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Changes in Osmolality Modulate Voltage-gated Calcium Channels in Trigeminal Ganglion Neurons

Lei Chen^{1,2,4}, Changjin Liu¹, and Lieju Liu^{1,3}

¹ Department of Physiology, Tongji Medical College, Huazhong University of Science and Technology, Wuhan, P.R. China

² Department of Neurobiology, Duke University, Durham, North Carolina, USA

³ Department of Anesthesiology, Duke University, Durham, North Carolina, USA

⁴ Department of Physiology, Nanjing Medical University, Nanjing, P. R. China

Abstract

Voltage-gated calcium channels (VGCCs) participate in many important physiological functions. However whether VGCCs are modulated by changes of osmolarity and involve in anisotonicity-induced nociception are still unknown. For this reason by using whole cell patch clamp techniques in rat and mouse trigeminal ganglion (TG) neurons we tested the effects of hypo and hypertonicity on VGCCs. We found that high voltage-gated calcium current (I_{HVA}) was inhibited by both hypo and hypertonicity. In rat TG neurons, the inhibition by hypotonicity was mimicked by Transient Receptor Potential Vanilloid 4 receptor (TRPV4) activator but hypotonicity did not exhibit inhibition in TRPV4^{-/-} mice TG neurons. Concerning the downstream signaling pathways, antagonism of PKG pathway selectively reduced the hypotonicity-induced inhibition, whereas inhibition of PLC- and PI3K-mediated pathways selectively reduced the inhibition produced by hypertonicity. In summary, although the effects of hypo- and hypertonicity show similar phenotype, receptor and intracellular signaling pathways were selective for hypo- versus hypertonicity induced inhibition of I_{HVA} .

Keywords

voltage-gated calcium channels; osmolality; TRPV4; nociception; intracellular signal transduction

1. Introduction

Osmolarity is an important factor determining the excitability of neural tissue and neurotransmitter release (Andrew et al., 1989; Azouz et al., 1997; Baraban and Schwartzkroin, 1998; Kilb et al., 2006; Saly and Andrew, 1993). Changes in osmolarity can induce many diseases and symptoms such as brain edemas (Ayus et al., 1996; Diringier and Zazulia, 2006), epilepsy (Ramsey et al., 2006; Schwartzkroin et al., 1998) and pain (Alessandri-Haber et al., 2005; Alessandri-Haber et al., 2003). In the laboratory, injecting both hypotonic and hypertonic saline can stimulate C-fiber afferent and consequently produce pain-related behavior

Corresponding author: Lieju Liu, Department of Physiology, Tongji Medical College, Huazhong University of Science and Technology No. 13, Hangkong Road, Wuhan, P.R. China, 430030, Ph: 86-27-8369-2622, FAX: 86-27-8369-2608, E-mail: chentlj@hotmail.com.

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(Alessandri-Haber et al., 2005; Alessandri-Haber et al., 2003; Guler et al., 2002). In the clinic, pain is often induced by contrast media which are commonly used for examination and treatment (Gomi, 1992; Manke et al., 2003). Additionally, the nociception induced by pH (Hamamoto et al., 2000), capsaicin (Liu et al., 2007), carrageenan (Alessandri-Haber et al., 2006) and mechanical stimulus (Alessandri-Haber et al., 2004) can be facilitated by hypo and hypertonicity. These evidences suggest that anisotonicity is involved in the inflammatory and neurophathic pain, but the mechanism remains unclear. Recently, it is found that hypo- and hypertonicity can modulate many channels and receptors, such as sensitizing the function of Transient Receptor Potential Vanilloid 1 receptor (TRPV1) (Liu et al., 2007). So, the studies on the modulation of ion channels will provide us new information to explore the mechanisms of anisotonicity induced nociception.

Voltage-gated calcium channels (VGCCs) are distributed in the nervous systems and play an important role in many physiological functions such as regulation of membrane excitability, intracellular signal transduction and synaptic neurotransmitter release. VGCCs are commonly divided into two classes: low-voltage-activated (LVA) and high-voltage-activated (HVA) channel depending on their voltage dependent activation character (Ikeda and Matsumoto, 2003; Lacinova, 2005). HVA channels have been implicated in a variety of neuronal processes including neurotransmitter release and action potential depolarization. It is reported that blockade of calcium channels in the spinal cord could affect neurotransmitter release from primary afferents or interneurons, or calcium influx through the postsynaptic membrane (Llinás, 1989; Miller, 1987; Spedding and Paoletti, 1992). However, whether VGCCs can be modulated by changes in osmolality and participate in the anisotonicity-induced pain are still unknown.

TRPV4, one of Transient Receptor Potential Vanilloid (TRPV) family members, is a calcium permeable channel and regarded as one of the most important osmolality cellular sensors (Clapham, 2003; Liedtke et al., 2003). Besides osmolality, TRPV4 receptor can be activated by moderate heat, mechanical stimuli, synthetic activators such as 4 α -phorbol-12,13-didecanoate (4 α -PDD) and endogenous agonists derived from arachidonic acid and its metabolites et al (Liedtke and Kim, 2005; Watanabe et al., 2002). TRPV4 receptors are expressed in trigeminal ganglion (TG) and dorsal root ganglion (DRG) (Alessandri-Haber et al., 2003; Suzuki et al., 2003). Several approaches, including the knockdown of TRPV4 with gene-disruption or antisense oligonucleotides, have led to reports that TRPV4 accounts for anisotonicity-induced nociception and plays a crucial role in inflammatory and neurophathic pain. In this study, we investigated whether high voltage-gated calcium current (I_{HVA}) was modulated by hypo and hypertonicity in TG cultured neurons and further explored the possible role of TRPV4 receptor and intracellular pathways in the modulation.

2. Results

2.1 Changes in osmolality decrease I_{HVA}

Hypotonic stimulus—Figure 1A shows that I_{HVA} was reduced reversibly after exposure to hypotonicity (260mOsm). On the average, I_{HVA} was inhibited by 38.3 $\pm$ 10.6% from -28.6 $\pm$ 6.0 pA/pF to -18.4 $\pm$ 4.0 pA/pF (n=33, paired *t* test, *P*<0.01). The current recovered to -23.3 $\pm$ 3.9 pA/pF after hypotonicity was washed out for 3 min. We also found that G–V curve did not significantly shifted before and after hypotonicity treatment (paired *t* test, *P*>0.05) (Fig. 1C). Figure 1D shows the effect on inactivation–voltage curve in the presence of hypotonicity. When the extracellular solution was changed from 300mOsm to 260mOsm, the inactivation–voltage curve significantly shifted (15 mV) to the hyperpolarizing direction.

Hypertonic stimulus—Different from the inhibition of hypotonicity, I_{HVA} was inhibited by hypertonicity (322mOsm) irreversibly. On the average, I_{HVA} was inhibited by 37.1 $\pm$ 9.0%

from -28.6 ± 2.9 pA/pF to -17.8 ± 2.0 pA/pF ($n=22$, paired t test, $P<0.05$) when the extracellular solution was changed from 300mOsm to 322mOsm. The current slightly recovered to -20.6 ± 3.9 pA/pF upon a 3 min washout (Fig. 2A). Also unlike the effect of hypotonicity, neither G–V curve nor inactivation–voltage curve significantly shifted when the extracellular solution was changed from 300mOsm to 322mOsm (paired t test, $P>0.05$) (Fig. 2C and 2D).

The dose-dependent inhibition of I_{HVA} under anisotonic conditions was presented in figure 2E. The curve exhibited “V” shape, that is, larger response was found in the presence of larger osmotic gradient. Since 260mOsm and 322mOsm were mild anisotonicity and exhibited significant inhibition, these concentrations were used in all subsequent experiments.

In this study, we also found there was no significant difference in I_{HVA} when the extracellular solution was changed from 300mOsm to Krebs-Henseleit solution (KH) ($n=6$, paired t test, $P>0.05$).

2.2 Effect of TRPV4 receptor agonist, 4 α -PDD, on I_{HVA}

TRPV4 receptor has been reported as osmosensitive receptor (Liedtke et al., 2003; Liedtke, 2005). To explore the role of TRPV4 receptor in the effect of anisotonicity, we tested the effect of TRPV4 receptor agonist, 4 α -PDD, on I_{HVA} in rat TG neurons. As shown in figure 3A, application of 4 α -PDD (1 μ M) inhibited I_{HVA} by $31.7 \pm 9.8\%$ from -28.2 ± 2.4 pA/pF to -19.7 ± 2.5 pA/pF ($n=8$, paired t test, $P<0.01$). The inhibition of I_{HVA} recovered to -24.6 ± 3.6 pA/pF after 4 α -PDD was washed out. Similar to the effect of hypotonicity, in the presence of 1 μ M 4 α -PDD G–V curve did not shift (paired t test, $P>0.05$) but inactivation–voltage curve markedly shifted to the hyperpolarizing direction (Fig. 3C and 3D). The concentration-dependent inhibition of I_{HVA} by 4 α -PDD was shown in figure 3E. I_{HVA} was inhibited $7.1 \pm 5.3\%$ and $77.2 \pm 10.0\%$ by 0.03 and 100 μ M 4 α -PDD, respectively. The dose–response curve was fitted by Hill equation with IC50 being 1.9 μ M.

2.3 Effect of hypo- and hypertonicity on I_{HVA} in TG neurons from TRPV4 wild type (TRPV4^{+/+}) and TRPV4 knockout (TRPV4^{-/-}) mice

To further determine whether TRPV4 receptor was involved in the effects of hypo or hypertonicity, we tested the effects of anisotonicity on I_{HVA} in TG neurons from TRPV4^{+/+} and TRPV4^{-/-} mice with soma diameters ranging between 10–25 μ m (Fig. 4).

