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The C-Type Natriuretic Peptide Induces Thermal Hyperalgesia through a Noncanonical Gβγ-dependent Modulation of TRPV1 Channel

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Natriuretic peptides (NPs) control natriuresis and normalize changes in blood pressure. Recent studies suggest that NPs are also involved in the regulation of pain sensitivity, although the underlying mechanisms remain essentially unknown. Many biological effects of NPs are mediated by guanylate cyclase (GC)-coupled NP receptors, NPR-A and NPR-B, whereas the third NP receptor, NPR-C, lacks the GC kinase domain and acts as the NP clearance receptor. In addition, NPR-C can couple to specific Gβγ-mediated intracellular signaling cascades in numerous cell types. We found that NPR-C is coexpressed in transient receptor potential vanilloid-1 (TRPV1)-expressing mouse dorsal root ganglia (DRG) neurons. NPR-C can be coimmunoprecipitated with Gβγ1, and C-type natriuretic peptide (CNP) treatment induced translocation of protein kinase Cδ (PKCe) to the plasma membrane of these neurons, which was inhibited by pertussis toxin pretreatment. Application of CNP potentiated capsaicin- and proton-activated TRPV1 currents in cultured mouse DRG neurons and increased their firing frequency, an effect that was absent in DRG neurons from TRPV1−/− mice. CNP-induced sensitization of TRPV1 activity was attenuated by pretreatment of DRG neurons with the specific inhibitors of Gβγ, phospholipase C-β (PLCβ), or PKC, but not of protein kinase A, and was abolished by mutations at two PKC phosphorylation sites in TRPV1. Furthermore, CNP injection into mouse hindpaw led to the development of thermal hyperalgesia that was attenuated by administration of specific inhibitors of Gβγ or TRPV1 and was also absent in TRPV1−/− mice. Thus, our work identifies the Gβγ–PLCβ–PKC-dependent potentiation of TRPV1 as a novel signaling cascade recruited by CNP in mouse DRG neurons that can lead to enhanced nociceptor excitability and thermal hypersensitivity.

Introduction

Natriuretic peptides (NPs) mediate natriuresis to provide homeostatic control of body water, Na⁺ and K⁺ electrolytes, and fat, as well as playing a critical role in normalizing changes in blood pressure (Suzuki et al., 2001; Pandey, 2005b; Moro and Berlan, 2006; Potter et al., 2009). These events are mediated via downstream cellular signaling events, resulting from the activation of guanylate cyclase (GC)-coupled natriuretic peptide receptors, NPR-A and NPR-B, by atrial-type (ANP), brain-type (BNP), and C-type (CNP) natriuretic peptides (Pandey, 2005b; Potter et al., 2006, 2009). Additionally, NPs have been implicated in sensory nerve fiber sprouting and bifurcation (Kishimoto et al., 2008; Schmidt et al., 2009), as well as regulation of pain sensitivity (Schmidtko et al., 2008a; Zhang et al., 2010; Heine et al., 2011). Activation of protein kinase G (PKG) downstream of BNP/NPR-A in dorsal root ganglia (DRG) neurons has been shown to mediate the negative regulation of nociceptive transmission via modulation of presynaptic BKCa channel activity in the dorsal horn of the spinal cord (Zhang et al., 2010). Conversely, a recent report showed enhanced mechanical hypersensitivity in rats upon intrathecal injection of CNP (Heine et al., 2011). The canonical NPR-A- and NPR-B-mediated downstream cGMP/PKG signaling pathways mediate all these effects of BNP and CNP, respectively (Schmidtko et al., 2008a; Zhang et al., 2010; Heine et al., 2011). NPR-C is an additional NP receptor that, unlike NPR-A and NPR-B, lacks the GC kinase domain and thereby serves as the NP clearance receptor (Pandey, 2005a; Potter et al., 2006). However, NPR-C has also been shown to signal through the pertussis toxin (PTX)-sensitive Gαi protein in a variety of cell types, in which its activation by CNP (and ANP) leads to specific Gαi/βγ downstream signaling, including Gβγ-mediated phospholipase C-β (PLCβ) activation, phosphatidylinositol-4,5-bisphosphate (PIP2) hydrolysis, and subsequent activation of protein kinase C (PKC) (Levin, 1993; Murthy and Makhlof, 1999; Murthy et al., 2000; Anand-Srivastava, 2005).

