Changes in Osmolality Sensitize the Response to Capsaicin in Trigeminal Sensory Neurons

Lieju Liu, Lei Chen, Wolfgang Liedtke, and S. A. Simon

Departments of Anesthesiology, Neurobiology, and Neurology and Center of Neuroengineering, Duke University, Durham, North Carolina

Submitted 20 August 2006; accepted in final form 2 January 2007


Changes in osmolality can activate nociceptors and produce pain. Under local inflammatory conditions, nociceptive response behavior evoked by anisotonic stimuli is selective for hypo- versus hypertonicity-induced tuning of capsaicin-activated currents. Specifically, antagonism of PKA- and PI3K-activated pathways appreciably reduced the hypertonicity-induced sensitization of I_{caps}. Confocal immunolocalization studies also revealed a modest anisotonicity-mediated redistribution of TRPV1 towards the plasma membrane of TG neurons. With respect to downstream signaling pathways, toxicity-induced sensitization of I_{caps} was dependent on whether hypo- or hypertonic stimuli were applied. Antagonism of PKA- and PI3K-activated pathways appreciably reduced the hypertonicity-induced sensitization of I_{caps}, whereas inhibition of PKC-mediated pathways selectively reduced the sensitization produced by hypotonic solutions. In summary, whereas the overall effects of hypo- and hypertonicity resulted in a similar pattern of potentiation of I_{caps}, intracellular signaling pathways were selective for hypo- versus hypertonicity-induced tuning of capsaicin-activated currents.

INTRODUCTION

Cells strive to maintain homeostasis while adapting to environmental changes. One environmental change with fundamental relevance for all life is osmolality. Maintenance of osmotic balance is of particular significance for nervous systems of metazoan animals. For sensory transduction of noxious stimuli, toxicity changes in the microenvironment of peripheral nerve terminals surrounding nociceptors will produce, or at least enhance, a nociceptive response. Therefore it is important to understand the molecular basis of how nociceptors and some of their associated ion channels respond to changes in osmotic pressure such as may occur during injury, edema, or inflammation, all of which can lead to marked changes in tonicity (e.g., within abscesses) (Wiese 1994; Wiese et al. 1999).

Several ion channels that are responsive to changes in tonicity or osmotic pressure have been identified. These include members of the TRPV family, such as TRPV4, TRPV2, and TRPV1, as well as the two-pore K^{+} channel family (Alloui et al. 2006; Birder et al. 2001, 2002; Liedtke and Kim 2005; Lyall et al. 2005; Muraki et al. 2003). In this investigation we focus mainly on the osmotic responses of TRPV1 channels. With respect to changes in toxicity, anisotonic solutions were previously shown to activate nociceptors and cause pain (Carstens et al. 1998; Green and Gelhard 1989; Wang et al. 1993). In this regard, Alessandri-Haber and colleagues (2003, 2005, 2006) recently examined whether the trpv4 gene participates in nociceptive response behavior evoked by anisotonic stimuli. Using trpv4^{-/-} mice they found that trpv4 was necessary for nociceptive behavior elicited by subcutaneous injection of mild hypertonic and hypotonic saline solutions.

With respect to TRPV1 channels, a recent study demonstrated that trpv1^{-/-} mice exhibit defects in systemic osmoregulation (Sharif Naeini et al. 2006). This study showed that a TRPV1 variant with an N-terminal deletion was expressed in osmosensitive magnocellular neurons of the hypothalamus. Although this variant was not identified at the molecular level, magnocellular neurons expressing this variant were not activated by capsaicin and not inhibited by capsazepine, a TRPV1 antagonist. However these neurons were responsive to hypertonic solutions in wild-type (wt), but not in trpv1^{-/-} mice (Sharif Naeini et al. 2006). A similar behavior was reported for neurons isolated from the organum vasculosum laminae terminalis, a sensory circumventricular organ (Ciura and Bourque 2006). Other experiments showed that hypertonic concentrations of ethanol sensitize the responses of TRPV1 receptors to capsaicin, protons, and heat (Geppetti and Trevisani 2004; Trevisani et al. 2002). Similar sensitizing responses of ethanol to capsaicin were also found in recordings from rat taste cells and their associated neurons (Lyall et al. 2005). Cultured urothelial cells isolated from wt mice were found to respond to capsaicin or hypotonic buffers by releasing NO and ATP. Secretion of these pro-nociceptive mediators was largely inhibited by capsazepine or in cells derived from trpv1^{-/-} mice, thereby suggesting that TRPV1 channels may function in signal transduction of bladder epithelial cells in response to hypotonicity (Birder et al. 2001, 2002). Taken together these results indicate that trpv1 and trpv4 gene products participate in the response to hypotonic and hypertonic stimuli.

Given capsaicin’s well-known role in chemically induced pain, inflammation, and thermal hyperalgesia (Caterina and Park 2006; Davis et al. 2000a), we have first investigated how capsaicin-activated TRPV1 receptors, expressed on dissociated trigeminal neurons, are affected by changes in osmotic pressure and then determined the intracellular pathways that may

Address for reprint requests and other correspondence: Lieju Liu, 327 Bryan Research Building, 101 Research Drive, Duke University Medical Center, Durham NC 27710 (E-mail: lieju@neuro.duke.edu).


The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
modulate their activation. We found that although the overall effects of anisotonicity are both large and similar, intracellular signaling pathways are selective for hypotonic and hypertonic stimuli.

**METHODS**

**Cell culture**

Trigeminal ganglion (TG) neurons from adult Sprague–Dawley rats were excised and cultured as described previously (Liu and Simon 1996a). Briefly, trigeminal ganglia were dissected aseptically and collected in modified Hank’s balanced salt solution (mHBSS). After washing in mHBSS, the ganglia were diced into small pieces and incubated in mHBSS for 30–50 min at 37°C in 0.1% collagenase (Type Xl-S). Individual cells were dissociated by triturating the tissue through a fire-polished glass pipette, followed by a 10-min incubation at 37°C in 10 μg/ml DNase I (Type IV) in F-12 medium (Life Technologies, Gaithersburg, MD). After they were centrifuged three times with F-12, 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 10% CO2. The cell diameters (in micrometers) were measured with a calibrated eyepiece under phase-contrast illumination. Neurons having projected soma diameters ranging between 18 and 31 μm were used. All experiments were carried out at room temperature (22–24°C).

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 recordings**

Whole cell patch-clamp recordings were obtained using an Axopatch-200B patch-clamp amplifier (Axon Instruments, Foster City, CA) whose output was digitized with a Digidata 1322A converter (Axon Instruments). In these experiments we used N-51A borosilicate glass pipettes (Drummond Scientific, Broomall, PA) with resistances (Axon Instruments). In these experiments we used N-51A borosilicate glass pipettes (Drummond Scientific, Broomall, PA) with resistances of 1–5 MΩ. When the extracellular solution was Krebs–Henseleit (KH; see Table 1), the intracellular solutions contained (in mM): KCl 140 (or 130 K-aspartate), CaCl2 1, MgCl2 2, EGTA 10, HEPES 10, and Tris-ATP 5 at pH 7.3 (% in = 285 ± 5 mOsm/kg) (Liu and Simon 1996b). When the extracellular buffer was other than KH, the intracellular buffer contained (in mM) KCl 148, CaCl2 1, MgCl2 2, EGTA 10, HEPES 10, and ATP-Tri 5 at pH 7.3 and % in = 300 mOsm/kg. The osmolality was measured using a vapor pressure osmometer (Model 3300, Advanced Instruments, Norwood, MA).

Liquid junction potentials were compensated before patching. When the osmolality of external solutions was changed from 300 to 260 or to 348 mOsm/kg, measurements of the changes in liquid junction potentials were <2 mV and were not corrected.

For current-ramp measurements we followed previous protocols (Liu et al. 2006). Briefly, after many ramp cycles in which the main voltage-dependent channels were diminished, the resting membrane potential was adjusted to ~80 mV and the membrane potential was ramped from −80 to 80 mV in 1.5 s, whereupon it was returned to −80 mV.

**Chemicals and justification of concentrations used**

Capsaicin, capsazepe, CPT-cAMP, H-89 [dihydrochloride, N-(2-(p-bromocinnamylamino)ethyl)-5-isooquinoline sulfonamide, Di-HCl salt], LY294002 [2-(4-morpholinyl)-8-phenyl-4H-1-benzopyran-4-one], Wortmannin, sphingosine (trans-α-erythro-2-amino-4-octadecene-1,3-diol), ruthenium red, and NMDG-Cl (N-methyl-D-glucamine chloride) were purchased from Sigma Chemical (St. Louis, MO). The TRPV4 agonist 4α-PDD (4α-phorbol-12, 13-didecanoate), U-73122 (1-((17β-3-methoxyestra-1,3,5(10)-tri-en-17-yl)amino)hexyl)-1H-pyrrole-2,5-dione), BIM (bisindolylmaleimide), and PKI 5 (protein kinase A inhibitor 14–22 amide, cell-permeable myristoylated), and mannitol were purchased from Calbiochem (San Diego, CA). Capsaicin, capsaazepine, H-89, LY290022, Wortmannin, sphingosine, 4α-PDD, BIM, PKI, and U-73122 were prepared as stock solutions in DMSO. The final DMSO concentrations in the bath chamber and in pipette solution was <0.1%.

Concentrations of the above-listed chemicals were chosen for the following reasons: 10 μM H-89 because it was tested on TRPV1 responses in HEK-293 cells (Liu et al. 2005) and also because at this concentration it is an effective inhibitor of protein kinases (Davis et al. 2000b); 1 μM Wortmannin because it prevented TRPV1 channels from recovering from desensitization (Liu et al. 2005); 10 μM PKI because it was used to modulate TRPV1 recovery from desensitization (Liu et al. 2005); 50 μM LY290022 because it was tested on TRPV1 channels’ propensity to recover from desensitization (Liu et al. 2005) and because it is a commonly used inhibitor of PI3K (Davis et al. 2000b) and affects TRPV1 responses (Zhou et al. 2001); 20 μM sphingosine, which inhibits PKC and because it activates TRPM3 currents (Grimm et al. 2005) and inhibits voltage-gated calcium channels (Titienny et al. 1998); 10 μM U73122 because it blocked CCl4-induced sensitization of TRPV1 responses (Zhang et al. 2005) and because it attenuated capsaicin induced currents evoked from hTRPV1 receptors (Marshall et al. 2003); and 1 μM BIM was chosen because it was shown to inhibit capsaicin-evoked currents in DRGs; (deCharms et al. 2005).

Dose–response plots for capsaicin were fit using the Hill equation: $I/I_{\text{max}} = (1 + [(osmolality/EC_{50})^n]^{-1}$, where $I_p$ is the peak current evoked at a particular osmolality, $n$ is the Hill coefficient, and $EC_{50}$ is the half-maximal excitatory concentration. Data were analyzed for statistical significance using, where appropriate, either paired or unpaired t-tests. Statistical significance is defined as $P < 0.05$. All data are presented as means ± SE.

**TABLE 1. Extracellular solutions (in mM)**

<table>
<thead>
<tr>
<th></th>
<th>mOsm/kg</th>
<th>NaCl</th>
<th>Mannitol</th>
<th>NMDGCl</th>
<th>KCl</th>
<th>CaCl2</th>
<th>MgCl2</th>
<th>Glucose</th>
<th>HEPES</th>
</tr>
</thead>
<tbody>
<tr>
<td>KH</td>
<td>300</td>
<td>147</td>
<td>0</td>
<td>0</td>
<td>5</td>
<td>2</td>
<td>1</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>300M</td>
<td>300</td>
<td>88</td>
<td>106</td>
<td>0</td>
<td>5</td>
<td>2</td>
<td>1</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>348M</td>
<td>348</td>
<td>88</td>
<td>142</td>
<td>0</td>
<td>5</td>
<td>2</td>
<td>1</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>326M</td>
<td>326</td>
<td>88</td>
<td>118</td>
<td>0</td>
<td>5</td>
<td>2</td>
<td>1</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>279M</td>
<td>279</td>
<td>88</td>
<td>94</td>
<td>0</td>
<td>5</td>
<td>2</td>
<td>1</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>260M</td>
<td>260</td>
<td>88</td>
<td>70</td>
<td>0</td>
<td>5</td>
<td>2</td>
<td>1</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>219M</td>
<td>219</td>
<td>88</td>
<td>25</td>
<td>0</td>
<td>5</td>
<td>2</td>
<td>1</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>300 NMDGCl</td>
<td>300</td>
<td>88</td>
<td>0</td>
<td>120</td>
<td>5</td>
<td>2</td>
<td>1</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>348 NMDGCl</td>
<td>338</td>
<td>88</td>
<td>0</td>
<td>80</td>
<td>5</td>
<td>2</td>
<td>1</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>260 NMDGCl</td>
<td>260</td>
<td>88</td>
<td>0</td>
<td>42</td>
<td>5</td>
<td>2</td>
<td>1</td>
<td>10</td>
<td>10</td>
</tr>
</tbody>
</table>

In all solutions the pH was 7.3.
Measurement of intracellular $Ca^{2+}$

Briefly, cells were loaded with 5 μM fura-2-AM (Invitrogen/Molecular Probes) for 30 min at 37°C, followed by 20-min deesterification at room temperature that took place in the dark. After obtaining control values with 300M or 300KH, the toxicity was changed. The ratiometric calcium signals were recorded with a PTI IC-200 camera (Photon Technology International, Monmouth Junction, NJ). Sampling was performed by Imaging Workbench 5.1 (INDEC BioSystem, Santa Clara, CA). Intracellular calcium was obtained from the fura-2–emitted fluorescence by excitation at 340 and 380 nm and by the ratio of the emission intensities at 511-nm emission wavelength sampled at 1 Hz.

Immunofluorescence using confocal scanning microscopy

TG neurons were dissociated as detailed above. The adhering cells were treated with hypotonic buffer (260 mOsm/kg) or hypertonic buffer (348 mOsmol/kg) for 10 min, fixed in 10% neutral-buffered formalin for 20 min, and then washed with PBS-0.1% Tween-20. For the blocking of unspecific binding, the cells were then incubated in 5% normal serum derived from the same species as the secondary antibody (see following text) for 2 h at room temperature, then incubated overnight at 4°C with primary antibody [anti-TRPV1 C-terminal antibody (Neuromics, Edina, MN), raised in guinea pigs, 1:1,000 dilution; anti-IL-1RI antibody (Abcam), raised in rabbits, 1:500 dilution]. The cells were then washed three times, incubated for 1 h at room temperature in goat anti-guinea-pig/anti-rabbit antibody, conjugated to the fluorophor Alexa-fluor 488 (Invitrogen/Molecular Probes, Pittsburgh, PA). Sections were inspected and imaged on a Leica inverted microscope confocal imaging station (Leica Microsystems TCS SP2), driven by Leica software, using a 63 × oil-immersion objective. Stimulation controls used were isotonic buffer supplementing hypotonic and hypertonic stimulation, histochemistry controls were preimmune serum from guinea pig and rabbit and primary antibody preabsorbed with TRPV1 C-terminal peptide. Confocal scans were analyzed with ImageJ freeware (http://rsb.info.nih.gov/ij/download.html) by line-scanning neuronal cross sections and subjecting the line to densitometric analysis. Outer-cell membrane densities were averaged as were densities recorded in the soma (Fig. 5C). Averages per cell were compiled for each toxicity group, which consisted of 24 cells, derived from three rats (eight cells per animal).

RESULTS

Changes in toxicity acutely sensitize responses to capsaicin

Figure 1A shows that when the osmotic pressures of the intracellular ($\pi_{\text{in}}$) and extracellular ($\pi_{\text{ex}}$) (300M) buffers were matched to 300 mOsm/kg, the application of 1 μM capsaicin evokes a small inward current (see Table 1). In this and subsequent experiments, unless explicitly stated, the holding potential was −60 mV. Removing mannitol from the 300M buffer to make it hypotonic (260M) did not evoke changes in the holding current. However, in 260M, a significantly larger current was evoked on the reapplication of 1 μM capsaicin. (We call this type of sensitization “acute” to distinguish it from the sensitization that occurs on repeated applications under anisotonic conditions; see Fig. 6.) When the buffer was again changed to 300M, after 3 min the capsaicin response was reduced to nearly control levels (Fig. 1A). On average, the response to 1 μM capsaicin increased from −23 ± 6 pA/pF ($n = 8$) in 300M to −149 ± 27 pA/pF ($n = 8$) in 260M. A similar sensitizing behavior was observed when the extracellular buffer was changed from isotonic 300M to hypertonic 348M (Fig. 1B). We found that, as for 260M, changing the buffer to 348M also did not evoke a current (see also Fig. 4). On average, we found the response to 1 μM capsaicin increased from −20 ± 5 pA/pF ($n = 7$) in 300M to −137 ± 25 pA/pF ($n = 7$) in 348M.

In a separate set of experiments in which the initial response is reported, we obtained the response to 1 μM capsaicin in the various solutions indicated on the abscissa in Fig. 1C. The mean changes in the capsaicin-evoked currents plotted as a function of osmolality exhibit a parabolic shape with a minimum of the current at isotonicity.

Because toxicity changes sensitize the responses to 1 μM capsaicin, we then obtained the capsaicin–hypotonic and capsaicin–hypertonic response relationships. Figure 2A shows the response to capsaicin obtained previously under conditions when the extracellular buffer was KH ($\pi_{\text{ex}} = 300$ mOsm/kg) and the intracellular osmotic pressure ($\pi_{\text{in}}$) was about 285 mOsm/kg. Under this condition, fitting the response to the Hill equation yielded values of $EC_{50}$ and $n$ of 0.63 μM and 1.4, respectively (Liu and Simon 1996a). However, on increasing $\pi_{\text{in}}$ from nearly 285 to 300 mOsm/kg (by adding KCl) such that $\pi_{\text{ex}} = \pi_{\text{in}}$, the $EC_{50}$ of the dose–response curve increased to 2.6 μM and the response became more cooperative ($n =$...
To determine whether the toxicity-evoked increases in capsaicin-induced currents are through TRPV1 receptors, we performed voltage-ramp experiments to determine whether the current–voltage (I–V) relationship remained the same as that under isotonic conditions. We also used the TRPV1 antagonist, capsaicin, to see whether the responses would be inhibited. As seen in Fig. 3, when the extracellular buffer contained 300M, 260M, and 348M, the I–V responses were outwardly rectifying and had reversal potentials of −2.9 ± 1.3 mV (n = 8), −3.3 ± 1.3 mV (n = 8), and −2.1 ± 1.1 mV (n = 7), respectively. These numbers do not significantly differ (P > 0.05), nor do they differ from those obtained in KH buffer under anisotonic conditions (Liu and Simon 1996b). This suggests that the shift in the dose–response curve with osmolality gradients seen at −60 mV does not arise from a marked change in ion selectivity. Concerning whether changes in toxicity would result in changes in voltage dependency, we compared the ratios of the currents at ±60 mV. The I(−60 mV)/I(+60 mV) ratios were not statistically different (P > 0.05); at 300M, 260M, and 348M ratios were 0.48 ± 0.03 (n = 8), 0.42 ± 0.03 (n = 8), and 0.44 ± 0.03 (n = 7), respectively. We also found that in the 348M and 260M buffers, 10 μM capsazepine inhibited the evoked currents by 92 ± 4.3% (n = 5) and 94 ± 3.7% (n = 5), respectively (data not shown). These results indicate that the increased sensitivity to capsaicin evoked by changes in toxicity arises primarily from currents through TRPV1 receptors rather than through capsazepine-insensitive, hypertonically activated, TRPV1 variants (Ciura and Bourque 2006; Sharif Naeini et al. 2006).

**Currents evoked by changes in toxicity**

Previously we noted that on changing the toxicity from 300M to 348M a current was not evoked. Figure 4A shows this absence of response on a more sensitive current scale than was shown in Fig. 1. With respect to hypotonic stimulation, we found that in only four of 19 neurons tested under this condition were inward currents evoked when \( \Delta \pi_{in} \) was changed from 300M to 260M. Moreover, in these neurons even larger currents were evoked at a lower (220M) toxicity (Fig. 4B). These osmosensitive responses also exhibit an I–V relation that is outwardly rectifying and has a reversal potential of 0.9 ± 1.4 mV (n = 4). Pertinent to the regulation of \( I_{\text{caps}} \) by osmotic pressure gradients these data indicate that activation of these currents is not necessary for toxicity-induced sensitizing responses to capsaicin.