Hypotonic stimulus—Figure 4A shows the effect of hypotonicity on I_{HVA} in TG neurons from TRPV4^{+/+} and TRPV4^{-/-} mice. Similar to the results found in rat TG neurons, I_{HVA} was reduced reversibly by $43.6 \pm 9.1\%$ from -36.4 ± 3.9 pA/pF to -20.6 ± 2.7 pA/pF in TRPV4^{+/+} mice TG neurons when the extracellular solution was changed from 300mOsm to 260mOsm ($n=18$, paired t test, $P<0.01$). However, I_{HVA} was reduced only by $4.9 \pm 3.6\%$ from -37.2 ± 6.2 pA/pF to -33.5 ± 5.7 pA/pF ($n=18$, paired t test, $P>0.05$) in TG neurons from TRPV4^{-/-} mice. The inhibitions of I_{HVA} by hypotonicity in TRPV4^{+/+} and TRPV4^{-/-} mice were statistically different (unpaired t test, $P<0.05$).

Hypertonic stimulus—Figure 4B shows that I_{HVA} was inhibited irreversibly after exposure to hypertonicity (322mOsm). In TG neurons from TRPV4^{+/+} mice, I_{HVA} was reduced by $44.7 \pm 8.9\%$ from -37.3 ± 9.4 pA/pF to -21.8 ± 6.5 pA/pF ($n=14$, paired t test, $P<0.01$). In TG neurons from TRPV4^{-/-} mice, I_{HVA} was inhibited by $48.0 \pm 9.8\%$ from -35.5 ± 10.1 pA/pF to -18.9 ± 5.4 pA/pF ($n=14$, paired t test, $P<0.01$). However, there was no statistical difference in the inhibitions of I_{HVA} by hypertonicity between TRPV4^{+/+} and TRPV4^{-/-} mice (unpaired t test, $P>0.05$).

In summary, these data showed changes in tonicity yielded similar effect on I_{HVA} in TG neurons from rats and TRPV4^{+/+} mice. However, the inhibition of hypotonicity seen in TRPV4^{+/+} mice was significantly reduced in TRPV4^{-/-} mice, suggesting the involvement of TRPV4 receptor in hypotonic induced inhibition.

2.4 Intracellular pathways involve in the effects of anisotonicity on I_{HVA}

In this study, we tested several intracellular pathways in rat TG neurons to determine whether they participated in the inhibitions of I_{HVA} by hypo and hypertonicity.

G-protein system—Studies have reported that G-protein can inhibit HVA via direct as well as indirect (involving a second messenger) pathway (Herlitze, et al., 1996; Hille, 1994; Hirning, et al., 1990; Ikeda, 1996). In the present study, we firstly studied G-protein system by using GTP- γ s (G-protein agonist, non-hydrolyzable GTP analog) and GDP- β s (G-protein antagonist, non-hydrolyzable GDP analog). In the isotonic solution, a 10min pre-incubation of 0.3 mM GTP- γ s or 0.3 mM GDP- β s reduced I_{HVA} by $52.3 \pm 5.3\%$ ($n=26$, paired t test, $P<0.01$) and $19.2 \pm 8.0\%$ ($n=19$, paired t test, $P<0.01$), respectively (see Table 1). Figure 5A shows the effects of 0.3 mM GTP- γ s and 0.3 mM GDP- β s on the inhibitions of hypo and hypertonicity. There was no significant difference between the inhibitions of I_{HVA} by anisotonicity in the presence of GTP- γ s or GDP- β s and those in the normal pipette solution (unpaired t test, $P>0.05$). These data indicated that G-protein was not responsible for the inhibition of I_{HVA} under anisotonic conditions.

PKA system—PKA is one of the downstream pathways of G-protein and activation of PKA has been reported to increase the function of VGCCs (Kavalali et al., 1997). We then performed the experiment to further test this pathway. Here, in the presence of 300mOsm, application of PKA agonist, 1mM 8-Br-cAMP 3 min, increased I_{HVA} by $13.6 \pm 8.4\%$ ($n=12$, paired t test, $P<0.05$). Pre-incubation of PKA antagonist, 10 μ M H-89, inhibited I_{HVA} by $12.9 \pm 3.0\%$ ($n=17$, paired t test, $P<0.05$) (see Table 1). The effects of H-89 on the inhibitions of hypo and hypertonicity were shown in figure 5B. Following pre-incubation with H-89, the inhibition neither by hypotonicity nor by hypertonicity was markedly reversed (unpaired t test, $P>0.05$), which eliminated the involvement of PKA in the modulation of I_{HVA} by anisotonic stimuli.

PKC system—We also performed the experiment on PKC pathway. PKC has been demonstrated to exert a direct and (or) indirect effect on L- and N-type VDCC, resulting in PKC-dependent increase of channel activity (Barrett et al., 2000; Puri et al., 1997; Swartz, 1993). Consistently, in the present study, application of PKC agonist, 1 μ M PMA 3min increased I_{HVA} by $15.9 \pm 4.6\%$ ($n=7$, paired t test, $P<0.05$), while application of PKC antagonist, 1 μ M BIM 3min decreased I_{HVA} by $12.9 \pm 5.8\%$ ($n=10$, paired t test, $P<0.05$) (see Table 1). Figure 5C shows that BIM did not statistically affect the inhibition of hypotonicity or hypertonicity (unpaired t test, $P>0.05$). This result suggested that inhibition of PKC was not required for the blockage of I_{HVA} under anisotonic conditions.