Vascular endothelial cells secrete elevated levels of CNP during stimulation by inflammatory mediators, such as interleu-
kin-1α/β (IL-1α/β), tumor necrosis factor-α (TNF-α), and lipo-
poly saccharide (Suga et al., 1993). Furthermore, increased serum 
CNP levels were found in human patients with rheumatoid ar-
thritis and systemic sclerosis (Olewick-Gawlak et al., 2010a,b).
These findings suggest that CNP plays a role in vascular inflamma-
tion (Ahuwalia and Hobbs, 2005) and possibly in inflammatory 
pain sensitization. The transient receptor potential vanilloid-1 
(TRPV1) channel on sensory afferents is a key mediator of periph-
eral sensory detection and transduction of inflammatory pain. 
Mechanistically, this process involves diverse phosphorylation-
dependent sensitization and proton-activation/potentiation of 
TRPV1 channel activity by a variety of pro/inflammatory mediators 
(Hucho and Levine, 2007; Patapoutian et al., 2009; Gold and Geh-
hart, 2010; Kuner, 2010; Ren and Dubner, 2010). Here we demon-
strate that CNP induces acute sensitization of TRPV1 channel activity 
through a non-canonical G_{0/1}G_{βγ}-mediated downstream 
activation of PKC and phosphorylation of the channel protein, 
resulting in enhanced nociceptor firing. We also show that CNP injec-
tion leads to the development of thermal hyperalgesia that is 
dependent on G_{βγ} signaling and TRPV1.

Materials and Methods

All experiments involving the use of mice and the procedures followed 
therein were approved by the University of Iowa Institutional 
Animal Care and Use Committee and in strict accordance with the NIH Guide for 
the Care and Use of Laboratory Animals. Every effort was made to mini-
nimize the number of mice used and their suffering.

Primary cultures of mouse DRG neurons. DRGs were isolated from adult 
C57BL/6 mice of both TRPV1+/- and TRPV1-/- genotypes and cultured on 
poly-L-ornithine- and laminin-coated glass coverslips, as described pre-
viously (Schnizier et al., 2008). Briefly, isolated DRGs were digested with 
collagenase for 20 min followed by neutralization, centrifugation, and tritu-
ration before further digestion with pronase for 10 min. Cells were then 
pelleted by centrifugation and resuspended in DMEM (Invitrogen) supple-
mented with 10% fetal bovine serum, before additional trituration with 
fire-polished glass Pasteur pipettes. Neurons were plated onto poly-L-
ornithine- and laminin-coated glass coverslips. After 90 min incubation at 
37°C in a 5% CO2 incubator, the culture media was changed to TNB media 
(Invitrogen). For recordings in neurons, 1 μM tetrodotoxin was added to the extracel-
ular buffer to block fast-activating Nav currents. For extracellular buffers 
used in experiments with proton-activated currents (pH 5.8, 5.4, and 
4.8), HEPS was substituted with MES. All the activators/drugs were 
diluted in the extracellular buffer, from a stock solution, to achieve final 
concentrations. All control and agonist/drug-containing extracellular 
buffers were applied locally onto the cells under recording, with a sepa-
rate Teflon tubing-connected glass multiple-barrel perfusion system.

Cultures were recorded with an Axopatch 200B patch-clamp amplifier 
connected to a Digidata 1440A data acquisition system ( Molecular De-
vices), with a sample rate of 2 kHz and filtering at 1 kHz. pClamp 10 
software (Molecular Devices) was used for the acquisition of currents, 
and Clampfit 10 (Molecular Devices) and Origin 7.0 (OriginLab) soft-
ware were used for the analysis of currents and preparing traces/figures. 
Data are presented as means ± SEM or fitted value ± SE of the Hill 
equation fit for the pH dose–response relationship.

Capsaicin- and proton-induced action potential (AP) firings in small 
and medium-diameter cultured mouse DRG neurons were recorded under 
the current-clamp mode at room temperature following similar procedures 
used in cultured hippocampal neurons (Mohapatra et al., 2009). Neurons 
were bathed in extracellular buffer containing the following (in mM): 1 NaCl, 5 KCl, 1 MgCl2, 10 HEPES, and 10 glucose, pH 7.3. All 
the experiments were performed using whole-cell recording with a 
separate Teflon tubing-connected glass multiple-barrel perfusion system.
fractionated in 0.7% agarose gel electrophoresis and stained with ethidium bromide, to visualize the DNA bands and verify the respective predicted product sizes.

Assay for intracellular cGMP production in DRG neurons. Quantification of ANP/BNP/CNP-induced intracellular cGMP production in freshly isolated mouse DRG neurons was performed using a direct ELISA kit (Enzo Life Sciences) following the instructions of the manufacturer. