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The modulation of voltage-gated potassium channels by anisotonicity in trigeminal ganglion neurons

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Abstract

Voltage-gated potassium channels (VGPCs) play an important role in many physiological functions by controlling the electrical properties and excitability of cells. Changes in tonicity in the peripheral nervous system can activate nociceptors and produce pain. Here, using whole cell patch clamp techniques, we explore how hypo and hypertonicity modulate VGPCs in cultured rat and mouse trigeminal ganglion (TG) neurons. We found that hypo and hypertonicity had different effects on slow-inactivating K^+ current (I_K) and fast-inactivating K^+ current (I_A): hypotonicity increased I_K but had no effect on I_A while hypertonicity depressed both I_K and I_A. The increase of I_K by hypotonicity was mimicked by Transient Receptor Potential Vanilloid 4 (TRPV4) receptor activator (4α-PDD) but hypotonicity did not exhibit increase in TRPV4$^{-/-}$ mice TG neurons, suggesting that TRPV4 receptor was involved in hypotonicity induced response. We also found that inactivation of PKC selectively reversed the increase of I_K by hypotonicity, whereas antagonism of G-protein selectively rescued the inhibitions of I_K and I_A by hypertonicity, indicating that different intracellular signaling pathways were required for the modulation by hypo and hypertonicity. In summary, changes in osmolality have various effects on I_K and I_A and different receptors and second messenger systems are selective for the modulation of VGPCs induced by hypo versus hypertonicity.

Keywords

voltage-gated potassium channels; hypotonicity; hypertonicity; TRPV4; intracellular signaling pathway; second messenger system

Many studies have shown that osmolarity plays an important role in the regulation of neurons excitability and neurotransmitter release (Andrew et al., 1989; Azouz et al., 1997; Baraban and Schwartzkroin, 1998; Kilb et al., 2006; Saly and Andrew, 1993; Traynelis and Dingledine, 1989). Through this regulation, changes in osmolality can cause many disorders, such as

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epilepsy (Kilb et al., 2006; Schwartzkroin et al., 1998) in the central nerve system and pain in the peripheral nerve system (Alessandri-Haber et al., 2005; Alessandri-Haber et al., 2004; Alessandri-Haber et al., 2003; Suzuki et al., 2003). Recently, there are evidences that both hypo and hypertonic stimuli can activate C-fiber afferent and induce pain (Alessandri-Haber et al., 2005; Alessandri-Haber et al., 2004; Alessandri-Haber et al., 2003; Suzuki et al., 2003). Besides this, hypo and hypertonicity also facilitate the nociceptive signal transductions induced by pH (Hamamoto et al., 2000), capsaicin (Liu et al., 2007), carrageenan (Alessandri-Haber et al., 2006) and mechanical stimuli (Alessandri-Haber et al., 2004; Alessandri-Haber et al., 2006) and are involved in inflammatory and neuropathic pain (Alessandri-Haber et al., 2006; Alessandri-Haber et al., 2005; Alessandri-Haber et al., 2004). Transient Receptor Potential Vanilloid 4 (TRPV4) is one of the members of Transient Receptor Potential Vanilloid (TRPV) family, which can be activated by hypotonicity, modest heat, mechanical stimulus, synthetic activators like 4α-phorbol-12,13-didecanoate (4α-PDD) and endogenous agonists derived from arachidonic acid and its metabolites (Liedtke and Kim, 2005; Voets et al., 2002; Vriens et al., 2004). Many studies report that TRPV4 receptor is involved in hypo and hypertonicity induced nociception (Alessandri-Haber et al., 2005; Alessandri-Haber et al., 2004; Alessandri-Haber et al., 2003; Suzuki et al., 2003).

Voltage-gated potassium channels (VGPCs) comprise 12 families (named K_v1 to K_v12) and each family consists of several subunits (Gutman et al., 2005; Misonou and Trimmer, 2004). It is reported that more and more α subunits as well as β subunit of VGPCs have been found in rat trigeminal ganglion (TG) (Gutman et al., 2005; Rasband and Trimmer, 2006). VGPCs show significant diversity in their biophysical and pharmacologic properties and there are at least two types of VGPC currents in sensory neurons: slow-inactivating potassium current (I_K), displaying slow or sometimes incomplete inactivation and fast-inactivating potassium current (I_A) exhibiting fast inactivation kinetics (Catacuzzeno et al., 2003; Gold et al., 1996; Grunewald, 2003; Stewart et al., 2003). VGPCs participate in the action potential (AP) and determine the excitability of cells by regulating the resting membrane potential, the threshold of AP and the frequency of AP firings (Rasband and Trimmer, 2006; Yost, 1999). Studies show that VGPCs are involved in the nociceptive signal transduction induced by inflammatory mediators and nerve injury in primary sensory neurons (Everill and Kocsis, 1999; Harriott et al., 2006; Ishikawa et al., 1999; Rasband et al., 2001; Sculptoreanu et al., 2004; Stewart et al., 2003; Takeda et al., 2006; Xu et al., 2006; Yoshimura and de Groat, 1999) and are considered as important targets in the development of new analgesic (Ocana et al., 2004). In this study we tested whether VGPCs were modulated by hypo and hypertonicity and whether TRPV4 receptor and specific second messenger pathway were involved in these effects.

EXPERIMENTAL PROCEDURES

Cell culture

Trigeminal ganglion (TG) neurons from male Sprague–Dawley rats (180–200 g) were cultured as described previously (Liu et al., 2004). TG neurons from C57BL/6 wild type and TRPV4 knockout (Liedtke and Friedman, 2003) mice were cultured using similar method for adult rats. Briefly, trigeminal ganglions were dissected aseptically and washed with cold (4 °C) modified Hank's balanced salt solution (mHBSS). 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 10μg/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 h 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.

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 > 5mV 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 15–30 μm were used.

The voltage-dependent activation curve (G–V curve) was measured by a series of depolarizing pulses (500 ms) from −100 mV to +50 mV stepping by 10 mV with interval time of 5 s. The voltage–dependent inactivation curve (inactivation–voltage curve) was measured by double pulses: precondition pulses (2 s for I_K and 500 ms for I_A) ranging from −120 mV to +50 mV by stepping 10 mV and following +50 mV test pulse (500 ms) with internal time of 5 s. 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 MΩ when filled with pipette solution composed of (in mM): K-aspartate 120, KCl 20, $CaCl_2$ 1.0, $MgCl_2$ 2.0, Tris-ATP 5.0, HEPES 10, EGTA 10 at pH 7.2 and osmolality 300mOsm. The external solution at pH 7.4 was shown in table 1. I_K and I_A were selectively recorded with 4-AP or TEA-Cl in the extracellular solution. Hypo and hypertonic external solutions were obtained by adjusting the concentration of D-Mannitol. The osmolality was measured using a vapor pressure osmometer (Model 3300, Advanced Instruments, Norwood, MA).

Data analysis

I_K and I_A were measured at the peak outward 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 ± SEM 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, which $I_{peak}=I_{peakmax}/[1+(IC_{50}/C)n]$, with n as the Hill coefficient, and IC_{50} as the concentration producing 50% effect.

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-((17β-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), staurosporine, GTP-γs (Guanosine 5′-O-(3-thiotriphosphate) tetralithium salt), GDP-βs(Guanosine 5′-[β-thio]diphosphate trilithium salt), Wortmannin, LY-294002 (2-(4-Morpholinyl)-8-phenyl-1(4H)-benzopyran-4-one hydrochloride), U73122 and 4α-PDD were prepared as stock solutions in DMSO. The final concentrations in external solution or pipette solution were ≤0.1%.

GTP-γs, GDP-βs, H-89, KT5823, U73122, Wortmannin, LY294002 and staurosporine, were present in the pipette solution. Whereas 4α-PDD, 8-Br-cAMP, 8-Br-cGMP, BIM, PMA were applied in the external solution.

RESULTS

Effect of anisotonicity on I_K and I_A

Hypotonic treatment—In the present study, I_K and I_A were recorded in TG neurons with diameter between 15–30 μm for it has been reported that the small and medium size "nociceptive" TG and DRG neurons and associated Aδ- and C-fiber afferents are critical for detecting noxious stimuli and initiating pain sensation (Cardenas et al., 1995; Harper and Lawson, 1985; Slugg et al., 2000).

Figure 1A shows that I_K was increased reversibly upon exposure to hypotonicity (220mOsm). On the average, I_K was increased by 34.52±8.58% from 513.13±51.10 pA/pF to 687.53±38.76 pA/pF (n=37, paired t test, $P<0.01$). After hypotonicity was washed out the current recovered to 523.34±22.72 pA/pF. We also found that G–V curve did not shift (Fig. 1C) but inactivation–voltage curve significantly shifted (32 mV) to the depolarizing direction when the extracellular solution was changed from 300mOsm to 220mOsm (Fig. 1D) (Table 2).

Figure 2A shows that hypotonicity (220mOsm) had almost no effect on I_A. On the average, I_A was 294.46±39.76 pA/pF and 302.84±17.34 pA/pF before and during 220mOsm treatment (n=10, paired t test, $P>0.05$). As shown in figure 2C and 2D, neither G–V nor inactivation–voltage curve of I_A significantly shifted under hypotonic treatment (Table 2).

Hypertonic treatment—Different from the effect of hypotonicity, both I_K and I_A were inhibited irreversibly in the presence of hypertonicity (350mOsm) (Figs. 3 and 4). On the average, I_K was reduced by 34.54±8.21% from 495.18±30.18 pA/pF to 309.11±28.57 pA/pF (n=18, paired t test, $P<0.01$) when the extracellular solution was changed from 300mOsm to 350mOsm (Fig. 3A). I_A was inhibited by 27.55±6.17% from 294.29±18.22 pA/pF to 210.47 ±13.25 pA/pF (n=17, paired t test, $P<0.01$) in the presence of 350mOsm (Fig. 4A). Neither I_K (318.59±54.26 pA/pF) nor I_A (214.98±11.29 pA/pF) recovered to the control level after hypertonicity was washed out. It was also shown that for I_K and I_A, G–V curve as well as inactivation–voltage curve significantly shifted to the hyperpolarizing direction when exposed to hypertonicity (Figs. 3D and 4D) (Table 2).

The dose–dependent modulations of VGPCs by anisotonic treatment are presented in figure 5, in which hypertonic stimuli had the similar phenotype on I_K and I_A whereas hypotonicity had different effects by increasing I_K and leaving I_A unaffected. Since 220mOsm and 350mOsm produced significant effects on VGPCs: 220mOsm increased I_K by 34.52% while 350mOsm

decreased I_K and I_A by 34.54% and 27.55% respectively, these concentrations were used in all subsequent experiments.

Effect of 4α-PDD on I_K and I_A

TRPV4 receptor is regarded as an important osmotic cellular sensor (Liedtke and Friedman, 2003; Liedtke and Kim, 2005). To test the hypothesis that TRPV4 receptors may be involved in the effect of anisotonicity on VGPCs, in rat TG neurons we tested the effect of TRPV4 receptor agonist, 4α-PDD, on I_K and I_A.

Similar to the effect of hypotonicity, I_K was enhanced reversibly by 4α-PDD. On the average, application of 3.0 μM 4α-PDD increased I_K by 43.58±8.05% from 513.26±30.69 pA/pF to 736.93±22.71 pA/pF (n=9, paired t test, $P<0.01$). The increase was recoverable (>90%) after 4α-PDD was washed out. In the presence of 4α-PDD, G–V curve, representing the voltage dependence of activation, did not shift (n=9, paired t test, $P>0.05$) but inactivation–voltage curve significantly shifted to the depolarizing direction (Fig 6, B and C) (Table 2). The concentration–dependent increase of I_K by 4α-PDD is shown in figure 6A. I_K was increased 6.20±3.60% and 81.40±8.80% by 0.03 μM and 30 μM 4α-PDD, respectively. The dose–response curve was fitted by Hill equation with IC_{50} being 4.01 μM.

We also tested the effect of 3.0 μM 4α-PDD on I_A. This concentration was chosen because in our previous studies and here, 4α-PDD at this concentration had significant effects on voltage-gated sodium channels (VGSCs), voltage-gated calcium channels (VGCCs) (unpublished results) and I_K. We found that I_A was 290.17±20.04 pA/pF and 287.13±25.14 pA/pF before and during 3.0 μM 4α-PDD treatment, respectively (n=8, paired t test, $P>0.05$). Moreover, 4α-PDD had no effect on G–V curve or inactivation–voltage curve of I_A (Fig. 6, D and E) (Table 2). These data indicated that 4α-PDD mimicked the effect of hypotonicity on VGPCs.

Hypo- and hypertonicity induced modulations on VGPCs 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 changing the osmolality on VGPCs in TG neurons from TRPV4$^{+/+}$ and TRPV4$^{-/-}$mice with soma diameters ranging between 10–25 μm.

Hypotonic treatment—Figure 7A shows the effects of hypotonicity (220mOsm) on I_K obtained from TRPV4$^{+/+}$ and TRPV4$^{-/-}$ mice. Similar to the results found in rat TG neurons, I_K was increased reversibly from 523.22±10.32 pA/pF to 675.30±16.13 pA/pF after hypotonicity treatment in TRPV4$^{+/+}$ mice TG neurons (n=15, paired t test, $P<0.05$). In contrast, I_K was 486.42±35.52 pA/pF and 493.75±31.43 pA/pF before and during hypotonicity application in TRPV4$^{-/-}$ mice TG neurons (n=17, paired t test, $P>0.05$). Significant difference was found in the increase of I_K by hypotonicity between TRPV4$^{+/+}$ (29.21±7.80%) and TRPV4$^{-/-}$ mice (1.41±2.72%) (unpaired t test, $P<0.01$).

Figure 7B shows that hypotonicity had no effect on I_A in either TRPV4$^{+/+}$ (n=14, paired t test, $P>0.05$) or TRPV4$^{-/-}$ mice TG neurons (n=13, paired t test, $P>0.05$).

Hypertonic treatment—I_K was inhibited irreversibly when the extracellular solution was changed from 300mOsm to 350mOsm. In TRPV4$^{+/+}$ and TRPV4$^{-/-}$ TG neurons, I_K was reduced by 38.82±7.25% (n=14, paired t test, $P<0.01$) and 38.52±6.38% (n=16, paired t test, $P<0.01$) respectively, which was not statistically different (unpaired t test, $P>0.05$) (Fig 7C).

Similar to the inhibition of I_K, in the presence of hypertonicity, I_A was inhibited irreversibly by 30.68±7.16% (n=14, paired t test, $P<0.05$) and 33.31±6.61% (n=14, paired t test, $P<0.05$)

in TRPV4$^{+/+}$ and TRPV4$^{-/-}$ TG neurons, respectively (Fig 7D). No statistical difference was found in the reduction of I_A by hypertonicity between TRPV4$^{+/+}$ and TRPV4$^{-/-}$ mice (unpaired t test, P>0.05).

In summary, these data indicated that anisotonicity had similar effect on VGPCs in TG neurons from rats and TRPV4$^{+/+}$ mice. However, it was obvious that the increase of I_K by hypotonicity seen in TRPV4$^{+/+}$ mice was markedly reduced in TRPV4$^{-/-}$ mice, suggesting the involvement of TRPV4 receptor in the increase of I_K by hypotonic stimulus.

Second messenger systems involved in the modulation of anisotonicity on I_K and I_A

It is known that VGPCs, as well as VGCCs and VGSCs, can be modulated by many intracellular pathways through phosphorylation or dephosphorylaion. In this study, we tested several intracellular pathways in rat TG neurons to determine which, if any, of them takes part in the modulation of I_K and I_A by hypo and hypertonicity.

PKC system—We firstly tested whether PKC system was involved in the action of anisotonicity on I_K and I_A. In this study, after exposure to1.0 μM phorbol-12, 13-dibutyrate (PMA, the agonist of PKC) for 3 min, I_K was inhibited by 10.01±3.12% (n=19, paired t test, P<0.05) but I_A was not apparently affected (n=12, paired t test, P>0.05). Application of 1.0 μM Bisindolylmaleimide II (BIM, PKC antagonist) and 1.0 μM staurosporine (PKC antagonist) increased I_K by 8.17±3.11% (n=17, paired t test, P<0.05) and 9.11±4.39% (n=9, paired t test, P<0.05) respectively, leaving I_A unchanged. As shown in figure 8, application of BIM and staurosporine significantly blocked the increase of I_K by hypotonicity whereas unaffected the inhibitions of I_K or I_A by hypertonicity. These results suggested the selective involvement of PKC system in the increase of I_K by hypotonicity.

G-protein system—We then studied G-protein system using GTP-γs (non-hydrolyzable GTP analog) and GDP-βs (non-hydrolyzable GDP analog). With 0.3 mM GTP-γs or 0.3 mM GDP-βs in the pipette solution, I_K was reduced by 9.90±1.54% (n=27, paired t test, P<0.05) and 10.49±2.60% (n=24, paired t test, P<0.05) respectively, whilst I_A by 19.00±3.02% (n=10, paired t test, P<0.01) and 19.36±5.74% (n=10, paired t test, P<0.01), respectively. Neither GTP-γs nor GDP-βs apparently affected the increase of I_K by hypotonicity (Fig. 9A). However, both GTP-γs and GDP-βs reversed the inhibitions of I_K and I_A by hypertonicity (Fig. 9B). This result suggested that G-protein system was selectively required for the inhibitions of VGPCs by hypertonicity.

PKA system—We next explored PKA system by using 8-Br-cAMP (a kind of membrane permeable analogue of cAMP, agonist of PKA) and H-89 (antagonist of PKA). After exposure to 1.0 mM 8-Br-cAMP, I_K and I_A were decreased by 9.16±2.16% (n=11, paired t test, P<0.05) and 10.25±4.47% (n=8, paired t test, P<0.05), respectively. After pre-application of 10 μM H-89 for 10 min, I_K and I_A were enhanced by 9.60±2.39% (n=13, paired t test, P<0.05) and 10.26±5.85% (n=13, paired t test, P<0.05), respectively. As shown in figure 10A, pre-application of H-89 had no significant effect on the increase of I_K by hypotonicity when compared with that in normal pipette solution (unpaired t test, P>0.05). Also, H-89 did not affect the inhibitions of I_K or I_A by hypertonicity (unpaired t test, P>0.05). These data indicated that PKA system was not necessary for the actions of anisotonic stimuli on VGPCs.

Lipids cascade—Phosphatidylinositol 3-kinase (PI3K) is a kind of lipid kinase and reported to modulate potassium current amplitude and its development (Oliver et al. 2004). It has been reported that phosphatidylinositol-4′5′-bisphosphate (PIP_2) can modulate the function of VGPCs (Wu et al. 1998). Here, LY294002 (PI3K inhibitor) and Wortmannin (PI3K and PI4K inhibitor) were used to test whether the actions on I_K or I_A at different tonicities could be

affected. With 2.0 μM Wortmannin or 50 μM LY294002 in the pipette solution, I_K was reduced by 27.81±2.39% (n=22, paired t test, $P<0.01$) and 14.94±6.94% (n=18, paired t test, $P<0.05$) respectively, while I_A by 22.61±7.06% (n=22, paired t test, $P<0.01$) and 27.84±5.64% (n=14, paired t test, $P<0.01$) respectively. We found that neither LY294002 nor Wortmannin reversed the increase of I_K by hypotonicity (unpaired t test, $P>0.05$). Similarly, neither of them blocked the inhibitions of I_K or I_A by hypertonicity (unpaired t test, $P>0.05$) (Fig. 10B).

U73122 is the PLC inhibitor which can increase the concentration of PIP_2 and decrease the production of IP_3 and DAG. With 10 μM U73122 in the pipette solution, I_K and I_A were reduced by 16.78±6.82% (n=22, paired t test, $P<0.05$) and 20.09±6.97% (n=12, paired t test, $P<0.01$), respectively. Figure 10B shows that pre-application of U73122 didn't affect the actions on I_K or I_A when treated with anisotonicity. These data indicated that lipids cascade was not involved in the actions on VGPCs under hypo or hypertonic conditions.

PKG system—We also performed the experiment to investigate whether PKG system was responsible for the modulations of I_K or I_A by changes in osmolality. Exposure to 1.0 mM 8-Br-cGMP (a kind of membrane permeable analogue of cGMP, agonist of PKG) for 3 min had no effect on I_K (n=12, paired t test, $P>0.05$) but decreased I_A by 15.03±3.33% (n=10, paired t test, $P<0.05$). After pre-incubation with 10 μM KT5823 (antagonist of PKG), I_A was increased by 9.77±2.09% (n=14, paired t test, $P<0.05$), while I_K was unaffected (n=24, paired t test, $P>0.05$). Figure 10C shows that KT5823 did not markedly affect the actions on I_K or I_A when the extracellular osmolality deviated from isotonicity, indicating that PKG system was not required for the effects of anisotonic stimuli on VGPCs.

DISCUSSION

Modulation of VGPCs by anisotonicity

TG neurons are primary afferent neurons that carry sensory signals from the face, oral cavity, and nasal cavity to the medulla (Sessle, 1999). Many nociceptive stimuli are known to change the neuronal excitability at peripheral nerve endings of the TG neurons (Davies, 1988; Lazarov, 2002), so in the present study, TG neurons were used as a pain model to study the tonicity induced tuning of VGPCs. We demonstrate that VGPCs, including I_K and I_A, could be modulated by the changes of osmolality in cultured TG neurons. The modulation of anisotonicity on VGPCs is type-dependent. Both I_K and I_A were inhibited by hypertonic solutions, but after exposure to hypotonicity I_K was markedly increased whilst I_A was left unaffected. In this study, we also performed the experiment by using the calcium buffer and found the increase of I_K by hypotonicity and inhibition of I_K and I_A by hypertonicity (data not shown). So it was indicated that the modulation of VGPCs by anisotonicity was calcium independent. We then explored the role of TRPV4 receptor and found that TRPV4 might underlie the modulation of I_K by hypotonicity. Considering the downstream signaling pathways, different intracellular messenger systems were selective for the modulation of VGPCs induced by hypo versus hypertonicity.

Participation of VGPCs in hypertonicity-induced nociception

VGPCs play fundamental roles, such as regulation of membrane repolarization, resting membrane potential, and frequency of firing and neurotransmitter release (Mathie et al., 1998; Yellen, 2002). Studies show that the inhibition of I_K can depolarize the cell membrane, prolong AP duration, reduce the amplitude of afterhyperpolarization and lower the threshold for AP firing (Birinyi-Strachan et al., 2005; Safronov et al., 1996), while the inhibition of I_A can induce repetitive firing (Amir et al., 2002; Storm, 1988). It is widely accepted that a decrease of VGPCs, including I_K and I_A, can increase the excitability of cells (Birinyi-Strachan et al., 2005; Yost, 1999). Recent evidences suggest that VGPCs participate in nociceptive signal

transduction induced by inflammatory mediators (Harriott et al., 2006; Sculptoreanu et al., 2004; Stewart et al., 2003; Takeda et al., 2006; Yoshimura and de Groat, 1999; Xu et al., 2006) and nerve injury (Everill and Kocsis, 1999; Ishikawa et al., 1999; Rasband et al., 2001) in primary sensory neurons. For example, it is found that in temporomandibular joint inflammation the excitability of rat trigeminal root ganglion neurons is enhanced via the decrease of I_A (Takeda et al., 2006) and the excitability of masseter muscle afferents is increased by inhibiting VGPCs in inflammation (Harriott et al., 2006). In the present study, we found that both I_K and I_A were blocked by hypertonicity and larger response was found in the presence of larger osmotic gradient (Fig. 5). In addition to the reduction in current amplitude, inactivation–voltage curves of both I_K and I_A significantly shifted to the hyperpolarizing direction, which could contribute to the observed decreases of I_K and I_A (Figs. 3 and 4). In contrast, no reduction of I_K or I_A was found under hypotonic treatment (Figs. 1 and 2). So it was suggested that the inhibitions of I_K and I_A selectively participated in the nociception induced by hyper but not hypotonicity.

Involvement of TRPV4 receptor in the modulation of I_K by hypotonicity

In the peripheral nervous system, C-fiber afferents can be activated by both hypo and hypertonic stimuli (Alessandri-Haber et al., 2005; Alessandri-Haber et al., 2003; Matran et al., 1989; Viana et al., 2001). Moreover, changes in osmolality can facilitate the nociceptive signal transduction induced by many stimuli (Alessandri-Haber et al., 2006; Alessandri-Haber et al., 2004; Hamamoto et al., 2000; Liu et al., 2007), so it is proposed that anisotonicity plays an important role in the process of nociception. Knockdown of TRPV4 either by gene-disruption or antisense oligonucleotides has proved that TRPV4 is involved in mechanical stimulus as well as hypo and hypertonicity-induced nociception in rodents (Alessandri-Haber et al., 2005; Alessandri-Haber et al., 2004; Alessandri-Haber et al., 2003; Suzuki et al., 2003). In the present study, we found that the increase of I_K by hypotonicity was well confirmed by TRPV4 agonist (4α-PDD), that is, the increase is reversible and accompanied with inactivation–voltage curve depolarization shifting (Figs. 1 and 6). Furthermore, we found that hypotonicity did not exhibit increase of I_K in TRPV4-/- mice TG neurons. By contrast, the inhibitory effect of I_K or I_A by hypertonicity was unaffected in TRPV4$^{-/-}$ mice TG neurons (Fig. 7). These data indicated that TRPV4 receptor selectively participated in the increase of I_K by hypotonicity. In this study, we found that I_A was unaffected by hypotonicity in TRPV4$^{-/-}$ mice TG neurons, but we were also aware that I_A was not affected by hypotonicity in TRPV4$^{+/+}$ mice and was not changed by 4α-PDD or hypotonicity in rat TG neurons. These results therefore indicated that TRPV4 had no modulatory effect on I_A.

Besides the hypotonic solution, TRPV4 has been shown to be activated by other stimuli. Many studies report the role of TRPV4 as the thermosensitive receptor and TRPV4 can be activated by warm temperature (27–35 ºC) (Gao et al., 2003; Guler et al, 2002; Todaka et al., 2004). Given the fact that TRPV4 can be activated at a temperature of 35°C, it is possible that TRPV4 might become sensitized in the brain because the body core temperature is ~37 °C. Therefore we suggest that the hypotonicity induced tuning of I_K might be facilitated at physiological condition (37 °C) or sometimes at pathological conditions (for instance, at a temperature of 40 °C during a fever).

In the present study, it was noted that effects of hypotonicity and TRPV4 receptor were complicated. On one hand, hypotonicity and TRPV4 could induce the inward current (Alessandri-Haber et al., 2003; Vriens et al., 2004) and enhance the capsaicin-induced current (Liu et al., 2007) which facilitate the depolarization of AP and increase the excitability of neurons, resulting in algetic effect. On the other hand, they facilitate I_K and inhibit VGCCs and VGSCs (unpublished results), which decreases the excitability of neurons and leads to the analgesic effect. If the hypotonicity induced algetic effect is more profound than the analgesic

effect, nociception will be induced as an integrated result. As is known that the abnormal excitability of nociceptor is mediated by changes in function of many ion channels including voltage-gated channels and receptors, therefore the nociception induced by hypertonicity just as that by hypotonicity is very likely an integrative result.

Here, it was also noteworthy that although both hypo and hypertonic stimuli can induce pain behavior, the mechanism may be different. Considering the modulation of VGPCs, first, hypo and hypertonicity exhibit different phenotype: hypotonicity increased I_K but had no effect on I_A, whereas hypertonicity decreased both I_K and I_A and second, the increase of I_K by hypotonicity was TRPV4 dependent, while the inhibitions of I_K and I_A by hypertonicity was TRPV4 independent.

Given that TRPV4 was not involved in the inhibition of VGPCs by hypertonicity, there might be other receptors that mediate hypertonic response. In fact, there are other possibilities underlying the inhibition induced by hypertonic solution. For instance, the shrinking membrane by hypertonicity might exert lateral pressure on VGPCs, which causes incomplete opening of channels. However, in many cases, hypertonic shrinkage is partially rescued by regulatory volume increase (RVI), so in order to properly define the role of membrane mechanism, actual membrane tension will have to be measured during the experiment. Another possibility is that the inhibition of VGPCs by hypertonicity was mediated through intracellular messengers or regulator substance. Next we will discuss some candidates accounting for the modulation of VGPCs by anisotonic treatment.

Involvement of specific intracellular pathways in the modulations of VGPCs by hypo versus hypertonicity

VGPCs are modulated by numerous second messenger pathways, such as G-protein (ffrench-Mullen et al., 1994; Galeotti et al., 1999; Galeotti et al., 2001; Ocana et al., 2004), cAMP-PKA (Akins and McCleskey, 1993; Hampson et al., 1995; Jonas and Kaczmarek, 1996; Mason et al., 2002; Mu et al., 2000), cGMP-PKG (Liu and Simon, 2003; Moreno et al., 2001; Ocana et al., 2004), PKC (Doerner et al., 1988; Jonas and Kaczmarek, 1996; Lotan et al., 1990; Sun et al., 2003) and lipids cascade (Sun et al., 2003; Sun et al., 2004). Our present study shows that the activities of I_K and I_A are modulated by the above intracellular pathways.

G-protein is an important modulator for VGPCs. Intracellular dialysis with G-protein antagonist (GDP-βs) can significantly diminish the Carbachol-induced inhibition of I_K (ffrench-Mullen et al., 1994). Recently it is reported that the modulation of G-protein on VGPCs underlies the antinociceptive effects for tricycles antidepressants and H1-antihistamines (Galeotti et al., 1999; Galeotti et al., 2001; Ocana et al., 2004). In this study, we found that the inhibitions of I_K and I_A by hypertonicity were significantly reversed by pretreatment with G-protein antagonist (GDP-βs) but the increase of I_K by hypotonicity was unaffected, indicating the selective involvement of G-protein in hypertonic induced response (Fig. 8).

PKC also plays an important role in modulating the activities of VGPCs. For example, PKC is involved in the inhibition of angiotensin II on delayed rectifier potassium current (Du et al., 2004; Pan et al., 2001) and the modulation of delayed rectifier potassium channels by alpha1-adrenergic activation (Kim et al., 2005). Consisting with the report in hippocampus neurons (Doerner et al., 1988), activation of PKC selectively reduced I_K but had no effect on I_A. Here we found that the increase of I_K by hypotonicity was markedly reversed by PKC antagonists (BIM and staurosporine), whereas the inhibitions of I_K and I_A by hypertonicity were not affected, implying the selective participation of PKC system in hypotonic induced response. Collectively, these data demonstrated that specific and different intracellular pathways were involved in the modulation of VGPCs by hypo versus hypertonicity (Fig. 9). However, we are

also aware that antagonism of G-protein and PKC did not reverse the modulation of VGPCs by hypo or hypertonicity completely, so there might be other mechanisms, such as some factors that can be affected by changes in the cell volume, contributing to the modulations.

CONCLUSION

Our present study demonstrated that changes in osmolality were one of the important regulators of VGPCs. TRPV4 receptor was selectively involved in the increase of I_K by hypotonicity and different intracellular pathways were required for the actions on I_K and I_A induced by hypo versus hypertonicity. We also found that both I_K and I_A were inhibited by hypertonicity and this probably contributes to hypertonicity-induced nociception. Given the obvious tonicity-induced tuning of VGPCs, it is suggested that besides the anisotonicity-induced nociception, changes in osmolality may participate in many physiological and pathological functions via the modulation of VGPCs.

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Abbreviations used in this manuscript

AP
: action potential

BIM
: Bisindolylmaleimide II

DRG
: dorsal root ganglion

4α-PDD
: 4α-phorbol-12,13-didecanoate

8-Br-cAMP
: 8-Bromoadenosine 3′,5′-cyclic monophosphate

8-Br-cGMP
: 8-Bromoguanosine-3′,5′-cyclomonophosphate sodium salt

GDP-βs
: Guanosine 5′-[β-thio]diphosphate trilithium salt

GTP-γs
: Guanosine 5′-O-(3-thiotriphosphate) tetralithium salt

G–V curve
: voltage dependent activation curve

H-89
: N-[2-(p-Bromocinnamylamino)ethyl]-5-isoquinolinesulfonamide dihydrochloride

I_A
: fast-inactivating K+ current

I_K

slow-inactivating K+ current

inactivation–voltage curve
voltage dependent inactivation curve

KT5720, (9S,10S,12R)-2,3,9,10,11,12-Hexahydro-10-hydroxy-9-methyl-1-oxo-9,12-e poxy-1H-diindolo[1,2,3-f
3′,2′,1′-kl]pyrrolo[3,4-i][1,6]benzodiazocine-10-carboxylic acid hexyl ester

LY290022
2-(4-Morpholinyl)-8-phenyl-1(4H)-benzopyran-4-one hydrochloride

mHBSS
modified Hank's Balanced Salt solution

PMA
Phorbol 12-myristate 13-acetate

RVI
regulatory volume increase

TG
trigeminal ganglion

TRPV4
Transient Receptor Potential Vanilloid 4

TRPV
Transient Receptor Potential Vanilloid

U73122
1-[6-((17β-3-Methoxyestra-1,3,5(10)-trien-17-yl) amino) hexyl]-1H-pyrrole-2,5-dione)

VGPCs
voltage-gated potassium channels

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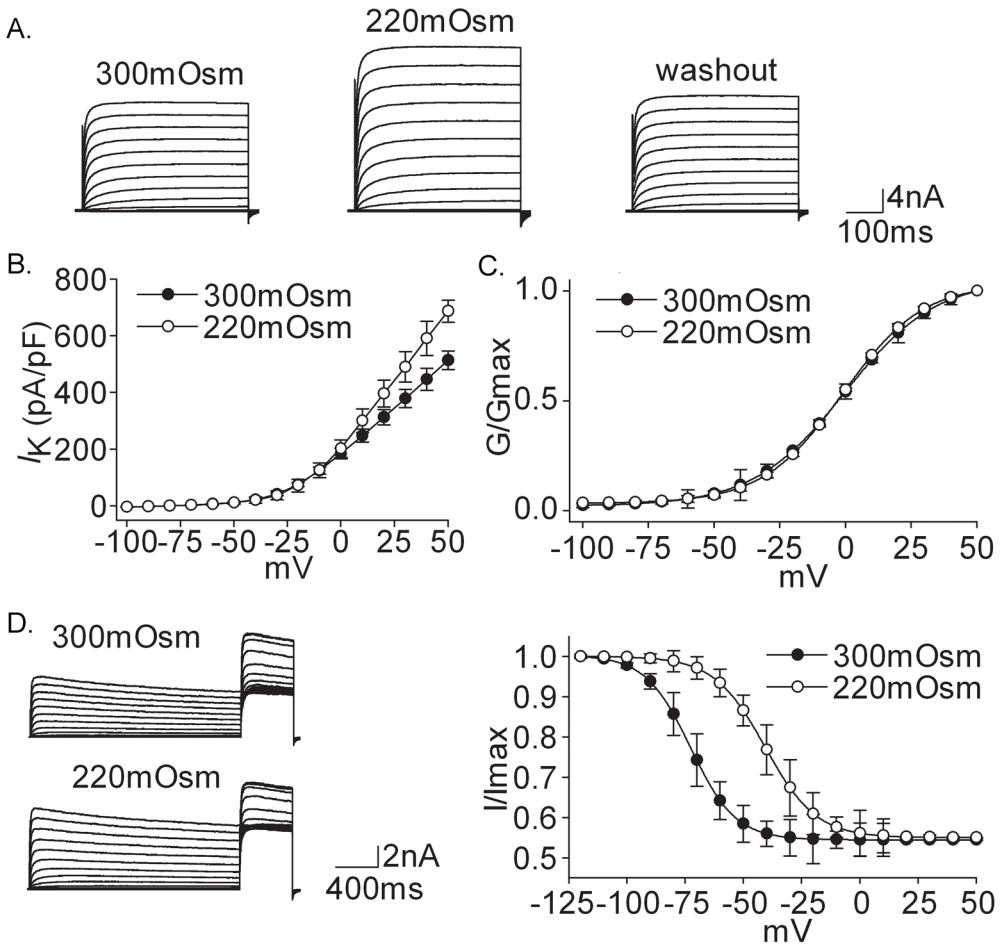

Figure 1. Effect of hypotonicity on I_K

A. The typical recordings show that I_K, evoked by voltage step to +50 mV, was increased from 4.57 nA to 6.38 nA when the external solution was changed from 300mOsm to 220mOsm for 3min and the current recovered to 4.77 nA after washout. **B.** The peak current-voltage relationship (I–V) was shown before and during hypotonicity treatment. **C.** In the presence of hypotonicity, G–V curve did not shift significantly. $V_{0.5}$ was −0.93±1.30 mV and −0.90±0.71 mV (n=8, paired t test, P>0.05) for 300mOsm and 220mOsm, respectively; k was 16.46±1.13 and 16.07±0.41 (n=8, paired t test, P>0.05), respectively. Data were transformed from the I–V data shown in B. **D**. The typical recordings show that hypotonicity increased I_K and inactivation–voltage curve significantly shifted to depolarizing direction. $V_{0.5}$ was −72.67 ±1.54 mV and −40.55±5.25 mV (n=8, paired t test, P<0.05) for 300mOsm and 220mOsm, respectively; k was −9.67±1.32 and −10.98±4.61 (n=8, paired t test, P>0.05), respectively.