PKG system—Many studies show that VGCCs can be modulated by cGMP-PKG system (Hirooka et al., 2000; Kim et al., 2000). However, the effect of PKG varies based on different subtypes and location of VGCCs. In this study, incubation with PKG agonist, 1 mM 8-Br-cGMP 3min (a kind of membrane permeable analogue of cGMP) decreased I_{HVA} by $16.1 \pm 2.1\%$ ($n=7$, paired t test, $P<0.05$). Pre-incubation with PKG antagonists, 10 μ M KT5823 or 1 μ M Rp-8-Br-PET-cGMPs for 10 min increased I_{HVA} by $11.7 \pm 4.1\%$ ($n=13$, paired t test, $P<0.05$) and $13.2 \pm 3.9\%$ ($n=10$, paired t test, $P<0.05$), respectively (see Table 1). It was noted that pre-incubation with 10 μ M KT5823 or 1 μ M Rp-8-Br-PET-cGMPs 10 min significantly reduced the inhibition by hypotonicity (unpaired t test, $P<0.01$), while leaving the inhibition by hypertonicity unaffected (unpaired t test, $P>0.05$) (Fig. 6).

We further tested the effect of PKG antagonists on the inhibition of 4 α -PDD and found that in the presence of KT5823 or Rp-8-Br-PET-cGMPs, the inhibition of 1 μ M 4 α -PDD (31.7 $\pm$ 9.8%) was reduced to 10.1 $\pm$ 3.4% (n=7) (unpaired *t* test, *P*<0.05) and 8.9 $\pm$ 4.1% (n=8) (unpaired *t* test, *P*<0.05), respectively. These data suggested that cGMP-PKG system was selectively involved in the modulation of I_{HVA} by hypotonicity.

Lipids system—PIP₂, via its degradation as well as its resynthesis, has been shown to modulate VGCCs (Lechner et al., 2005; Wu et al., 2002). Pre-incubation with 2 μ M Wortammin (PI3-4K antagonist) or 50 μ M LY294002 (PI3K antagonist) 10min reduced I_{HVA} by 24.5 $\pm$ 8.4% (n=13, paired *t* test, *P*<0.05) and 36.0 $\pm$ 3.9% (n=16, paired *t* test, *P*<0.05), respectively. Pre-incubation with PLC antagonist, 10 μ M U73122 reduced I_{HVA} by 16.7 $\pm$ 5.6% (n=10, paired *t* test, *P*<0.05) (see Table 1). Figure 7 shows that after pre-incubation with Wortmannin, LY294002 or U73122, the inhibition by hypertonicity was significantly reversed, whereas the inhibition by hypotonicity was left unaffected (unpaired *t* test, *P*>0.05). These data suggested that lipids cascade was selectively involved in the inhibition of I_{HVA} by hypertonicity.

3. Discussion

3.1 Inhibitions of hypo- and hypertonicity on I_{HVA} are mediated by different receptors

In this study, we found that osmolality was an important modulator on VGCCs. I_{HVA} was inhibited by hypo and hypertonicity with V shape dose–response curve (Figs. 1 and 2). Such V shape dose–response curve was also found in the modulation of capsaicin induced-current by hypo and hypertonicity (Liu et al., 2007). Although I_{HVA} was inhibited by both hypo and hypertonicity, different receptors accounted for their effects. Firstly, the inhibition by hypotonicity was well mimicked by TRPV4 activator (4 α -PDD), that is, the inhibition was reversible and the inactivation–voltage curve significantly shifted to hyperpolarizing direction (Figs. 1 and 3). Secondly, hypotonicity did not yield inhibition of I_{HVA} in TRPV4^{−/−} mice TG neurons. By contrast, the inhibition induced by hypertonicity was not affected in TRPV4^{−/−} mice TG neurons. In addition, the inhibitions of both hypotonicity and 4 α -PDD were reversed by PKG antagonists. These data suggested that TRPV4 receptor might be selectively involved in the hypotonicity induced inhibition.

The mechanism how TRPV4 receptor underlies the inhibition by hypotonicity is still not clear. TRPV4 is commonly accepted as an ionotropic receptor. However, studies show that, at room temperature, the inward current induced by anisotonicity and the elevation of intracellular calcium concentration are detected only in a small part of TG neurons (Alessandri-Haber et al., 2003; Liu et al., 2007; Viana et al., 2001). Here, we also found that I_{HVA} was significantly reduced but in the same TG neurons no detectable hypotonicity-induced inward current was recorded (data not shown). So it is suggested that channel opening was not essential for the hypotonicity induced inhibition of I_{HVA} and there must be other mechanism responsible for the modulation. One possible mechanism is the so-called “non-conduction functions” (Kaczmarek, 2006). Recently, it is reported that many ion channels can per se influence biochemical events in ways that do not depend on their function as ion channels. For example, channels can directly activate enzymes linked to cellular signal pathways which serve as cell adhesion molecules or components of the cytoskeleton and then their activation can alter the expression of specific genes. This kind of “non-conduction function” has been found in some TRP channels such as TRPM2, TRPM6 and TRPM7 (Perraud et al., 2001; Runnels et al., 2001; Schlingmann et al., 2002; Schmitz et al., 2005).

