly affected (unpaired t -test, $P>0.05$), which eliminated the involvement of PKG in inhibition of TTX-R current by anisotonic stimuli.

Lipid cascade—It is reported that in striatal neurons inositol 1, 4, 5-triphosphate receptors antagonistically modulate VGSCs (Hourez et al., 2005). Here PI3K inhibitor (Wortmannin and LY294002) and PLC inhibitor (U73122) were used to test whether lipid cascade participated in the inhibition of TTX-R current by anisotonicity.

TTX-R current was reduced from -135.1 ± 16.6 pA/pF to -91.3 ± 14.5 pA/pF ($n=15$, paired t -test, $P<0.05$) and from -139.1 ± 15.1 pA/pF to -112.7 ± 9.5 pA/pF ($n=17$, paired t -test, $P<0.05$) by pre-application of 2 μ M Wortmannin or 50 μ M LY294002 for 10 min in isotonic solution, respectively. Fig. 5D shows the different effects of PI3K inhibitors on the inhibitions of TTX-R current by hypo- and hypertonicity. In the presence of Wortmannin or LY294002, the inhibition of TTX-R current by hypertonicity was $14.9 \pm 3.1\%$ and $16.6 \pm 7.8\%$ respectively, which was markedly different from that in normal pipette solution (unpaired t -test, $P<0.05$). In contrast, the inhibition by hypotonicity was unaffected by either Wortmannin or LY294002 (unpaired t -test, $P>0.05$).

Under isotonic condition, TTX-R current was not changed in the presence of 10 μ M U73122 (300mOsm: -138.0 ± 16.1 pA/pF, U73122: -134.1 ± 13.1 pA/pF, $n=13$, paired t -test, $P>0.05$). Although pre-application of U73122 did not affect the inhibition by hypotonicity, it did statistically attenuate the inhibition by hypertonicity (unpaired t -test, $P>0.05$) (Fig. 5D). Collectively, these data suggested that lipid cascade was selectively responsible for the inhibition of TTX-R current by hypertonicity.

Discussion

TTX-R current is inhibited by hypo- and hypertonicity

It has been previously shown that TG and DRG neurons with small- and medium-sized somas have characteristics of nociceptors (Cardenas et al., 1995; Liu et al., 2001). That is, they have broad action potentials with humps on their repolarization phase and are activated by capsaicin (Liu et al., 2001). Therefore, in the present study, small- to medium-sized TG neurons were used to study the role of TTX-R current in the anisotonicity-induced nociception. As shown in Fig. 2E, TTX-R current was inhibited by hypo- and hypertonicity and similar dose–response curve was also found in the effect of anisotonicity on capsaicin-induced current (Liu et al., 2007). However, different from the sensitization on capsaicin-induced current by changes in osmolality, the modulation of TTX-R current was marked when the external osmolality was changed within a small range (± 40 mOsm). Concerning the maximal responses, TTX-R current was reduced by 32% and 39% after exposure to hypo- and hypertonicity respectively. In this study, we found different effect of hypo- and hypertonicity on the activation and inactivation kinetics of TTX-R current, that is, both G–V curve and inactivation–voltage curve shifted to the hyperpolarizing direction under hypertonic stimulus, whereas only inactivation–voltage

curve shifted under hypotonic stimulus (Figs.1 and 2). This difference indicated that different mechanisms might underlie the modulation of TTX-R current induced by hypo- versus hypertonicity (Fig. 6).

The inhibition of TTX-R current by hypotonicity is mediated through TRPV4 receptor

Although both hypo- and hypertonicity decreased TTX-R current, the inhibitions by hypo- and hypertonicity were mediated by different receptors. First, the inhibition by hypotonicity was well confirmed by TRPV4 activator. In the presence of 4 α -PDD, TTX-R current was reversibly inhibited and only inactivation-voltage curve shifted to the hyperpolarizing direction (Fig. 3). Second, the inhibition of TTX-R current by hypotonicity was markedly attenuated by TRPV4 antagonist ruthenium red and in TRPV4^{-/-} mice TG neurons, whereas that by hypertonicity not (Fig. 4). These data indicate that TRPV4 receptor is selectively involved in the inhibition of TTX-R current by hypotonicity.