**Trafficking of TRPV1 receptors under anisotonic conditions**

To obtain a better understanding of the cellular mechanisms underlying the observed toxicity-induced sensitization of capsaicin-evoked currents, we used confocal laser scanning microscopy (CLSM) after incubation for 10 min with isotonic (300M), hypertonic (348M), or hypotonic (260M) solutions. Quantitatively, under anisotonic conditions, densitometric measurements of cellular cross sections served as a surrogate for TRPV1 and IL-1RI receptor abundance in the outer cellular membrane versus the cytoplasm (Fig. 5). Although under
control conditions, anti-TRPV1-C-terminus immunoreactivity was slightly enriched in the plasma membrane, this fraction increased significantly under both hypotonic and hypertonic conditions (Fig. 5, A and B). In addition, under hypertonic conditions we observed a more punctate pattern of outer-membrane fluorescence, indicative perhaps of TRPV1 channel complex aggregation. The changes for TRPV1 could be observed for all TRPV1-C-terminal positive neurons in the 18- to 31-μm range. For hypotonic stimulation, we calculated the mean membrane density (as surrogate marker for TRPV1 protein abundance) as a percentage of total cellular density and found that it increased from 55 to 65%. Such quantification using line-scan densitometry of a confocal image of an immunolabeled for TRPV1 TG neuron, seen in Fig. 5C, shows for hypotonic stimulation an increase in density from 55 to 73%. For hypertonic stimulation the corresponding cytoplasmic density decreased from 45 to 35%, whereas for hypertonic stimulation, there was a corresponding reduction from 45 to 27%. For TRPV1, all differences reached the level of statistical significance (P < 0.05, ANOVA test) (Fig. 5B).

As a control we tested whether in response to toxicity stimuli a different transmembrane receptor would be redistributed in a similar manner as are TRPV1 receptors. Because we recently showed that the IL-1β receptor, IL-1RI, is expressed in a vast majority of small trigeminal neurons (Liu et al. 2006), we tested whether IL-1RI would redistribute under the same anisotonic conditions used with TRPV1. We found no significant change under conditions of anisotonicity for IL-1RI (Fig. 5, A and B; Fig. 5C illustrates the methodology for 5B). This negative result for IL-1RI reiterates the specificity of the changes obtained for TRPV1 receptors.

Capsaicin responses in a subpopulation of TG neurons sensitize on repeated applications in anisotonic buffers

It was previously established that on repeated applications of capsaicin, responses (currents) decrease on sequential applications (Koplas et al. 1997). This process—called tachyphylaxis or desensitization—is clinically relevant, as evidenced by the fact that capsaicin is sold over the counter for treatment of painful peripheral neuropathy (Krause et al. 2005). Tachyphylaxis has primarily been observed in electrophysiological measurements on cultured TG neurons, when the external buffer has a composition akin to that of KH and in the presence of mild gradients in tonicity (∆π = 10–20 mOsm/kg) (Liu and Simon 1996a,b). In Fig. 6A, we show that tachyphylaxis is also observed when the composition of the external buffer is 300M and π_ex = π_in = 300 mOsm/kg and capsaicin is applied every 3 min. In contrast, in the continued presence of significantly larger osmotic gradients (i.e., ∆π ≡ ±50 mOsm/kg, where π_in = 300 mOsm/kg), the responses to 20-s applications of 1 μM capsaicin in 260M every 3 min sensitized in 8 of 11 neurons (Fig. 6B) as they did in 348M in 9 of 13 neurons (Fig. 6C). It is important to state that in every instance, after changing the tonicity from isotonic to anisotonic, the first capsaicin response always increased. In the minority of neurons (three for 260M and four for 348M) the large sensitizing current seen on the first application decreased after subsequent applications. An example of this behavior is shown in Fig. 7A and in other figures. Nevertheless it is important to point out that because of these two populations the average responses that are shown in the histograms in Fig. 6, B and C remain relatively constant on subsequent applications. Similar responses were observed when NMDG-Cl replaced mannitol to adjust the osmolality (data not shown). In summary, in the presence of osmotic gradients in the range ±40–50 mOsm/kg, the capsaicin-activated currents become sensitized on repeated applications.

Modulators of capsaicin’s responses to changes in tonicity

CHANGES IN INTRACELLULAR CALCIUM. We now explore several of the pathways that could be modulated by changes in osmolality in a manner to produce sensitization rather than desensitization. Before proceeding, however, we want to make the distinction between acute sensitization seen after the first capsaicin application in anisotonic media and the sensitization...
seen on repeated applications, also in anisotonic media. As stated, we found that when the osmolality was changed from 300M to 260M or 348M either small or no currents were evoked (HP = −60 mV; see Figs. 1 and 4). Such an absence of effect was also reported for hypertonic stimuli in DRGs (Sharif Naeini et al. 2006). We therefore investigated whether changing the extracellular osmolality (\(\pi_{\text{ex}}\)) per se would produce an increase in intracellular calcium [indicating the activation of an osmotically sensitive calcium permeable pathway such as one involving osmosensitive TRPV4 channels (Liedtke and Kim 2005)]. Changing the extracellular buffer from 300M to 260M did not produce increases in intracellular calcium either in capsaicin-sensitive (Fig. 7A, \(n = 10\)) or capsaicin-insensitive (\(n = 9\), data not shown) neurons. Similarly, changing the buffer from 300M to 348M did not evoke changes in intracellular calcium in either capsaicin-sensitive (\(n = 12\); Fig. 7B) or capsaicin-insensitive (\(n = 7\)) neurons (data not shown). These results confirm previous studies that showed that in most (DRG) TG neurons, mild (±50 mOsm/kg) changes in tonicity do not evoke changes in intracellular calcium (Alessandri-Haber et al. 2003; Sharif Naeini et al. 2006; Viana et al. 2001). They show, as did Viana et al. (2001), that mannitol does not prevent calcium from entering the cells that are activated by capsaicin. In summary, whatever changes resulted in the increased responses to capsaicin in anisotonic media, they apparently do not require a tonicity-induced increase in intracellular calcium.

Because marked changes in intracellular calcium were not observed on changing the tonicity over the 260- to 348-mOsm range, and because TRPV4 channels were previously shown to be activated by changes in osmolality, we tested whether capsaicin-sensitive TG neurons could be activated by the TRPV4 agonist 4\(\alpha\)-PDD. We tested 21 TG neurons for their response to 5 \(\mu\)M 4\(\alpha\)-PDD. Fifteen were capsaicin-sensitive and six were capsaicin-insensitive. Of the 15 capsaicin-sensitive neurons, we found four that were responsive to 4\(\alpha\)-PDD (Fig. 7C). None of the six capsaicin-insensitive neurons was responsive. Taken together, these data show that although TRPV4 receptors that are responsive to 4\(\alpha\)-PDD are present in a subpopulation of capsaicin-sensitive neurons, the observed responses of tonicity tuning were found to be elicited in neurons that are not activated by 4\(\alpha\)-PDD. This suggests that activation of TRPV4, in the sense of an inward current or a rise in intracellular calcium, is not necessary to observe the effects of tonicity on TRPV1-evoked responses. Nevertheless, we cannot eliminate the possibility that, like some other TRP channels (e.g., TRPM2, TRPM6, TRPM7), TRPV4 can activate intracellular pathways even in a nonconducting state (Kaczmarek 2006).

**PKA- AND PKC-MEDIATED PATHWAYS.** We next inquired as to whether the activation of other intracellular pathways could account for the sensitization seen when the osmolality is changed away from isotonicity. We initially explored the effect of CTP-cAMP in 300KH buffer where, through PKA activation, it was shown to sensitize (or reduce desensitization) TRPV1 receptors to capsaicin (Bhave et al. 2002; Lopshire and Nicol 1998). We confirmed that under isotonic conditions [\(\pi_{\text{ex}}\) (KH) = \(\pi_{\text{in}}\) = 300 mOsm/kg], the addition of 0.3 mM CTP-cAMP also sensitizes the responses to repeated capsaicin applications (Fig. 8A). Under this condition where \(\pi_{\text{ex}}\) (KH) = 300 mOsm/kg the application of 1 \(\mu\)M capsaicin produced a small inward current (~32 pA) that is not observed at this current scale. However, in the presence of CTP-cAMP, a significant increase in the current was evoked on the next capsaicin application. In the example shown, the responses to all subsequent capsaicin applications decreased, although as seen in the histogram, after the initial increase (second capsaicin application) the response on subsequent applications remained essentially unchanged since some responses increased whereas others decreased. These data suggest an involvement of activation of the cAMP–PKA pathway in the tonicity-dependent sensitizing effects to capsaicin. However, although these sensitizing responses are similar to those seen in changes in tonicity, on repeated applications they attenuate rather than increase. Although the differences are not understood they could arise as a consequence of the presence of CTP-cAMP and/or the higher NaCl concentration seen under this condition.

Further exploration of this effect in anisotonic buffers revealed that the hypotonic responses could be separated from the hypertonic responses, at least, by the presence of the PKA antagonists H-89 and PKI. In the presence of both of these PKA antagonists, the capsaicin responses were virtually eliminated in the presence of hypertonic (348M) buffers (Fig. 8C). This can be most easily noted by comparing the currents evoked for the first capsaicin application after the buffer was exchanged from 300M to 348M (control: \(-2.9 \pm 0.7\) nA, \(n = 13\); H-89: \(-0.26 \pm 0.07\) nA, \(n = 6\); PKI: \(-0.59 \pm 0.29\) nA, \(n = 7\)). Note that values for control in this and subsequent comparisons were taken from the data in Fig. 6 because the experiments were performed in the same manner.

A somewhat more complex response was observed under hypotonic conditions (Fig. 8B). In the presence of H-89 the
Capsaicin responses were dramatically sensitized on the second capsaicin application (the first in 260M) when they decreased and then monotonically increased (data not shown but see histogram). A different result was found in the presence of PKI (Fig. 8B). Although the response on the second capsaicin application (260M: \(-0.84 \pm 0.52 \text{nA}, n = 4\)) was significantly \((P < 0.05, \text{paired } t\)-test\) greater than the response in 300M (\(-0.24 \pm 0.11 \text{nA}, n = 4\)), it was significantly \((P < 0.05)\) smaller than the response evoked in the presence of H-89 (260M: \(-4.7 \pm 1.2 \text{nA}, n = 6\)).

Importantly, in the presence of PKI the magnitude of the evoked current in 260M (\(-0.84 \pm 0.52 \text{nA}, n = 4\)) was not significantly different from that in 348M (\(-0.59 \pm 0.29 \text{nA}, n = 7\)). Moreover the subsequent responses in the presence of PKI did not exhibit the biphasic responses seen in the presence of H-89, but rather showed a small desensitization on subsequent applications. In summary, inhibiting PKA eliminates the sensitizing response observed in hypertonic buffers, whereas in hypotonic buffers the evoked responses are dependent on the particular PKA antagonist.

Previous studies indicated that activation of PKC sensitizes TRPV1 responses (Cesare and McNaughton 1996; Premkumar and Ahern 2000; Riddall et al. 2006). For this reason we tested two different PKC antagonists, 20 \(\mu\text{M sphingosine and 1 \(\mu\text{M BIM, on their ability to modulate responses to capsaicin in anisotonic conditions. As with the PKA antagonists described above, similar but not identical results were obtained for both antagonists of PKC. In the presence of 260M the first capsaicin application evoked a smaller response than in 300M, but the subsequent response was not significantly different from the first. The evoked currents in 348M were significantly smaller than in 260M, indicating a desensitization.}

![Confocal laser scanning microscopy (CLSM) of dissociated trigeminal neurons immunofluorescently labeled for TRPV1 and IL-1RI (control). A, top panels: representative micrographs of dissociated trigeminal neurons that were subjected to TRPV1-C-terminal–specific and IL-1RI–specific fluorescent antibody labeling and then followed by CLSM. Neurons were exposed to either hypotonic (260M) or hypertonic (348M) for 10 min, fixed for 20 min, and then immunolabeled as described in METHODS. Note the increased intensity of the label on the plasma membrane under conditions of anisotonicity for TRPV1, in contrast a lack of change for IL-1RI, thus emphasizing the specificity of the observed TRPV1 redistribution. Redistribution pattern of TRPV1 receptors appears perhaps more striking for hypertonicity, where a punctate labeling of membrane fluorescence is apparent. B: histogram showing results of densitometry. Twenty-four neurons from each condition (300M, 260M, and 348M) derived from 3 rats were imaged. Cross sections of the imaged cells were line-scanned and subjected to densitometry (see METHODS and C, right). TRPV1 outer membrane fluorescence (black bars) and intracellular fluorescence (white bars) were averaged for each cell and then averaged for the respective cohort [mean \(\pm\) SD: same procedure for IL-1RI (membrane dark-gray bars, cytoplasm light-gray bars)]. Sum of soma and membrane fluorescence was calculated as 100% of TRPV1-C-term response. IL-1RI immunofluorescence equivalent per neuron. Note the increasing distribution of TRPV1 immunoreactivity toward the plasma membrane when going from isotonic to hypotonic or to hypertonic buffers, and a lack thereof for IL-1RI. For TRPV1, both hypo- and hypertonic stimulation led to a statistically significant increase of membrane fluorescence when compared with isotonicity.](http://jn.physiology.org/)

FIG. 5. Confocal laser scanning microscopy (CLSM) of dissociated trigeminal neurons immunofluorescently labeled for TRPV1 and IL-1RI (control). A, top panels: representative micrographs of dissociated trigeminal neurons that were subjected to TRPV1-C-terminal–specific and IL-1RI–specific fluorescent antibody labeling and then followed by CLSM. Neurons were exposed to either hypotonic (260M) or hypertonic (348M) for 10 min, fixed for 20 min, and then immunolabeled as described in METHODS. Note the increased intensity of the label on the plasma membrane under conditions of anisotonicity for TRPV1, in contrast a lack of change for IL-1RI, thus emphasizing the specificity of the observed TRPV1 redistribution. Redistribution pattern of TRPV1 receptors appears perhaps more striking for hypertonicity, where a punctate labeling of membrane fluorescence is apparent. B: histogram showing results of densitometry. Twenty-four neurons from each condition (300M, 260M, and 348M) derived from 3 rats were imaged. Cross sections of the imaged cells were line-scanned and subjected to densitometry (see METHODS and C, right). TRPV1 outer membrane fluorescence (black bars) and intracellular fluorescence (white bars) were averaged for each cell and then averaged for the respective cohort [mean \(\pm\) SD: same procedure for IL-1RI (membrane dark-gray bars, cytoplasm light-gray bars)]. Sum of soma and membrane fluorescence was calculated as 100% of TRPV1-C-term response. IL-1RI immunofluorescence equivalent per neuron. Note the increasing distribution of TRPV1 immunoreactivity toward the plasma membrane when going from isotonic to hypotonic or to hypertonic buffers, and a lack thereof for IL-1RI. For TRPV1, both hypo- and hypertonic stimulation led to a statistically significant increase of membrane fluorescence when compared with isotonicity.
response was greater than that with 300M (Fig. 9A). Nevertheless the responses were significantly inhibited relative to what they were in the absence of these PKC antagonists (260M control: $-2.9 \pm 0.5$ nA, $n = 11$; 260M + BIM: $-0.43 \pm 0.09$ nA, $n = 6$; 260M + sphingosine: $-0.4 \pm 0.2$ nA, $n = 7$). In addition, sensitization responses were seen on subsequent applications.

We also found that in the presence of 348M, BIM and sphingosine acted similarly in the sense that they did not inhibit the sensitizing response found in the presence of hypertonic buffers and, in addition, on subsequent capsaicin applications the responses desensitized (Fig. 9B). That is, compared with the control response (348M control $-2.9 \pm 0.7$ nA, $n = 13$) similarly large responses were evoked in the presence of the two PKC antagonists (348M + BIM $-2.8 \pm 0.5$, $n = 6$; 348M + sphingosine to $-3.5 \pm 1.3$ nA, $n = 7$). In summary, only in the setting of $\pi_{\text{ex}} < \pi_{\text{in}}$ does inhibition of PKC-mediated pathways attenuate capsaicin’s sensitizing responses. For $\pi_{\text{ex}} > \pi_{\text{in}}$, PKC-mediated pathways eliminate the sensitization observed when repeatedly applying capsaicin in hypertonic buffers.

PTDINS(4,5)P2 (PIP2)-MEDIATED PATHWAYS. The role of PIP2 in modulating TRPV1 is controversial. PIP2 has been proposed to bias TRPV1 from opening (Chuang et al. 2001; Prescott and Julius 2003), open TRPV1 (Stein et al. 2006), and by its resynthesis, aid its recovery from desensitization (Liu et al. 2005). For these reasons as well as the recent finding that PI3K was shown to interact with TRPV1 receptors (Stein et al. 2006), we tested how inhibiting PI3K with either 50 M LY294002 (Fig. 10, A and B) or with 2 M Wortmannin (data not shown) would affect capsaicin-evoked responses under anisotonic conditions. We found that these two antagonists behaved similarly in that they did not significantly affect the hypotonicity-induced sensitization (Fig. 10A), but completely eliminated the sensitizing responses in the presence of 348M (Fig. 10B).

We also investigated whether the inhibition of PLC (with 10 M U73122 in the pipette) would affect the responses to capsaicin under hypo- and hypertonic conditions. This inhibition would decrease the production of IP3 and DAG and perhaps increase the concentration of PIP2. Figure 11 shows that under both hypotonic (Fig. 11A) and hypertonic (Fig. 11B)
Osmotic gradients sensitize responses to capsaicin

When TRPV1-containing TG neurons are exposed to osmotic gradients such that $\pi_{in} > \pi_{ex}$, as is commonly the case in patch-clamp experiments (Kim et al. 2006; Liu et al. 2005; Sharif Naeini et al. 2006), and also under some pathological conditions (e.g., confined edema, abscesses), then capsaicin will evoke larger currents than if the intra- and extracellular tonicities are matched (Figs. 1, 2, and 8–11). Our findings demonstrate that the sensitization of capsaicin-activated currents was carried by TRPV1 receptors. This is because their reversal potential remained unchanged from their value under isotonic conditions (Fig. 3), their voltage dependency, as determined by the ratios of the currents at ±60 mV, remained unchanged from what they were under isotonic conditions, and because they were inhibited by capsazepine.

The sensitizing responses to capsaicin were also observed when NMDG-Cl replaced mannitol in the extracellular solution to maintain the osmolality. This indicates that the sensitizing responses are not critically dependent on the chemistry of the osmolytes. Below we will discuss the nature of the tonicity-induced sensitization that normally causes tachyphylaxis. Before proceeding to this issue, however, we will first discuss other important aspects regarding changes in tonicity.

One aspect of TRPV1 responses noted by many investigators is that for a given capsaicin concentration and a similar holding potential, the magnitude of evoked current varies to a large extent (from pico- to nanoamperes). These results were rationalized by asserting that differences in the number of expressed functional TRPV1 channels will arise depending on the animal strain, culture conditions (e.g., the presence of NGF), and other variables including associated proteins and the extent of channel phosphorylation (Caterina and Park 2006; Geppetti and Trevisani 2004; Kim et al. 2006; Stein et al. 2006). Although these factors certainly can contribute powerfully to variability, in this study we identified and quantified the osmotic gradient as yet another factor that could contribute to the different magnitudes of responses and different values of $EC_{50}$ values (Figs. 1 and 2). In this regard, as seen in Fig. 2A, a small osmotic gradient generated by lowering the tonicity of the intracellular solution, while keeping the extracellular buffer KH, has a significant effect on the response to capsaicin. That is, a 10- to 20-mOsm decrease in the intracellular tonicity will cause a nearly threefold increase in capsaicin’s sensitivity ($EC_{50}$). Similar threefold changes in values were found on keeping the internal composition constant but changing the extracellular tonicity in such a manner that the extracellular ion content remained constant (Fig. 2B).

Currents activated in anisotonic conditions

We also investigated whether changing $\Delta \pi$ would activate some pathway(s) that in turn would sensitize TRPV1 channels responses to capsaicin. Changes in tonicity will obviously produce transient changes in neuron volume, which in various cells were shown to evoke currents and changes in the activity of intracellular pathways (Hoffmann and Dunham 1995; Vriens et al. 2004). In principle changes in cell volume could affect TRPV1 mechanical responses by affecting the interac-

**FIG. 7.** Changes in osmolality (260M and 348M) do not evoke marked changes in intracellular calcium. Following traces represent changes in fluorescence of fura 2–AM as indicated by the 340 nm/380 nm ratio. A: trace showing a neuron that was insensitive to a change in osmolality from 300M to 260M but responded to 10 μM capsaicin. B: example of a neuron that was insensitive to changes in osmolality from 300M to 348M but was responsive to 10 μM capsaicin. C: this tracing shows an example of neuron that was responsive to both 5 μM 4α-PDD, a TRPV4 agonist, and 10 μM capsaicin.
tion of its numerous ankyrin subunits with each other or with cytoskeletal elements. Although other researchers found that osmotic gradients greater than $\Delta \pi = 50 \text{ mOsm/kg}$ activate currents by gating TRPV4 receptors to induce increases in calcium in a subpopulation of sensory neurons (Alessandri-Haber et al. 2003, 2006; Vriens et al. 2004), in our experiments that were conducted at room temperature, the neurons tested at 348M did not evoke tonicity-activated currents and at 260M in only four of 19 neurons were currents evoked (see Figs. 1, 4, 5, and 7–11). We are aware that this lack of response does not eliminate a nonconducting state of TRPV4 as a part of the osmotransduction process (Kaczmarek 2006). However, we believe it likely that over this limited range of osmotic gradients, other factors including those intracellular modulators (see following text) that are changed by alterations in the cell volume, or by molecules directly attached to TRPV1 (Kim et al. 2006; Stein et al. 2006), and/or by TRPV1 trafficking to the plasma membrane (Brou et al. 2003; Sharif Naeini et al. 2006; Stein et al. 2006; Viana et al. 2001), likely contribute to the changes in sensitivity to capsaicin.

We also characterized, albeit to a limited extent, hypotonicity-evoked currents. For hypotonic stimuli, we observed currents whose magnitude increased with increasing hypertonicity (Fig. 4). These currents were found to exhibit several characteristics. They have an outwardly rectifying current–voltage relationship and a reversal potential near 0 mV (Fig. 4). Moreover, in a subpopulation of capsaicin-sensitive neurons, $4\alpha$-PDD produced an influx of calcium (Fig. 5). From this information, it is reasonable to attribute these currents, at least in part, to the activation of TRPV4 channels (Vriens et al. 2004). In future studies this tonicity-sensitive current will be more fully characterized.

Evoked responses from bladder urothelial cells from wild-type mice were found to be significantly more sensitive to capsaicin (also capsazepine), to stretch, and to hypertonic buffers than those from trpv1$^{-/-}$ mice, suggesting that the trpv1 gene is functional in response to hypertonicity (Birder et al. 2001, 2002). Other studies in heterologously expressed TRPV1 receptors (Trevisani et al. 2002) or responses evoked from TRPV1 variants in rat taste cells (Lyall et al. 2005) showed in a dose-dependent manner that hypertonic concentrations of ethanol sensitize responses to capsaicin (in the presence of salt). Given the results presented in this study, we posit that the ethanol-evoked sensitization of TRPV1 likely arises from the...
changes in tonicity. In many experiments, capsaicin is dissolved in ethanol or some other solvent, like DMSO, which by itself may not induce currents or changes in Ca^{2+}, but could sensitize responses to TRPV1 receptors. It thus follows that the particular response to capsaicin that is obtained is dependent on many factors, including the osmotic pressure gradient.

**Tonicity-induced sensitization of responses to capsaicin**

There are several nonmutually exclusive mechanisms by which changes in tonicity can result in the sensitization of responses to capsaicin. Among these are: that more functional channels became incorporated in the plasma membrane (Bron et al. 2003; Ji et al. 2002; Stein et al. 2006), that channels already present in the plasma membrane become sensitized by tonicity-evoked intracellular pathways, that newly synthesized TRPV1 activators (Bron et al. 2003; Wang et al. 2006) are generated under anisotonic conditions, and that TRPV1 channels recover more rapidly from acute desensitization under mild anisotonic conditions.

**Changes in the location or trafficking of TRPV1 receptors**

In inflammatory hyperalgesia both TRPV1 phosphorylation and increases in TRPV1 surface expression are major events underlying nociceptor activation and sensitization (Bron et al. 2003; Caterina and Park 2006; Planells-Cases et al. 2005). In this regard we found that changing the osmotic pressure gradient in either direction from isotonicity produced a relocation of TRPV1 channels (see Fig. 5) from the cytoplasm to the plasma membrane. In both anisotonic conditions the density of label associated with the outer membrane increased 15% for hypotonic and 25% for hypertonic stimulations (Fig. 5, B and C). Given the roughly sevenfold increase in current seen under similar conditions (see Figs. 1 and 6), under these two conditions the observed increase in the plasma membrane density of TRPV1 is too small to fully account for the differences in currents. This assessment relies on the assumption that the newly incorporated channels have the same characteristics as those already present in the plasma membrane.

One final point regarding the outer plasma membrane distribution of TRPV1 channels under hypertonic conditions is that they were present in small aggregates. One possible explanation for this aggregation is that the channels became targeted to rafts (McIntosh and Simon 2006).

**Intracellular pathways and changes in tonicity**

Several intracellular pathways including cAMP–PKA, PLC and PI3K were previously shown to sensitize TRPV1 channels either acutely or on repeated applications (Caterina and Park 2006; Stein et al. 2006; Zhang and McNaughton 2006). A major finding of this study was to show that the sensitizing effects arising from tonicity changes, either acutely and/or on repeated applications, are pathway specific (Figs. 8–10, Table 2).

First we will discuss the role of the cAMP–PKA pathway. We found that, similar to changes in tonicity, CTP-cAMP can sensitize capsaicin-induced currents. This suggests that pathways involved in increasing cAMP (and likely PKA) would be
activated by changes in tonicity. In this regard, we found that inhibiting PKA with H-89 and PKI essentially eliminated the sensitizing effect of hypertonic stimuli (348M). In contrast, the sensitization remained in the presence of hypotonic stimuli (260M) (Fig. 8A). Under these hypertonic conditions, magnitudes of the currents evoked were much greater in the presence of H-89 than of PKI. There are many possible reasons for these differences including the selectivity of these antagonists at these particular concentrations.

Nonetheless it is important to mention that in heterologously expressed TRPV1 receptors in the presence of H-89, no changes in the sensitivity to capsaicin were observed. In these experiments, however, \( \pi_{ex} \) was greater than \( \pi_{in} \) and the intracellular buffer had a slightly different composition (Liu et al. 2005). Because these experiments were performed under different conditions than those found in primary cultures of TG neurons, we believe that the different behavior of TRPV1 channels should only be noted rather than compared. In summary, we have clearly demonstrated that the cAMP–PKA pathway is involved in the sensitizing response to capsaicin in the presence of hypertonic solutions.

Phosphorylation of TRPV1 by PKC was previously shown to sensitize responses to capsaicin and/or to reverse desensitization (Patapoutian et al. 2003; Premkumar and Ahern 2000; Vellani et al. 2001). For this reason we tested how inhibiting the PKC pathway would affect the tonicity-sensitive capsaicin-activated currents. We found that inhibiting the PKC pathway (with sphingosine and BIM) substantially reduced the sensitizing response to hypotonic stimulation (Fig. 8A) but not the initial sensitizing responses to hypertonic stimulation (Fig. 8B). For hypertonicity, regarding the desensitization on repeated application of capsaicin, PKC inhibition through sphingosine and BIM attenuated the elimination of desensitization on repeated application of capsaicin. Again, we found that hypotonic and hypertonic stimuli activate distinctive pathways.

Recent experiments showed that TRPV1 is sensitized by PIP2 (Stein et al. 2006). This is in contrast to previous studies indicating that the interaction of PIP2 with TRPV1 biases the channel to a nonconducting state (Chuang et al. 2001; Prescott and Julius 2003). In addition, it was shown that TRPV1 and PI3K are associated proteins and that inhibiting PI3K with Wortmannin abolished NGF-induced sensitization of TRPV1 responses that arose from its cytoplasm-to-plasma membrane translocation (Stein et al. 2006). Other investigations showed that the activation of PI3K sensitizes TRPV1 (Zhuang et al. 2006). Thus it follows that PI3K is a key regulator of TRPV1. Its activation will result in the generation of PIP3 (and the possible reduction of PIP2), whereas its inhibition will result in a reduction of PIP3 and a possible buildup of PIP2.

We found that inhibiting PI3K with either LY294002 (Fig. 10) or with 2 \( \mu \)M Wortmannin (not shown), which would involve decreasing PIP3 and downstream products, did not affect capsaicin’s sensitizing effect when \( \pi_{ex} < \pi_{in} \) (Fig. 10A), but it completely eliminated the sensitizing responses when \( \pi_{ex} > \pi_{in} \) (Fig. 10B). Because inhibiting PI3K has been shown to affect the upregulation of TRPV1 (Bron et al. 2003; Stein et al. 2006), it is possible that this factor could play a small role in the effect of these inhibitors on the hypertonic response. The absence of an effect under hypotonic conditions implies this pathway(s) is not an important factor in this condition.
before, similar studies were performed in heterologously expressed TRPV1 receptors in HEK293 cells under hypertonic conditions (Liu et al. 2005). Whereas both in the latter study as well as the current one, it was found that Wortmannin (1 μM) decreased the sensitivity to capsaicin, they did not find that LY294002 had a significant effect. They suggested, in fact, that PI4K activity is specifically required for recovery of TRPV1 from desensitization (see following text). In summary, although changes in tonicity away from the isotonic set point both act to sensitize responses to capsaicin, they do so by different intracellular pathways.

We also tested what would happen under anisotonic conditions when we used U473122 to inhibit PLC. This inhibition would be expected to reduce the concentration of IP3 and DAG and possibly increase the PIP2 concentration. When PLC was inhibited by U73122 (Fig. 11), we found that the responses were still sensitized, but compared with those observed under control anisotonic conditions the responses were decreased. It is clear that several signaling events, perhaps antagonistic, are occurring simultaneously with those that are sensitizing, so as to make the interpretation of the underlying effects quite difficult.

**Recovery from desensitization**

From a clinical perspective one of the most important aspects of the response to capsaicin is that when it is given repeatedly, its response monotonically decreases, a process known as desensitization (or tachyphylaxis) (Szallasi and Blumberg 1996). This process was extensively investigated in TRPV1 receptors and was shown to depend on many factors and can occur over different timescales (Caterina and Park 2006). In general, it was found that dephosphorylation of TRPV1 will promote tachyphylaxis, whereas TRPV1 phosphorylation slows down tachyphylaxis (Docherty et al. 1996; Szallasi and Blumberg 1996).

**TABLE 2. Summary of changes in the first (acute) responses to 1 μM capsaicin under anisotonic conditions, in the presence of intracellular modulators, relative to the control response**

<table>
<thead>
<tr>
<th>Sensitization $I_{caps}$</th>
<th>Targeted Pathway [Inhibitors Used]</th>
<th>Sensitivity $\pi_{on} &lt; \pi_{on}$</th>
<th>$\pi_{off} &gt; \pi_{on}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>PKA [H-89, PKI]</td>
<td>$[\pm 0.1] \downarrow \uparrow$</td>
<td>$[\pm 0.1] \downarrow \downarrow$</td>
<td>$[\pm 0.1] \downarrow \downarrow$</td>
</tr>
<tr>
<td>PI3K [LY29402, Wortmannin]</td>
<td>$[\pm 0.1] \downarrow \downarrow$</td>
<td>$[\pm 0.1] \downarrow \downarrow$</td>
<td>$[\pm 0.1] \downarrow \downarrow$</td>
</tr>
<tr>
<td>PKC [Sphingosine, BIM]</td>
<td>$[\pm 0.1] \downarrow \downarrow$</td>
<td>$[\pm 0.1] \downarrow \downarrow$</td>
<td>$[\pm 0.1] \downarrow \downarrow$</td>
</tr>
<tr>
<td>PLC [U73122]</td>
<td>$[\text{Decrease in magnitude}]$</td>
<td>$[\text{Decrease in magnitude}]$</td>
<td>$[\text{Decrease in magnitude}]$</td>
</tr>
</tbody>
</table>

**FIG. 11.** Inhibition of PLC does not affect sensitizing responses in anisotonic conditions, but reduces the response magnitude. A: in the presence of 300 μM, the addition of capsaicin activated a small inward current (−29 pA) that is not apparent on this current scale. Then in the presence of 260 M the response to repeated applications of 1 μM capsaicin produced a sensitizing response that did not decrease. B: this tracing is similar to the above except that the buffer was 348 μM. A sensitizing effect is apparent both in the exemplary trace and on average. HP = −60 mV.
Koplas et al. 1997; Mandadi et al. 2004; Rosenbaum et al. 2003; Tominaga 2006). We note that many of these experiments were performed under an osmotic gradient and with various intracellular concentrations of ATP, all of which could affect the recovery from desensitization (Liu et al. 2004, 2005). In this investigation we found that under isotonic conditions ($\pi_{dL} = \pi_{dC}$), repeated applications of capsaicin produced a marked tachyphylaxis (Fig. 6A). Yet, strikingly, in the presence of osmotic gradients, the responses not only recover from desensitization but rather, in a large majority of TG neurons, sensitize. These novel regulatory processes involve the activation of different pathways under conditions where the soma volume increases (hypotonic) or decreases (hypertonic).

In summary, we have uncovered a novel mechanism of how capsaicin-evoked currents in trigeminal sensory neurons, a molecular hallmark of pain transduction, can be modified by changes in tonicity. Anisotonicity in either direction not only sensitizes these currents, but it also leads to a striking reversal of the desensitization/tachyphylaxis after repeated capsaicin applications under isotonic conditions, even in the presence of extracellular calcium. Both hypo- and hypertonic tuning of the tonic-sensitivity currents are phenotypically similar, yet, at the level of intracellular signal transduction, we could observe a remarkable exclusiveness of the critical signaling pathways. Hypertonicity-induced tuning was critically dependent on PKA and PI3K activation, whereas hypotonicity-induced tuning depended on PKC. However, PKC also regulated the lack of desensitization on repeated application of capsaicin observed under hypertonic conditions. Finally, the inhibition of PLC reduced the responses under anisotonic conditions. Because changes in tonicity occur under pain-enhancing inflammatory conditions in vivo, our novel findings reported here bear direct implications for our understanding of pain and how to target it more rationally.

ACKNOWLEDGMENTS

We thank S. Halkiotis for comments on the manuscript.

GRANTS

This work was supported by National Institute of General Medical Sciences Grant GM-63577 to L. Liu and in part by grants from Philip Morris USA and Philip Morris International to S. A. Simon, National Institute of Mental Health Career Development Award K08, and by Duke University grant to W. Liedtke.

REFERENCES