3.2 Inhibitions of hypo- and hypertonicity on I_{HVA} are mediated through different intracellular messenger systems

VGCCs can be modulated by many intracellular pathways such as G-protein (De Waard et al., 2005; Zhu and Yakel, 1997), cAMP-PKA (Catterall, 2000; Kavalali et al., 1997), cGMP-PKG (Chen, 2000; Grassi et al., 2004), lipids cascade (Delmas et al., 2005; Gamper et al., 2004), and PKC systems (Titievsky et al., 1998; Zhu and Ikeda, 1994) and the modulations vary depending on different kinds of cells and subtypes of VGCCs (Felix, 2005). In this study we tested which, if any of them was involved in the effects of anisotonicity on I_{HVA} , and whether different and specific pathways were required for hypo- and hypertonicity induced inhibition. Table 1 showed that VGCCs could be modulated by many intracellular pathways. Here, two intracellular pathways, cGMP-PKG and lipid cascade, were particularly noted (Figs. 6 and 7). For other pathways, none of their antagonists significantly reversed the inhibition by hypo or hypertonicity, indicating that G-protein, PKA and PKC were not involved in the modulation of I_{HVA} by anisotonic stimuli (Fig. 5).

Many studies show that VGCCs are modulated by cGMP-PKG pathway, which accounts for the modulation on Ca(v)1 and Ca(v)2.2 channels by nitric oxide (Grassi et al., 2004) and L-type channel by muscarinic receptor (Chen, 2000). Similar to previous study (Kim et al., 2000), I_{HVA} was inhibited by PKG agonist (8-Br-cGMP) and increased by PKG antagonists (KT5823 and Rp-8-Br-PET-cGMPs) (Table 1). Here, it was found that KT5823 and Rp-8-Br-PET-cGMPs significantly reversed the inhibition by hypotonicity and 4 α -PDD, suggesting PKG system was selectively involved in the inhibition by hypotonicity but not hypertonicity (Fig. 6).

Lipids cascade plays an important role in modulating the function of VGCCs and phospholipids kinase C (PLC), PI3-kinase (PI3K) and PI4-kinase (PI4K) are important kinases in lipids pathway (Delmas et al., 2005). Our previous study shows that lipids cascade is involved in the facilitating effect of capsaicin-induced current induced by hypertonicity (Liu et al., 2007). In isotonic solution, I_{HVA} was decreased following pre-incubation with PLC antagonist (U73122) and PI3-4K antagonists (LY294002 and Wortammin) (Table 1). In this study, both PLC and PI3-4K antagonists significantly reversed the inhibition of hypertonicity, suggesting that lipids cascade selectively accounted for the inhibition of hypertonicity but not hypotonicity (Fig. 7).

Here, we are also aware that antagonism of PKG and lipids cascade did not reverse the inhibitions of I_{HVA} by hypo (260mOsm) or hypertonicity (322mOsm) completely, implying other factors such as those that can be affected by alterations in the cell volume, probably contribute to the process and we can not eliminate this possibility especially when exposed to greater osmotic gradients.

3.3 Physiological significance

VGCCs are highly versatile and ubiquitous ion channels and play fundamental role in cell excitability, neuron transmitter release and calcium induced modulations on many cellular functions (Catterall et al., 2005; Lacinova, 2005). In this study, by using culture TG neurons, we found that I_{HVA} was significantly inhibited by hypo and hypertonicity. Although we don't know yet whether the same modulatory effects could happen in other tissues, it is possible that changes in tonicity may participate in many physiological and pathological processes via modulating the function of VGCCs.

VGCCs are distributed in the peripheral and the central nervous systems. In the peripheral nervous system, HVA (including L-, N-, P/Q and R-type) and LVA channels have been identified in TG and DRG neurons (Borgland et al., 2001; Kim and Chung, 1999). In nociceptors, VGCCs contribute to the upstroke and duration of AP and when activated may

depolarize the nociceptor and release pro-inflammatory transmitters and peptides therefore, VGCCs are important targets for the treatment of pain (Bourinet and Zamponi, 2005; McGivern, 2006; Wallace, 2000), including acute (Ogasawara et al., 2001), chronic and neuropathic pain (Snutch, 2005). In this study, LVA current was found only in about 7% (54/744) small and medium size TG neurons (data not shown), so we focused our experiment on I_{HVA} . Our previous study shows that changes in osmolality can increase the capsaicin-evoked current, which may be one of mechanisms accounting for the nociception induced by anisotonicity (Liu et al., 2007). Here, our study found that I_{HVA} was inhibited by both hypo and hypertonic stimuli. It is known that the inhibition of VGCCs decreases nociceptor excitability and reduces the nociceptive signal transduction so changes in tonicity rather than sensitizing VGCCs might produce an analgesic effect that provides the information for better understanding the role of VGCCs in the nociception produced by anisotonicity.

4. Experimental Procedure

4.1 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., 2004). Briefly, trigeminal ganglions were dissected aseptically and washed with cold (4°C) modified Hank's balanced salt solution (mHBSS). The mHBSS solution contains (in mM): NaCl 130, KCl 5, KH_2PO_4 0.3, NaHCO_3 4, NaH_2PO_4 0.3, D-glucose 5.6, and EGTA 10, HEPES 10 at pH 7.4. The ganglia were diced into small pieces, and then incubated in 3 ml mHBSS with 0.1% collagenase (Type XI-S) for 20–40 min at 37 °C. 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 24 hr at 37 °C in a water saturated atmosphere with 5% CO_2 .