But it is still unknown how TRPV4 receptor contributes to hypotonicity-induced modulation of TTX-R current. In native cells, at the room temperature, hypotonicity (260mOsm)-induced current is small and only seen in a small subpopulation of cells (Alessandri-Haber et al., 2003; Liu et al., 2007). In the present study, we found that TTX-R current was markedly decreased, but no detectable hypotonic-induced current was recorded (data not shown). Therefore, it is indicated that the inhibition of TTX-R current by hypotonicity is not dependent on hypotonic-induced current *via* TRPV4 receptor. Recently, some TRP channels such as TRPM1, TRPP1 and TRPP2 have been reported to have biological effects independent of channel opening, which is referred as “non-conduction function” (Kaczmarek, 2006) and it was possible that TRPV4 modulated TTX-R current through this mechanism under hypotonic stimuli. TRPV4 receptor is a poly-activated receptor which is also sensitive to warm temperature besides anisotonic stimulation (Guler et al., 2002). In order to exclude the activation by heat, all the experiments were performed at room temperature (22–23 °C). At present, we did not do the experiment at higher temperature, but given the fact that TRPV4 can be activated at warm temperature, it is likely that TRPV4 might be sensitized *in vivo*.

Here, it is noted that hypotonicity-induced inhibition was not completely blocked in TRPV4^{-/-} mice TG neurons, whereas the inhibition by 4 α -PDD was completely blocked in TRPV4^{-/-} mice, indicating that besides TRPV4 receptor, there might be other factors underlying hypotonicity-induced modulation of TTX-R current.

The inhibitions of TTX-R current by hypo- and hypertonicity are mediated through different intracellular signaling pathways

VGSCs are modulated by many intracellular signaling pathways, such as PKA, PKC, PKG system and lipid cascade (Chahine et al., 2005). In the present study, we found that TTX-R current was inhibited by antagonists of PI3K, PKA and PKC systems (Wortmannin, LY294002, H-89, KT5720 and BIM) and increased by PKG antagonists (KT5823). So, it is obvious that TTX-R current can be modulated by the above intracellular signaling pathways in TG neurons. Then we tested which of them was involved in hypo or/and hypertonic response by using the antagonists because it was supposed that if certain intracellular system was involved in anisotonicity-induced modulation, antagonism of it would blocked the process (Perez et al., 2006). By comparing the effects of antagonists on anisotonicity-induced inhibition we found that after pre-incubation of PKC or PKG antagonist, the inhibition of TTX-R current by neither hypo- nor hypertonicity was markedly affected, indicating that PKC or PKG systems is not responsible for the modulation of TTX-R current under anisotonic conditions (Fig. 5, B and C).

In the present study, the inhibition of TTX-R current by hypotonicity was reversible, whereas that by hypertonicity not. One of the possible reasons is that different intracellular signaling pathways are involved in the action induced by hypo- versus hypertonicity and the effective time of different signaling pathway varies. To support this hypothesis, it is notable that PKA and lipid cascade were selective for hypo- versus hypertonicity-induced inhibition of TTX-R current. In the present study, hypotonicity-induced inhibition was markedly attenuated (about 70%) by PKA antagonists, implying that PKA system is selectively involved in hypotonic-response. But we should note that TTX-R current was inhibited 32% by hypotonicity in TRPV4^{+/+} mice and this inhibition was reduced to 14% in TRPV4^{-/-} mice, demonstrating that about 56% hypotonic-inhibition is mediated *via* TRPV4 receptor. Therefore, it is suggested that PKA antagonists affect not only the TRPV4-dependent inhibition but also the TRPV4-independent component under hypotonic conditions. In this study, hypertonicity-induced inhibition was selectively attenuated by PI3K and PLC inhibitors, indicating that lipid cascade is selectively responsible for hypertonic-response. Collectively, it is proposed that specific and different intracellular signaling pathways are required for the inhibition of TTX-R current by hypo- versus hypertonicity. Here we are also aware that inhibition of PKA and lipid cascade could not completely block the decrease of TTX-R current by anisotonicity, implying that other mechanisms might be involved in the process.