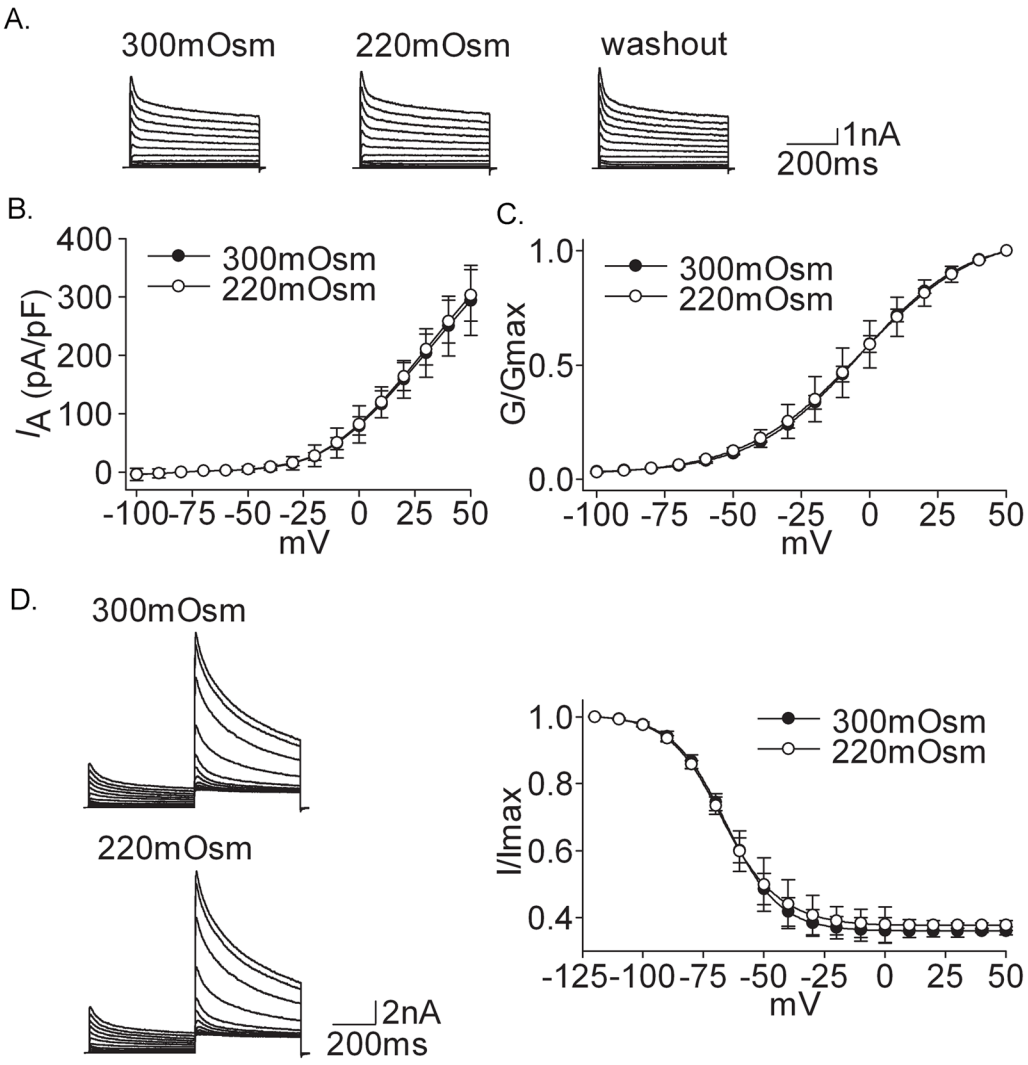

Figure 2. Effect of hypotonicity on I_A

A. After exposure to hypotonicity (220mOsm), I_A was not affected and the peak amplitude was 5.87 nA, 5.91 nA and 5.93 nA before, during and after hypotonicity application, respectively. **B.** I–V curve did not change before and during hypotonicity treatment. **C.** G–V curve did not shift in the presence of hypotonicity. $V_{0.5}$ was −3.57±0.18 mV and −3.33±0.72 mV (n=11, paired t test, P>0.05) for 300mOsm and 220mOsm, respectively; k was 19.47±1.20 and 21.08±0.75 (n=11, paired t test, P>0.05), respectively. Data were transformed from the I–V data shown in B. **D.** Hypotonicity had no effect on inactivation–voltage curve. $V_{0.5}$ was −68.47±0.23 mV and −69.42±0.21 mV (n=11, paired t test, P>0.05) for 300mOsm and 220mOsm, respectively; k was −9.37±0.20 and −9.67±0.15 (n=11, paired t test, P>0.05), respectively.

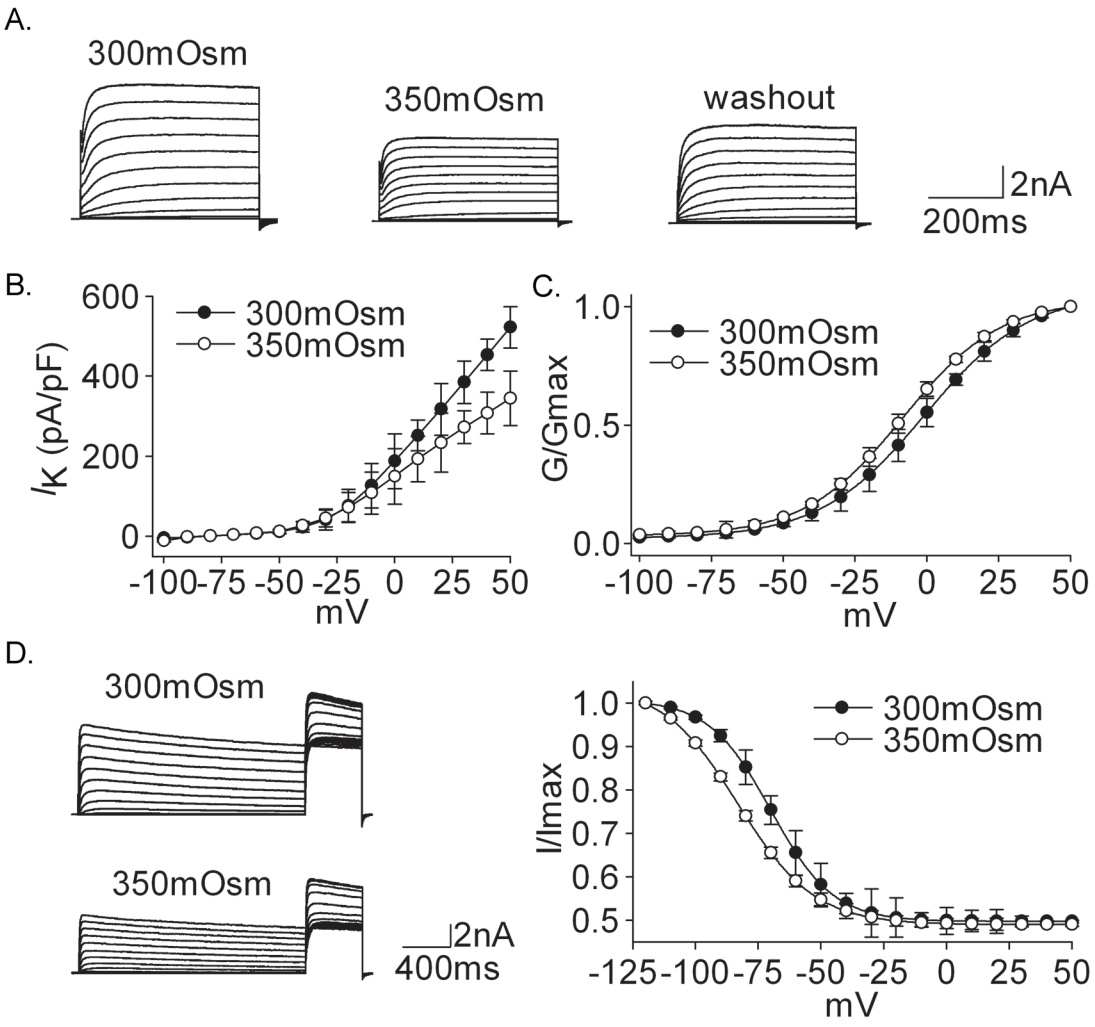

Figure 3. Effect of hypertonicity on I_K

A. The typical recordings show that I_K was reduced from 8.16 nA to 5.32 nA when the external solution was changed from 300mOsm to 350mOsm for 3 min but the current recovered only slightly (5.84 nA) after washout. **B.** I–V curve was shown before and during hypertonicity treatment. **C.** In the hypertonic solution G–V curve shifted to the hyperpolarization direction. $V_{0.5}$ was −0.92±1.23 mV and −8.16±1.02 mV (n=11, paired t test, $P<0.05$) for 300mOsm and 350mOsm, respectively; k was 18.13±1.08 and 17.63±0.95 (n=11, 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 I_K was reduced in the presence of hypertonic solution and inactivation–voltage curve shifted to hyperpolarizing direction with $V_{0.5}$ being −69.99±1.48 mV and −83.00±1.22 mV (n=12, paired t test, $P<0.05$) for 300mOsm and 350mOsm, respectively; k being −11.68±1.28 and −15.19±0.87 (n=12, paired t test, $P<0.05$), respectively.

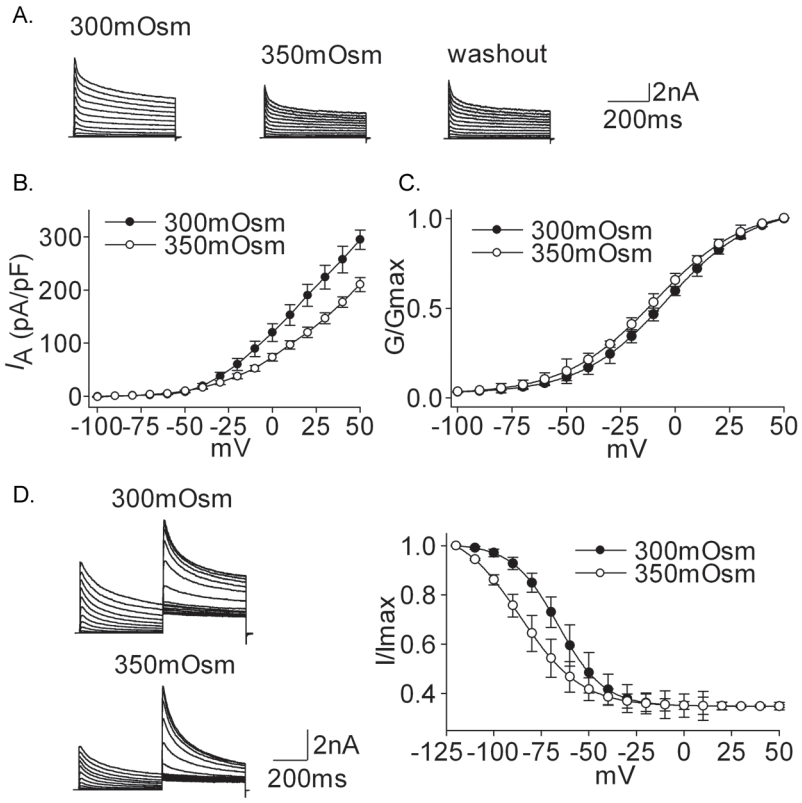

Figure 4. Effect of hypertonicity on I_A

A. The typical recordings show that I_A was reduced from 8.34 nA to 6.01 nA when the external solution was changed from 300mOsm to 350mOsm but the current almost did not recover (6.75 nA) after washout. **B.** I–V curve was shown before and during hypertonicity treatment. **C.** In the hypertonic solution G–V curve shifted to the hyperpolarization direction. $V_{0.5}$ was −3.98 ±0.25 mV and −9.43±0.99 mV (n=10, paired t test, $P<0.05$) for 300mOsm and 350mOsm, respectively; k was 19.16±0.81 and 20.38±0.94 (n=11, 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 I_A was inhibited upon exposure to hypertonic stimulus and inactivation–voltage curve shifted to the hyperpolarizing direction. $V_{0.5}$ was −69.45±0.27 mV and −86.32±0.47 mV (n=12, paired t test, $P<0.05$) for 300mOsm and 350mOsm, respectively; k was −9.93±0.24 and −16.37±0.28 (n=10, paired t test, $P<0.05$), respectively.

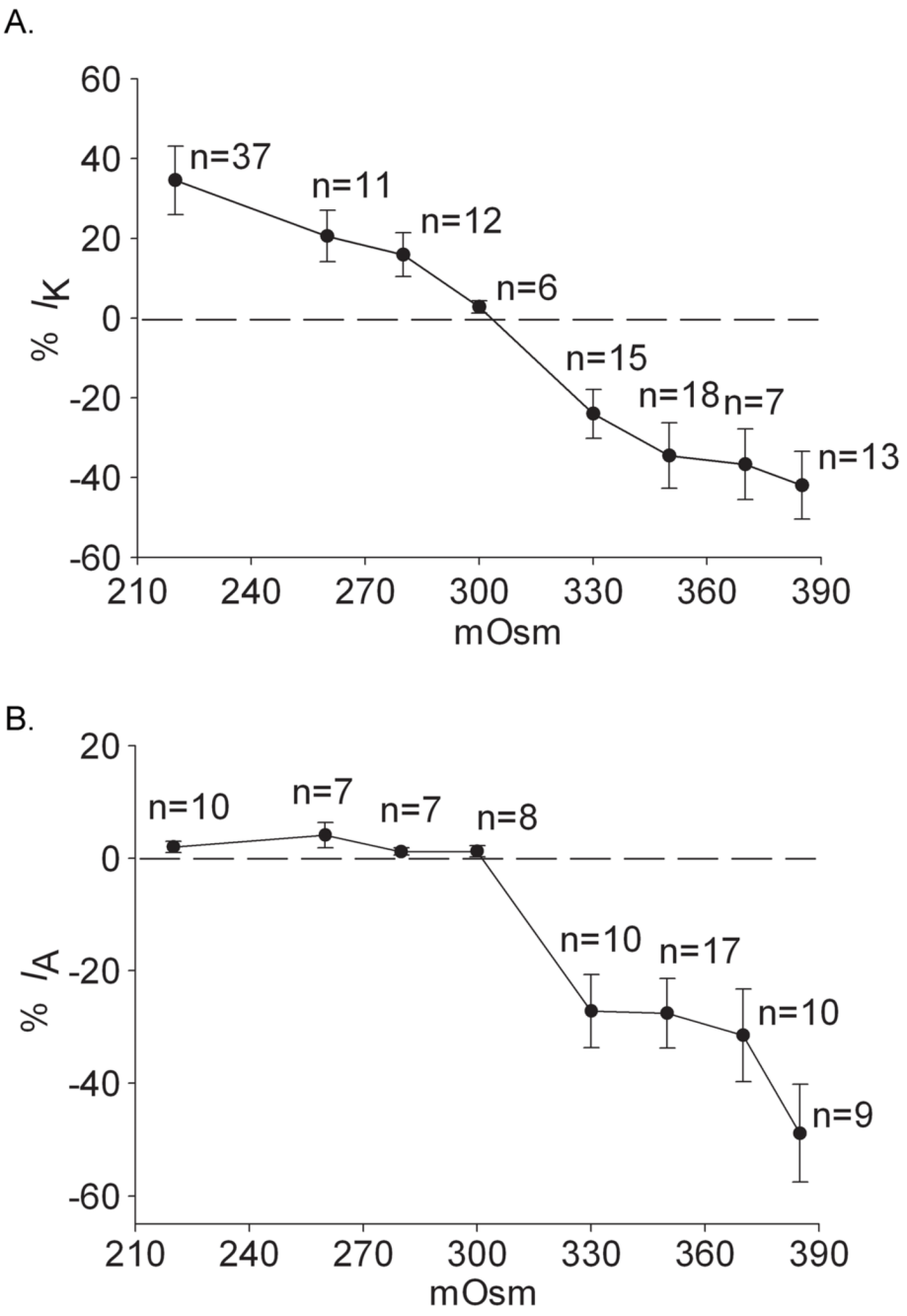

Figure 5. Effect of anisotonicity on I_K and I_A

A. I_K was enhanced by hypotonic stimuli and inhibited by hypertonic stimuli. **B.** I_A was not significantly affected when exposed to hypotonicity but inhibited by hypertonic stimuli.

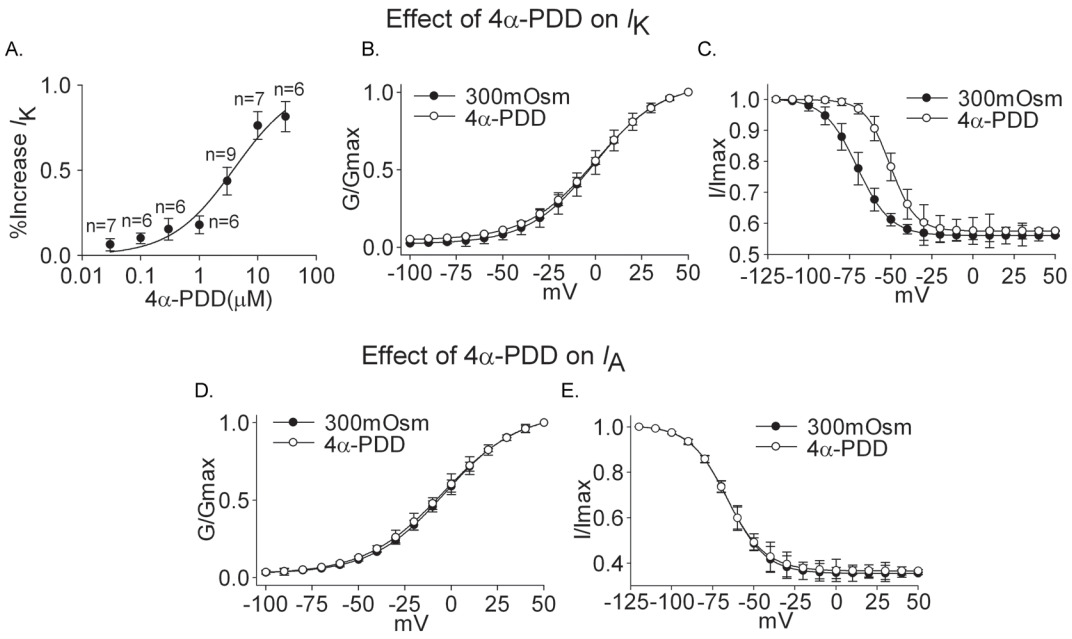

Figure 6. Effect of 4α-PDD on I_K and I_A

A. The plot shows the percentage in increase of I_K by 4α-PDD at concentrations of 0.03, 0.1, 0.3, 1.0, 3.0, 10 and 30 μM. The dose–response curve fits to Hill equation with IC_{50} being 4.01 μM and *n* being 0.80. **B.** G–V curve of I_K did not shift upon exposed to 3.0 μM4α-PDD. $V_{0.5}$ was −0.58±0.60 mV and −0.11±0.48 mV (n=9, paired t test, $P>0.05$) before and during 4α-PDD treatment, respectively; *k* was 17.83±0.53 and 18.38±0.42 (n=9, paired t test, $p>0.05$), respectively. **C.** After application of 3.0 μM 4α-PDD, inactivation–voltage curve of I_K significantly shifted to the depolarizing direction. $V_{0.5}$ was −70.44±0.10 mV and −50.40±0.42 mV (n=9, paired t test, $P<0.05$) before and during 4α-PDD treatment, respectively; *k* was −10.08±0.08 and −7.60±0.36 (n=9, paired t test, $P<0.05$), respectively. **D.** Application of 3.0 μM 4α-PDD had no effect on G–V curve of I_A (300mOsm: $V_{0.5}$=−3.46±0.17 mV, *k*=19.62 ±0.15; 4α-PDD: $V_{0.5}$=−4.42±0.25 mV, *k*=20.80±0.23, n=8, paired t test, $P>0.05$). **E.** Application of 4α-PDD had no effect on inactivation–voltage curve of I_A either (300mOsm: $V_{0.5}$=−65.93±0.29 mV, *k*=−11.49±0.25; 4α-PDD: $V_{0.5}$=−66.38±0.74 mV, *k*=−11.58±0.65, n=8, paired t test, $P>0.05$).