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.

4.2 Patch clamp recording

The cells were placed in a recording chamber mounted on the stage of an inverted microscope (Leica Inc. Germany) and perfused continually with extracellular solution at room temperature (21–22 °C) at the rate of 3ml/min. Whole-cell patch recordings were obtained using an Axopatch 200B amplifier (Axon Instruments, Foster City, CA, USA) and the output was digitized with a Digidata 1332A converter (Axon Instruments). Data were acquired at a sampling rate of 10 kHz and filtered at 5 kHz. In the experiments the capacitance was compensated and series resistance was compensated more than 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 isotonicity to hypo or to hypertonicity, measurements of the changes in liquid junction potentials were less than 2 mV and were not corrected. The cell diameter was measured with a calibrated eyepiece under phase contrast illumination. Neurons having projected soma diameters ranging between 15–30µm were used.

The voltage-dependent activation curve (G–V curve) was measured by a series of depolarizing pulses (200 ms) from –60 to +40 mV stepping by 10mV with interval time of 5 sec (Fig. 1A).

The voltage-dependent inactivation curve (inactivation–voltage curve) was measured by double pulses: precondition pulses (3 sec) ranging from -80 to $+20$ mV by stepping 10 mV and following $+10$ mV test pulse (200 ms) with internal time of 5 sec (Fig. 1B). For all experiments the holding potential was -80 mV.

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): CsCl 140, MgCl₂ 2, Na₂-ATP 5, TEA-Cl 2, HEPES 10, EGTA 10 at pH 7.2 and osmolality 300mOsm. The external solution was composed of (in mM): Choline-Cl 88, KCl 5, MgCl₂ 1, CaCl₂ 2.5, D-Mannitol 106, HEPES 10 at pH 7.4 and osmolality 300mOsm. Hypo and hypertonic external solutions were obtained by adjusting the concentration of D-Mannitol. When the external solution was Krebs-Henseleit solution (KH), it was composed of (in mM): Choline-Cl 145, KCl 5, MgCl₂ 1, CaCl₂ 2.5, 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).

4.3 Data analysis

The amplitude of I_{HVA} was calculated as peak current. Data were analyzed using pClamp (Axon Instruments, Foster City, CA) and SigmaPlot software (SPSS Inc., Chicago, IL). All of data were presented as mean $\pm$ SEM and the significance was indicated as $P < 0.05$ (*) and $P < 0.01$ (**) tested by paired or unpaired 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, which $I_{peak} = I_{peakmax}/[1 + (IC_{50}/C)^n]$, with n as the Hill coefficient, and IC_{50} as the concentration producing 50% inhibition.

4.4 Chemicals

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

8-Br-cAMP (8-Bromoadenosine 3',5'-cyclic monophosphate), 8-Br-cGMP (8-Bromoguanosine-3',5'-cyclomonophosphate sodium salt), H-89 (N-[2-(p-Bromocinnamylamino)ethyl]-5-isoquinolinesulfonamide dihydrochloride), KT5823, PMA (phorbol-12,13-dibutyrate), BIM (Bisindolylmaleimide II), GTP- γ s (Guanosine 5'-O-(3-thiotriphosphate) tetralithium salt), GDP- β s (Guanosine 5' [β -thio]diphosphate trilithium salt), Wortmannin, LY-294002 (2-(4-Morpholinyl)-8-phenyl-1(4H)-benzopyran-4-one hydrochloride), Rp-8-Br-cGMPs (Rp- β -Phenyl-1, N²-etheno-8-bromoguanosine 3',5'-cyclic monophosphorothioate sodium salt hydrate), U73122 and 4α -PDD were prepared as stock solutions in DMSO. The final concentration of DMSO in external solution or pipette solution was $\leq 0.1\%$.

GTP- γ s, GDP- β s, H-89, KT5823, U73122, Wortmannin, LY294002 were present in the pipette solution. Whereas 4α -PDD, 8-Br-cAMP, 8-Br-cGMP, BIM, PMA and Rp-8-Br-cGMPs were applied in the external solution.

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Figure 1. Hypotonicity reduces I_{HVA} in rat TG neurons

A The typical recordings show that I_{HVA} was reduced from -1.4 nA to -0.8 nA when the external solution was changed from 300mOsm to 260mOsm for 3 min and the current recovered to -1.2 nA after washout. The time-course curve was shown before, during and after hypotonicity treatment. **B.** The voltage-current relationship was shown in the I-V curve before and during application of hypotonicity. **C.** In the presence of hypotonicity, G-V curve did not significantly shift. $V_{0.5}$ were -17.3 ± 6.8 mV and -19.6 ± 2.8 mV ($n=10$, paired t test, $P>0.05$), k were 6.5 ± 5.9 and 6.8 ± 3.3 ($n=10$, paired t test, $P>0.05$) for 300mOsm and 260mOsm respectively. Data were transformed from the I-V data shown in B. **D.** In the presence of hypotonicity, inactivation-voltage curve significantly shifted to hyperpolarizing direction. $V_{0.5}$ were -31.3 ± 1.9 mV and -46.3 ± 2.2 mV ($n=10$, paired t test, $P<0.05$), k were -8.5 ± 2.2 and -10.8 ± 3.8 ($n=10$, paired t test, $P<0.05$) for 300mOsm and 260mOsm respectively.