Physiological significance

VGSCs, which produce the inward membrane current necessary for the generation of AP, play an important role in regulating neurons excitability (Amir et al., 2006; Black et al., 2004; Fang et al., 2005; Priest et al., 2005; Waxman and Hains, 2006). Many experimental and clinical observations have revealed a link between VGSCs and sensory neuron hyperexcitability producing pain (Devor, et al., 1992; Omana-Zapata, et al., 1997; Rizzo, 1997; Wood, et al., 2004). The electrical excitability of nociceptive neurons is an important component of the pain response. In the present study we found that TTX-R current was inhibited by changes in osmolality in TG neurons, which might result in decreased excitability of nociceptors. It is known that the generation, propagation and modulation of AP in nociceptors depend on the activity of a variety of receptors (such as TRPV1, P2X, 5-HT3, and GABA) and voltage-gated ion channels (such as Na⁺, Ca²⁺ and K⁺), and TTX-R current is not the sole target of anisotonicity (Liu et al., 2007). Therefore anisotonicity-induced nociception is likely the integrative result due, at least in part, to the complex modulation of a large number of ion channels on the membrane. Our present framework provides the new information for better understanding the role of TTX-R channels in anisotonicity-induced nociception.

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References

- Alessandri-Haber N, Dina OA, Yeh JJ, Parada CA, Reichling DB, Levine JD. Transient receptor potential vanilloid 4 is essential in chemotherapy-induced neuropathic pain in the rat. *J Neurosci* 2004;24:4444–4452. [PubMed: 15128858]
- Alessandri-Haber N, Joseph E, Dina OA, Liedtke W, Levine JD. TRPV4 mediates pain-related behavior induced by mild hypertonic stimuli in the presence of inflammatory mediator. *Pain* 2005;118:70–79. [PubMed: 16213085]
- Alessandri-Haber N, Yeh JJ, Boyd AE, Parada CA, Chen X, Reichling DB, Levine JD. Hypotonicity induces TRPV4-mediated nociception in rat. *Neuron* 2003;39:497–511. [PubMed: 12895423]

- Amaya F, Decosterd I, Samad TA, Plumpton C, Tate S, Mannion RJ, Costigan M, Woolf CJ. Diversity of expression of the sensory neuron-specific TTX-resistant voltage-gated sodium ion channels SNS and SNS2. *Mol Cell Neurosci* 2000;15:331–342. [PubMed: 10845770]
- Amir R, Argoff CE, Bennett GJ, Cummins TR, Durieux ME, Gerner P, Gold MS, Porreca F, Strichartz GR. The role of sodium channels in chronic inflammatory and neuropathic pain. *J Pain* 2006;7:S1–29. [PubMed: 16632328]
- Baker MD. Protein kinase C mediates up-regulation of tetrodotoxin-resistant, persistent Na⁺ current in rat and mouse sensory neurones. *J Physiol* 2005;567:851–867. [PubMed: 16002450]
- Baraban SC, Schwartzkroin PA. Effects of hyposmolar solutions on membrane currents of hippocampal interneurons and mossy cells in vitro. *J Neurophysiol* 1998;79:1108–1112. [PubMed: 9463467]
- Black JA, Liu S, Tanaka M, Cummins TR, Waxman SG. Changes in the expression of tetrodotoxin-sensitive sodium channels within dorsal root ganglia neurons in inflammatory pain. *Pain* 2004;108:237–247. [PubMed: 15030943]
- Cardenas CG, Del Mar LP, Cooper BY, Scroggs RS. 5HT₄ receptors couple positively to tetrodotoxin-insensitive sodium channels in a subpopulation of capsaicin-sensitive rat sensory neurons. *J Neurosci* 1997;17:7181–7189. [PubMed: 9295364]
- Cardenas CG, Del Mar LP, Scroggs RS. Variation in serotonergic inhibition of calcium currents in four types of rat sensory neurons differentiated by membrane properties. *J Neurophysiol* 1995;74:1870–1879. [PubMed: 8592180]
- Chahine M, Ziane R, Vijayaragavan K, Okamura Y. Regulation of Na^v channels in sensory neurons. *Trends Pharmacol Sci* 2005;26:496–502. [PubMed: 16125256]
- Devor M, Wall PD, Catalan N. Systemic lidocaine silences ectopic neuroma and DRG discharge without blocking nerve conduction. *Pain* 1992;48:261–268. [PubMed: 1589245]
- Fang X, Djouhri L, McMullan S, Berry C, Okuse K, Waxman SG, Lawson SN. trkA is expressed in nociceptive neurons and influences electrophysiological properties via Nav1.8 expression in rapidly conducting nociceptors. *J Neurosci* 2005;25:4868–4878. [PubMed: 15888662]
- Gold MS, Levine JD, Correa AM. Modulation of TTX-R INa by PKC and PKA and their role in PGE₂-induced sensitization of rat sensory neurons in vitro. *J Neurosci* 1998;18:10345–10355. [PubMed: 9852572]
- Gold MS, Reichling DB, Shuster MJ, Levine JD. Hyperalgesic agents increase a tetrodotoxin-resistant Na⁺ current in nociceptors. *Proc Natl Acad Sci USA* 1996;93:1108–1112. [PubMed: 8577723]
- Guler AD, Lee H, Iida T, Shimizu I, Tominaga M, Caterina MJ. Heat-evoked activation of the ion channel TRPV4. *J Neurosci* 2002;22:6408–6414. [PubMed: 12151520]
- Hourez R, Azdad K, Vanwalleghem G, Roussel C, Gall D, Schiffmann SN. Activation of protein kinase C and inositol 1,4,5-triphosphate receptors antagonistically modulate voltage-gated sodium channels in striatal neurons. *Brain Res* 2005;1059:189–196. [PubMed: 16168392]
- Ikeda M, Yoshida S, Kadoi J, Nakano Y, Mastumoto S. The effect of PKC activity on the TTX-R sodium currents from rat nodose ganglion neurons. *Life Sci* 2005;78:47–53. [PubMed: 1611720]
- Kaczmarek LK. Non-conducting functions of voltage-gated ion channels. *Nat Rev Neurosci* 2006;7:761–771. [PubMed: 16988652]
- Kim HC, Chung MK. Voltage-dependent sodium and calcium currents in acutely isolated adult rat trigeminal root ganglion neurons. *J Neurophysiol* 1999;81:1123–1134. [PubMed: 10085339]
- Liedtke W. TRPV4 plays an evolutionary conserved role in the transduction of osmotic and mechanical stimuli in live animals. *J Physiol* 2005;567:53–58. [PubMed: 15961428]
- Liedtke W, Choe Y, Marti-Renom MA, Bell AM, Denis CS, Sali A, Hudspeth AJ, Friedman JM, Heller S. Vanilloid receptor-related osmotically activated channel (VR-OAC), a candidate vertebrate osmoreceptor. *Cell* 2000;103:525–535. [PubMed: 11081638]
- Liedtke W, Kim C. Functionality of the TRPV subfamily of TRP ion channels: add mechano-TRP and osmo-TRP to the lexicon. *Cell Mol Life Sci* 2005;62:2985–3001. [PubMed: 16314934]
- Liu L, Chen L, Liedtke W, Simon SA. Changes in Osmolality Sensitize the Response to Capsaicin in Trigeminal Sensory Neurons. *J Neurophysiol* 2007;97:2001–2015. [PubMed: 17353553]
- Liu L, Oortgiesen M, Li L, Simon SA. Capsaicin inhibits activation of voltage-gated sodium currents in capsaicin-sensitive trigeminal ganglion neurons. *J Neurophysiol* 2001;85:745–758. [PubMed: 11160509]