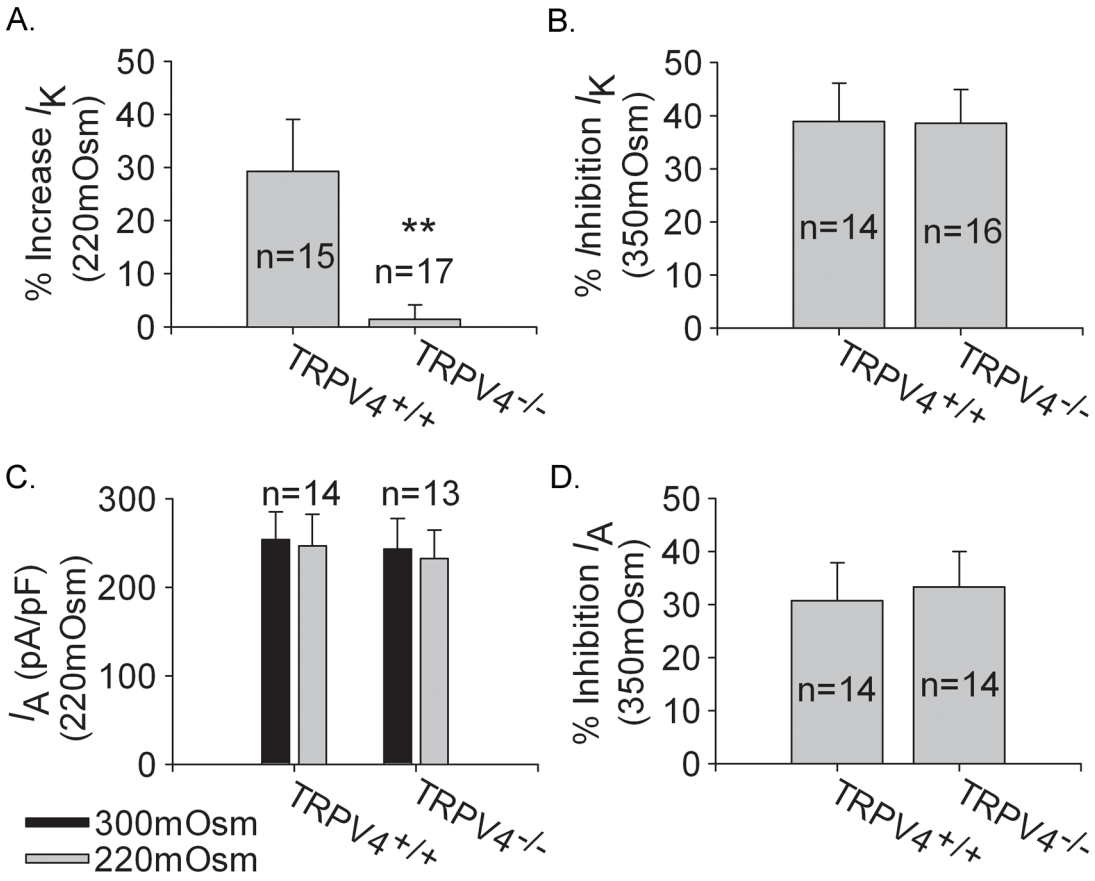

Figure 7. Effects of anisotonicity on I_K and I_A in TG neurons from TRPV4$^{+/+}$ and TRPV4$^{-/-}$ mice

A. Upon exposure to hypotonicity (220mOsm), I_K was increased by 29.21±9.81% (n=15) in TRPV4$^{+/+}$ mice TG neurons, which was significantly different from that in TRPV4$^{-/-}$ mice (1.41±2.72%, n=17) (unpaired t test, $P<0.01$). **B.** After exposure to hypotonicity (220mOsm), I_A was not significantly affected. On the average, in TRPV4$^{+/+}$ mice TG neurons, the amplitude was 253.75±31.43 pA/pF and 243.19±33.99 pA/pF (n=14, paired t test, $P>0.05$) before and during hypotonicity treatment, respectively, and in TRPV4$^{-/-}$ mice TG neurons I_A was 246.42 ±35.52 pA/pF and 232.13±32.58 pA/pF (n=13, paired t test, $P>0.05$), respectively. **C.** After exposure to hypertonicity (350mOsm), I_K was reduced by 38.82±7.25% (n=14) and 38.52 ±6.38% (n=16) in TRPV4$^{+/+}$ and TRPV4$^{-/-}$ TG neurons respectively (unpaired t test, $P>0.05$). **D.** After exposure to hypertonicity (350mOsm), the inhibition of I_A by hypertonicity was 30.68 ±7.16% (n=14) and 33.31±6.61% (n=14) in TRPV4$^{+/+}$ and TRPV4$^{-/-}$ mice TG neurons respectively (unpaired t test, $P>0.05$).

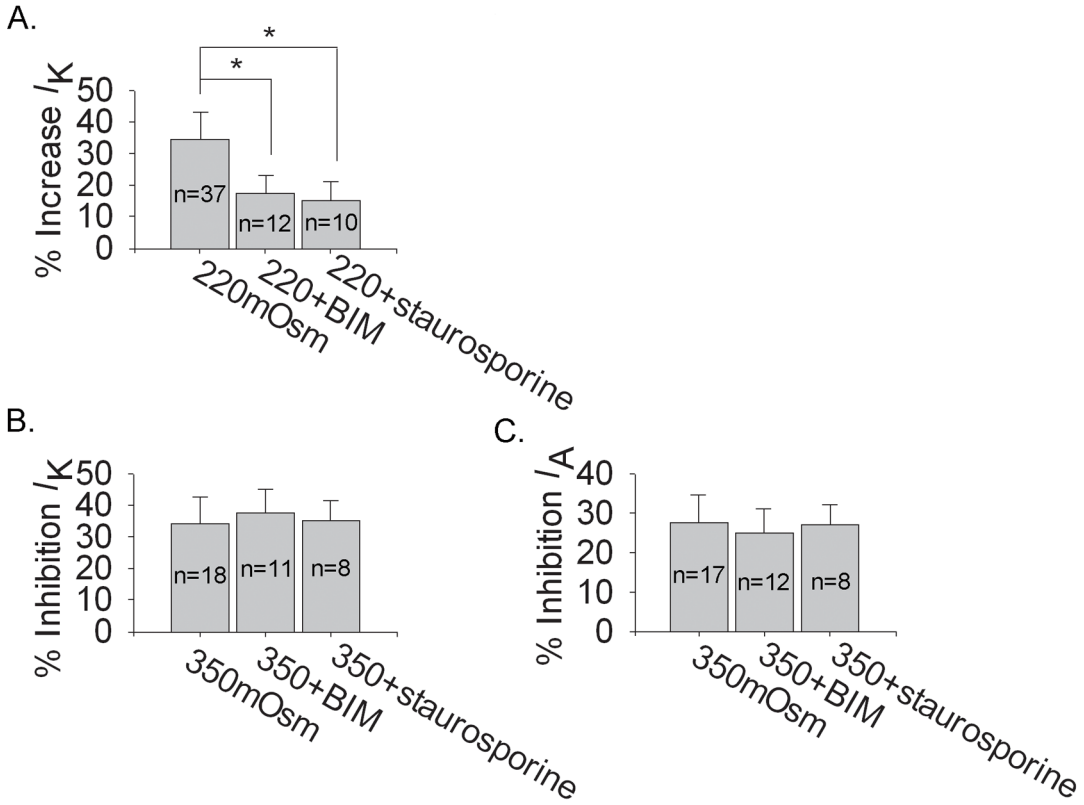

Figure 8. Modulation of PKC system on the effects induced by hypo and hypertonicity

A. Pre-incubation with PKC antagonists, BIM and staurosporine, significantly reversed the increase of I_K by hypotonicity from 34.52±8.58% to 17.48±5.59% (n=12) (unpaired t test, $P<0.05$) and 15.11±6.17% (n=10) (unpaired t test, $P<0.05$) respectively. **B.** Neither BIM nor staurosporine statistically affected the inhibitions of VGPCs by hypertonicity (unpaired t test, $P>0.05$). In the presence of BIM and staurosporine, I_K was inhibited by 37.88±7.38% (n=11) and 35.43±6.01% (n=8), respectively and I_A by 25.03±6.12% (n=12) and 27.13±5.05% (n=8), respectively.