Figure 2. Hypertonicity reduces I_{HVA} in rat TG neurons

A. The typical recordings show that I_{HVA} was reduced from -1.1 nA to -0.7 nA when the external solution was changed from 300mOsm to 322mOsm for 3min but the current did not recover (-0.8 nA) after washout. The time-course curve was shown before, during and after hypertonicity treatment. **B.** I-V curve was shown before and during application of hypertonicity. **C.** In the presence of hypertonicity G-V curve did not shift, in which $V_{0.5}$ were -17.8 ± 3.8 mV and -16.0 ± 4.8 mV ($n=6$, paired t test, $P>0.05$), k were 6.8 ± 4.1 and 6.0 ± 1.9 , ($n=6$, paired t test, $P>0.05$) for 300mOsm and 322mOsm respectively. Data were transformed from the I-V data shown in B. **D.** The typical recordings show that inactivation-voltage curve did not significantly shift with Boltzmann parameters $V_{0.5}$ being -28.6 ± 2.3 mV and -31.5 ± 3.4

mV (n=6, paired *t* test, $P>0.05$), k being -7.4 ± 0.9 and -8.8 ± 1.0 for 300mOsm and 322mOsm respectively (n=6, paired *t* test, $P>0.05$). **E.** I_{HVA} was inhibited by anisotonic stimuli with “V” shape dose–response curve.

Figure 3. 4α-PDD reduces I_{HVA} in rat TG neurons

A. The typical recordings show that I_{HVA} was reduced from -1.2 nA to -0.8 nA in the presence of $1 \mu M$ 4α-PDD for 3 min and the current recovered to -1.0 nA after washout. The time-course curve was shown before, during and after 4α-PDD application. **B.** I-V curve was shown before and during 4α-PDD treatment. **C.** There was no change in the G-V curve with $V_{0.5}$ being -18.3 ± 5.3 mV and -16.1 ± 5.0 mV ($n=8$, paired t test, $P>0.05$), k being 6.1 ± 3.6 and 6.4 ± 8.5 ($n=8$, paired t test, $P>0.05$) respectively before and during 4α-PDD treatment. Data were transformed from the I-V data shown in B. **D.** In the presence of 4α-PDD inactivation-voltage curve significantly shifted to hyperpolarizing direction. $V_{0.5}$ were -29.7 ± 1.2 mV and -36.2 ± 4.9 mV ($n=8$, paired t test, $P<0.05$), k were $2-8.5 \pm 3.1$ and -8.9 ± 3.1 ($n=8$, paired t test, $P<0.05$) before and during 4α-PDD treatment respectively. **E.** The plot showed the percentage inhibition of 4α-PDD at different concentrations of 0.03, 0.1, 0.3, 1.0, 3.0, 10, 30 and 100 μM . The dose-response curve fits to Hill equation with IC_{50} being $1.9 \mu M$ and n being 0.7.

Figure 4. Effects of hypo- and hypertonicity on I_{HVA} in TRPV4^{+/+} and TRPV4^{-/-} mice TG neurons

A. The typical recordings show the effect of hypotonicity on I_{HVA} . In TG neurons from TRPV4^{+/+} mice, after exposure to hypotonicity (260mOsm), I_{HVA} was reduced from -1.6 nA to -1.0 nA and recovered to -1.4 nA after a 3 min wash. In TG neurons from TRPV4^{-/-} mice, I_{HVA} were -1.37 nA, -1.29 nA and -1.35 nA before, during and after hypotonicity was applied. The inhibition by hypotonicity in TRPV4^{-/-} mice ($4.9 \pm 3.6\%$, $n=18$) is significantly different from that in TRPV4^{+/+} mice TG neurons ($43.6 \pm 9.1\%$, $n=18$) (unpaired t test, $P < 0.01$). **B.** The typical recordings show the effect of hypertonicity on I_{HVA} . In TRPV4^{+/+} mice TG neurons, after exposure to hypertonicity (322mOsm), I_{HVA} was reduced from -1.1 nA to -0.6 nA and recovered to -0.8 nA after washout. In TRPV4^{-/-} mice TG neurons, after exposure to hypertonicity, I_{HVA} was reduced from -1.1 nA to -0.6 nA and recovered to -0.7 nA after washout. On the average, I_{HVA} was reduced by $44.7 \pm 8.9\%$ ($n=14$) and $48.0 \pm 9.8\%$ ($n=14$) in TRPV4^{+/+} and TRPV4^{-/-} mice TG neurons respectively (unpaired t test, $P > 0.05$).