- Liu L, Yang T, Bruno MJ, Andersen OS, Simon SA. Voltage gated ion channels in nociceptors: modulation by cGMP. *J Neurophysiol* 2004;92:2323–2332. [PubMed: 15175369]
- Liu L, Yang TM, Liedtke W, Simon SA. Chronic IL-1 β signaling potentiates voltage-dependent sodium currents in trigeminal nociceptive neurons. *J Neurophysiol* 2006;95:1478–1490. [PubMed: 16319216]
- Matsuka Y, Spigelman I. Hyperosmolar solutions selectively block action potentials in rat myelinated sensory fibers: implications for diabetic neuropathy. *J Neurophysiol* 2004;91:48–56. [PubMed: 13679399]
- Novakovic SD, Tzoumaka E, McGivern JG, Haraguchi M, Sangameswaran L, Gogas KR, Eglén RM, Hunter JC. Distribution of the tetrodotoxin-resistant sodium channel PN3 in rat sensory neurons in normal and neuropathic conditions. *J Neurosci* 1998;18:2174–2187. [PubMed: 9482802]
- Omana-Zapata I, Khabbaz MA, Hunter JC, Bley KR. QX-314 inhibits ectopic nerve activity associated with neuropathic pain. *Brain Res* 1997;771:228–237. [PubMed: 9401743]
- Perez MF, White FJ, Hu XT. Dopamine D(2) receptor modulation of K(+) channel activity regulates excitability of nucleus accumbens neurons at different membrane potentials. *J Neurophysiol* 2006;96:2217–2228. [PubMed: 16885524]
- Priest BT, Murphy BA, Lindia JA, Diaz C, Abbadie C, Ritter AM, Liberator P, Iyer LM, Kash SF, Kohler MG, Kaczorowski GJ, MacIntyre DE, Martin WJ. Contribution of the tetrodotoxin-resistant voltage-gated sodium channel NaV1.9 to sensory transmission and nociceptive behavior. *Proc Natl Acad Sci USA* 102:9382–9387.
- Ramsey IS, Delling M, Clapham DE. An introduction to TRP channels. *Annu Rev Physiol* 2006;68:619–647. [PubMed: 16460286]
- Renganathan M, Cummins TR, Waxman SG. Nitric oxide blocks fast, slow, and persistent Na⁺ channels in C-type DRG neurons by S-nitrosylation. *J Neurophysiol* 2002;87:761–775. [PubMed: 11826045]
- Rizzo MA. Successful treatment of painful traumatic mononeuropathy with carbamazepine: insights into a possible molecular pain mechanism. *J Neurol Sci* 1997;152:103–106. [PubMed: 9395132]
- Rush AM, Cummins TR, Waxman SG. Multiple sodium channels and their roles in electrogenesis within dorsal root ganglion neurons. *J Physiol* 2007;579:1–14. [PubMed: 17158175]
- Schwartzkroin PA, Baraban SC, Hochman DW. Osmolarity, ionic flux, and changes in brain excitability. *Epilepsy Res* 1998;32:275–285. [PubMed: 9761327]
- Smith JA, Davis CL, Burgess GM. Prostaglandin E₂-induced sensitization of bradykinin-evoked responses in rat dorsal root ganglion neurons is mediated by cAMP-dependent protein kinase A. *Eur J Neurosci* 2000;12:3250–3258. [PubMed: 10998108]
- Suzuki M, Mizuno A, Kodaira K, Imai M. Impaired pressure sensation in mice lacking TRPV4. *J Biol Chem* 2003;278:22664–22668. [PubMed: 12692122]
- Voets T, Prenen J, Vriens J, Watanabe H, Janssens A, Wissenbach U, Boddling M, Droogmans G, Nilius B. Molecular determinants of permeation through the cation channel TRPV4. *J Biol Chem* 2002;277:33704–33710. [PubMed: 12093812]
- Vriens J, Watanabe H, Janssens A, Droogmans G, Voets T, Nilius B. Cell swelling, heat, and chemical agonists use distinct pathways for the activation of the cation channel TRPV4. *Proc Natl Acad Sci USA* 2004;101:396–401. [PubMed: 14691263]
- Watanabe H, Vriens J, Prenen J, Droogmans G, Voets T, Nilius B. Anandamide and arachidonic acid use epoxyeicosatrienoic acids to activate TRPV4 channels. *Nature* 2003;424:434–438. [PubMed: 12879072]
- Waxman SG, Cummins TR, Dib-Hajj S, Fjell J, Black JA. Sodium channels, excitability of primary sensory neurons, and the molecular basis of pain. *Muscle Nerve* 1999;22:1177–1187. [PubMed: 10454712]
- Waxman SG, Hains BC. Fire and phantoms after spinal cord injury: Na⁺ channels and central pain. *Trends Neurosci* 2006;29:207–215. [PubMed: 16494954]
- Wood JN, Boorman JP, Okuse K, Baker MD. Voltage-gated sodium channels and pain pathways. *J Neurobiol* 2004;61:55–71. [PubMed: 15362153]