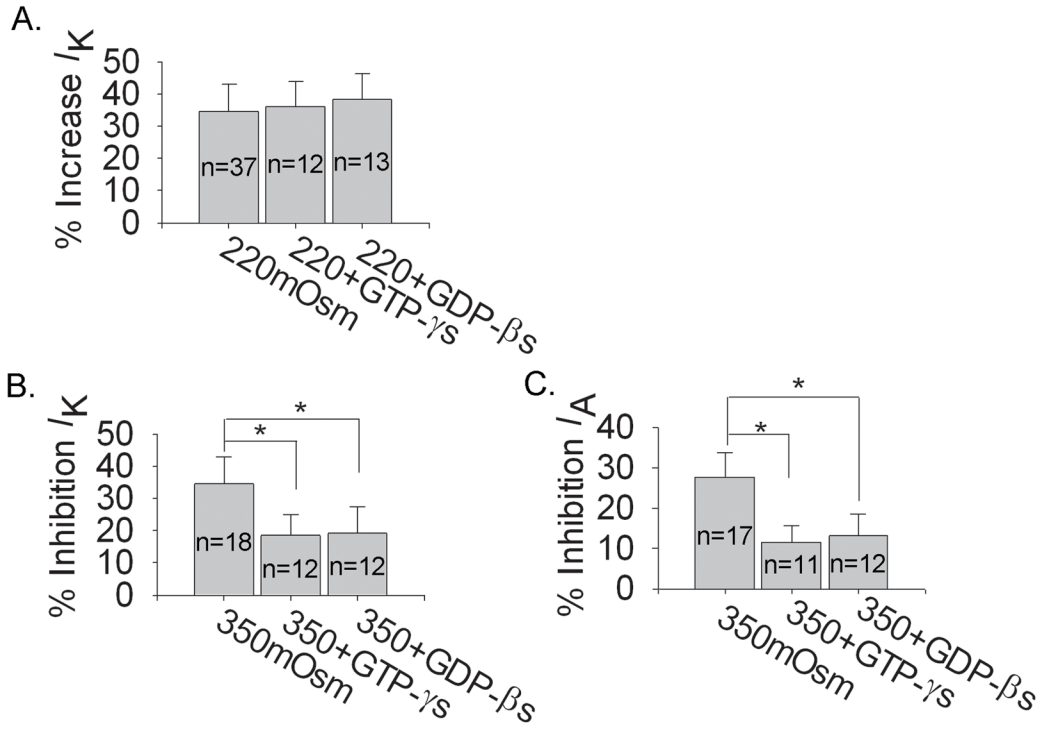

Figure 9. Modulation of G-protein system on the effects induced by hypo and hypertonicity

A. Neither GTP-γs nor GDP-βs had significant effect on the increase of I_K by hypotonicity (unpaired t test, $P>0.05$). In hypotonic solution (220mOsm) I_K was enhanced by 36.00±7.91% (n=12) and 38.23±8.01% (n=13) with GTP-γs and GDP-βs in the pipette solution, respectively. **B.** Both GTP-γs and GDP-βs markedly reversed the inhibitions of I_K (unpaired t test, $P<0.05$) and I_A (unpaired t test, $P<0.05$) by hypertonicity. In hypertonic solution (330mOsm) I_K was reduced by 18.73±5.90% (n=12) and 19.29±8.02% (n=12) in the presence of GTP-γs and GDP-βs, respectively and I_A by 11.53±4.19% (n=11) and 13.17±5.26% (n=12), respectively.

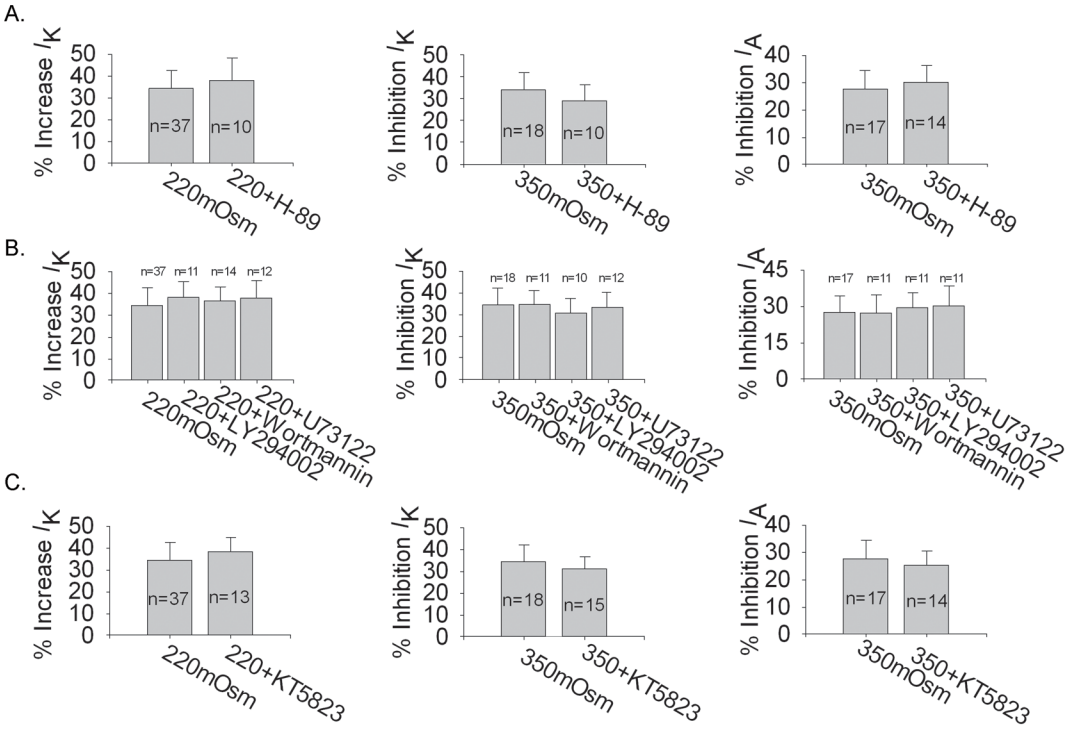

Figure 10. Modulation of PKA, lipid cascade and PKG systems on the effects induced by hypo and hypertonicity

A. For PKA system, pre-incubation with PKA inhibitor, H-89, had no significant effect on the modulations of VGPCs by hypo or hypertonicity (unpaired t test, $P>0.05$). In the presence of 10 μM H-89, I_K was increased 38.04±8.64% (n=10) by hypotonicity, while I_K and I_A were decreased 29.46±7.68% (n=10) and 30.00±6.64% (n=14) by hypertonicity respectively. **B.** For lipids cascade, in hypotonic solution, I_K was increased by 38.32±7.62% (n=11), 36.61±6.83% (n=14) and 37.86±8.59% (n=12) with Wortmannin, LY294002 and U73122 in the pipette solution, respectively. Compared with the inhibition of hypotonicity with normal pipette solution (34.52±8.58%, n=37), none of them was significantly different (unpaired t test, $P>0.05$). Similarly, pre-incubation with Wortmannin, LY294002 and U73122 did not affect the inhibition of I_K or IA by hypertonicity (unpaired t test, $P<0.05$). In the presence of Wortmannin, LY294002 and U73122, the inhibition of I_K by hypertonicity was 34.71±6.72% (n=11), 30.73±7.29% (n=10) and 33.23±7.55% (n=12) respectively and the inhibition of I_A was 27.25±8.02% (n=11), 29.50±6.61% (n=11) and 30.16±8.79% (n=11) respectively. **C.** For PKG system, KT5823 did not statistically alter the modulation of VGPCs by hypo or hypertonicity (unpaired t test, $P>0.05$). In the presence of KT5823, I_K was increased 38.33 ±7.15% (n=13) by hypotonicity, while I_K and I_A were inhibited 31.12±6.12% (n=15) and 25.17 ±5.67% (n=14) by hypertonicity, respectively.

Table 1

Extracellular solution (pH=7.4)

VGPCs	mOsm	Choline-Cl	KCl	$MgCl_2$	HEPES	Mannitol	TEA-Cl	4-AP	$CaCl_2$	$CdCl_2$
I_K	220	85	5.0	3.0	10	25	—	3.0		
	300	85	5.0	3.0	10	106	—	3.0		
	350	85	5.0	3.0	10	130	—	3.0		
	220-Ca	85	5.0	1.0	10	25	—	3.0	2.0	1.0
	300-Ca	85	5.0	1.0	10	106	—	3.0	2.0	1.0
	350-Ca	85	5.0	1.0	10	130	—	3.0	2.0	1.0
I_A	220	28	5.0	3.0	10	25	70	—		
	300	28	5.0	3.0	10	106	70	—		
	350	28	5.0	3.0	10	130	70	—		
	220-Ca	28	5.0	1.0	10	25	70	—	2.0	1.0
	300-Ca	28	5.0	1.0	10	106	70	—	2.0	1.0
	350-Ca	28	5.0	1.0	10	130	70	—	2.0	1.0

Table 2

Effect of anisotonicity and 4α-PDD on G–V and inactivation–voltage curve of VGPCs

		G–V curve				inactivation–voltage curve			
		$V_{0.5}$ (mV)		*k*		$V_{0.5}$ (mV)		*k*	
		300mOsm	Aniso/4α-PDD	300mOsm	Aniso/4α-PDD	300mOsm	Aniso/4α-PDD	300mOsm	Aniso/4α-PDD
Hypo (220mOsm)	I_K	−0.93±1.30	−0.90±0.71	16.46±1.13	16.07±0.41	−72.67±1.54	−40.55 ±5.25*	−9.67±1.32	−10.98±4.61
			n=8				n=8		
	I_A	−3.57±0.18	−3.33±0.72	19.47±1.20	21.08±0.75	−68.47±0.23	−69.42±0.21	−9.37±0.20	−9.67±0.15
			n=11				n=11		
4α-PDD (3.0μM)	I_K	−0.58±0.60	−0.11±0.48	17.83±0.53	18.38±0.42	−70.44±0.10	−50.40 ±0.42*	−10.08±0.08	−7.60±0.36*
			n=9				n=9		
	I_A	−3.46±0.17	−4.42±0.25	19.62±0.15	20.80±0.23	−65.93±0.29	−66.38±0.74	−11.49±0.25	−11.58±0.65
			n=8				n=8		
Hyper (350mOsm)	I_K	−0.92±1.23	−8.16±1.02*	18.13±1.08	17.63±0.95	−69.99±1.48	−83.00±. 122*	−11.68±1.28	−15.19 ±0.87*
			n=11				n=12		
	I_A	−3.98±0.25	−9.43±0.99*	19.16±0.81	20.38±0.94	−69.45±0.27	−86.32 ±0.47*	−9.93±0.24	−16.37 ±0.28*
			n=10				n=10		

Paired t test was used when comparing the effect of anisotonicity and 4α-PDD on $V_{0.5}$ and *k* in G–V and inactivation–voltage curve.

*$P<0.05$ *vs* 300mOsm.