Figure 5. The role of G-protein, PKA and PKC system in the inhibition of anisotonicity on I_{HVA}
A. For G-protein system, neither GTP- γ s nor GDP- β s had significant effect on the inhibitions induced by anisotonic stimuli. In hypotonic solution (260mOsm) I_{HVA} was reduced by $37.2 \pm 8.9\%$ (n=33) (unpaired t test, $P>0.05$) and $39.6 \pm 8.6\%$ (n=16) (unpaired t test, $P>0.05$) with GTP- γ s and GDP- β s in the pipette solution, respectively, while in hypertonic solution (322mOsm) I_{HVA} was reduced by $36.8 \pm 9.4\%$ (n=19) (unpaired t test, $P>0.05$) and $32.8 \pm 7.6\%$ (n=18) (unpaired t test, $P>0.05$), respectively. **B.** For PKA system, pre-incubation with H-89 10 min did not statistically alter the inhibitions of I_{HVA} by hypo or hypertonicity, with I_{HVA} being reduced by $33.5 \pm 8.1\%$ (n=14) (unpaired t test, $P>0.05$) and $35.5 \pm 7.2\%$ (n=16) (unpaired t test, $P>0.05$), respectively. **C.** For PKC system, application of BIM did not significantly affect the inhibition induced by hypo or hypertonicity. I_{HVA} was reduced by $45.0 \pm 7.0\%$ (n=13) (unpaired t test, $P>0.05$) and $31.3 \pm 6.6\%$ (n=23) (unpaired t test, $P>0.05$) with $1\mu\text{M}$ BIM in hypotonic and hypertonic solution, respectively.

Figure 6. Inhibition of PKG system selectively reverses the inhibition of hypotonicity on I_{HVA}
A. In the presence of PKG inhibitors KT5823 (10 μ M) or Rp-8-Br-PET-cGMPs (1 μ M), the inhibition of I_{HVA} by hypotonicity was significantly reduced to $13.8 \pm 5.4\%$ ($n=12$) (unpaired t test, $P<0.01$) and $11.2 \pm 3.1\%$ ($n=7$) (unpaired t test, $P<0.01$) respectively. **B.** Application of KT5823 and Rp-8-Br-PET-cGMPs had no significant effect on the inhibition by hypertonicity and I_{HVA} was reduced by $38.8 \pm 9.5\%$ ($n=22$) (unpaired t test, $P>0.05$) and $37.4 \pm 7.0\%$ ($n=8$) (unpaired t test, $P>0.05$), respectively.

Figure 7. Lipids system is selectively involved in the inhibition of hypertonicity on I_{HVA}
A I_{HVA} was reduced by $43.2 \pm 9.0\%$ ($n=15$), $41.2 \pm 6.1\%$ ($n=16$) and $42.0 \pm 7.9\%$ ($n=18$) in hypotonic solution with Wortmannin, LY294002 and U73122 in the pipette solution respectively. Compared with the inhibition by hypertonicity in normal pipette solution ($38.3 \pm 10.6\%$, $n=33$), none of them was significantly different (unpaired t test, $P > 0.05$). **B.** Pre-incubation with Wortmannin, LY294002 and U73122 significantly reversed the inhibition of I_{HVA} by hypertonicity from $37.1 \pm 9.0\%$, ($n=22$) to $22.5 \pm 10.3\%$ ($n=11$) (unpaired t test, $P < 0.05$), $18.9 \pm 6.5\%$ ($n=16$) (unpaired t test, $P < 0.01$) and $23.0 \pm 8.2\%$ ($n=16$) (unpaired t test, $P < 0.05$), respectively.

Table 1Effect of second messengers systems on I_{HVA}

Second messenger system		I_{HVA} (pA/pF)		n
		300mOsm	300+agonist (antagonist)	
G-protein	GTP- γ s (0.3 mM)	-29.5 $\pm$ 2.3	-13.8 $\pm$ 1.8**	26
	GDP- β s (0.3 mM)	-27.8 $\pm$ 3.7	-21.2 $\pm$ 3.5**	19
PKA	8-Br-cAMP (1 mM)	-29.1 $\pm$ 4.6	-33.0 $\pm$ 9.1*	12
	H-89 (10 μ M)	-29.5 $\pm$ 3.9	-25.4 $\pm$ 3.3*	17
PKC	PMA (1 μ M)	-28.2 $\pm$ 6.3	-32.6 $\pm$ 7.3*	7
	BIM (1 μ M)	-28.7 $\pm$ 1.9	-24.4 $\pm$ 1.4*	10
PKG	8-Br-GMP (1 mM)	-30.7 $\pm$ 1.1	-26.4 $\pm$ 2.0*	7
	KT5823 (10 μ M)	-28.4 $\pm$ 4.5	-31.5 $\pm$ 5.1*	13
Lipids system	Rp-8-Br-PET-cGMPs(1 μ M)	-27.9 $\pm$ 5.7	-32.1 $\pm$ 1.6*	10
	Wortmannin (2 μ M)	-29.5 $\pm$ 3.7	-20.6 $\pm$ 1.8*	13
	LY294002 (50 μ M)	-29.9 $\pm$ 2.3	-18.5 $\pm$ 1.6*	16
	U73122 (10 μ M)	-28.7 $\pm$ 3.8	-22.9 $\pm$ 2.4*	10

Paired t test was used when comparing the effect of agonists or antagonists on I_{HVA} in the isotonic solution (300mOsm).

* $P < 0.05$,

** $P < 0.01$.