Figure 1. Effect of hypotonicity on TTX-R current

(A) The typical recordings show that TTX-R current was reduced from -3.6 nA to -2.5 nA after the external solution was changed from 300mOsm to 260mOsm and the current recovered to -3.3 nA after washout. 4 α -PDD-induced current was recorded in the same TG neuron (*upper*). The time-course curve of TTX-R current was shown before, during and after hypotonicity treatment (*below*). (B) The voltage-current relationship (I-V curve) was shown in both isotonic and hypotonic solutions. (C) In the presence of hypotonicity, G-V curve did not markedly shift. $V_{0.5}$ was -9.0 ± 1.1 mV and -9.5 ± 0.9 mV ($n=17$, paired t -test, $P>0.05$) for 300mOsm and 260mOsm respectively; k was 5.8 ± 1.6 and 5.2 ± 0.6 ($n=17$, paired t -test, $P>0.05$), respectively. Data were transformed from the I-V data shown in B. (D) Hypotonic stimulus reduced the amplitude of TTX-R and inactivation-voltage curve markedly shifted to the hyperpolarizing direction. $V_{0.5}$ was -22.5 ± 3.0 mV and -30.8 ± 1.9 mV ($n=11$, paired t -test, $P<0.05$) for 300mOsm and 260mOsm respectively; k was -6.5 ± 0.7 and -6.9 ± 0.9 ($n=9$, paired t -test, $P>0.05$) respectively.

Figure 2. Effect of hypertonicity on TTX-R current

(A) The typical recordings show that TTX-R current was reduced from -3.5 nA to -2.3 nA after the external solution was changed from 300mOsm to 330mOsm but the current didn't recover (-2.5 nA) after washout. 4α-PDD-induced current was recorded in the same TG neuron (*upper*). The time-course curve of TTX-R current was shown before, during and after hypertonicity treatment (*below*). (B) I-V curve was shown before and during hypertonic treatment. (C) In the hypertonic solution, G-V curve shifted to the hyperpolarizing direction. $V_{0.5}$ was -9.1 ± 0.9 mV and -13.8 ± 1.2 mV ($n=16$, paired t -test, $P<0.05$) for 300mOsm and 330mOsm respectively; k was 5.4 ± 0.8 and 5.7 ± 1.2 ($n=16$, paired t -test, $P>0.05$), respectively. Data were transformed from the I-V data shown in B. (D) The typical recordings show that

the amplitude of TTX-R current was reduced in the presence of hypertonicity and the inactivation–voltage curve shifted to the hyperpolarizing direction. $V_{0.5}$ was -21.2 ± 1.7 mV and -28.1 ± 1.7 mV ($n=11$, paired t -test, $P<0.05$) for 300mOsm and 330mOsm respectively; k was -6.6 ± 0.6 and -8.1 ± 1.5 ($n=11$, paired t -test, $P<0.05$), respectively. (E) The amplitude of TTX-R was inhibited by anisotonic stimuli and the inhibition was conspicuous when the osmolality changed within a small range (± 40 mOsm).

Figure 3. Effect of 4 α -PDD on TTX-R current

(A) The typical recordings show that TTX-R current was reduced from -4.7 nA to -2.4 nA after exposed to $3 \mu\text{M}$ 4 α -PDD and the current recovered to -3.9 nA after washout. 4 α -PDD-induced current was recorded in the same TG neuron (*upper*). The time-course curve of TTX-R current was shown before, during and after 4 α -PDD treatment (*below*). (B) I-V curve was shown before and during 4 α -PDD treatment. (C) In the presence of 4 α -PDD G-V curve did not shift. $V_{0.5}$ was -9.2 ± 1.1 mV and -9.6 ± 2.7 mV ($n=7$, paired t -test, $P>0.05$) before and during 4 α -PDD treatment respectively; k was 5.6 ± 0.7 and 5.7 ± 1.0 ($n=7$, paired t -test, $P>0.05$), respectively. Data were transformed from the I-V data shown in B. (D) The amplitude of TTX-R current was reduced after exposed to $3 \mu\text{M}$ 4 α -PDD and the inactivation-voltage

curve markedly shifted to hyperpolarizing direction. $V_{0.5}$ was -20.3 ± 1.1 mV and -32.9 ± 0.9 mV ($n=9$, paired t -test, $P<0.05$) before and during 4 α -PDD treatment respectively; k was -5.0 ± 1.0 and -5.2 ± 0.8 ($n=10$, paired t -test, $P>0.05$), respectively. **(E)** The plot shows the inhibition of TTX-R current by 4 α -PDD at concentrations of 0.01, 0.03, 0.1, 0.3, 1, 3, 10, 30 and 100 μ M. The dose-response curve fits to Hill equation with IC_{50} being 2.4 μ M and n being 0.8.

Figure 4. Hypotonicity-induced inhibition of TTX-R current is markedly attenuated after blocking TRPV4 receptor and in TRPV4^{-/-} mice TG neurons

(A) After pre-application of RR for 3 min, the inhibition of TTX-R by hypotonicity was significantly attenuated (unpaired *t*-test, $P < 0.05$), while that by hypertonicity not (unpaired *t*-test, $P > 0.05$). (B) The inhibition by hypotonicity in TRPV4^{-/-} mice is markedly different from that in TRPV4^{+/+} mice TG neurons (unpaired *t*-test, $P < 0.05$). However, TTX-R current was similarly reduced in TRPV4^{+/+} and TRPV4^{-/-} mice TG neurons (unpaired *t*-test, $P > 0.05$).

Figure 5. Modulation of intracellular signaling pathways on the inhibition of TTX-R current by hypo- and hypertonicity

(A) For PKA system, after pre-incubation with H-89 (10 μ M) and KT5720 (1 μ M) the inhibition of TTX-R current by hypotonicity was markedly reduced from $31.7 \pm 7.2\%$ ($n=17$) to $7.8 \pm 3.1\%$ ($n=13$) (unpaired t -test, $P<0.05$) and $9.5 \pm 4.7\%$ ($n=14$) (unpaired t -test, $P<0.05$), respectively. H-89 and KT5720 had no marked effect on the inhibition by hypertonicity (unpaired t -test, $P>0.05$) and TTX-R current was reduced $40.9 \pm 6.5\%$ ($n=11$) and $39.8 \pm 6.5\%$ ($n=13$) by hypertonicity in the presence of H-89 and KT5720, respectively. (B) For PKC system, the inhibition by neither hypotonicity nor hypertonicity was affected in the presence of BIM (unpaired t -test, $P>0.05$). TTX-R current was reduced by $33.7 \pm 4.6\%$ ($n=13$) and 37.1

$\pm 3.9\%$ ($n=10$) with $1\ \mu\text{M}$ BIM in hypotonic and hypertonic solution respectively. **(C)** For PKG system, KT5823 did not statistically alter the inhibitions of TTX-R current by anisotonicity (unpaired t -test, $P>0.05$) and TTX-R current was reduced $29.9 \pm 4.7\%$ ($n=13$) and $40.1 \pm 7.1\%$ ($n=13$) by hypo- and hypertonicity, respectively. **(D)** For lipid cascade, upon exposure to hypotonicity TTX-R current was reduced by $33.2 \pm 8.7\%$ ($n=13$), $28.9 \pm 6.7\%$ ($n=12$) and $31.1 \pm 7.4\%$ ($n=11$) with Wortmannin, LY294002 or U73122 in the pipette solution, respectively. Compared with the inhibition by hypotonicity in normal pipette solution ($31.7 \pm 7.2\%$, $n=17$), none of them was markedly different (unpaired t -test, $P>0.05$). However, Wortmannin, LY294002 and U73122 markedly attenuated the inhibition of TTX-R current by hypertonicity from $38.8 \pm 8.5\%$ ($n=16$) to $14.9 \pm 3.1\%$ ($n=12$) (unpaired t -test, $P<0.05$), $16.6 \pm 7.8\%$ ($n=11$) (unpaired t -test, $P<0.05$) and $15.7 \pm 6.1\%$ ($n=11$) (unpaired t -test, $P<0.05$) respectively.

A

B

A

B

Figure 6. Possible mechanism underlying anisotonicity-induced inhibition of TTX-R current

Although TTX-R current is inhibited by changes in osmolality, receptor and intracellular signaling pathway are selective for hypo- versus hypertonic response: concerning receptors, TRPV4 receptor is selectively involved in hypotonic response; concerning intracellular pathways, PKA is selectively responsible for hypotonic response and lipid cascade for hypertonic response.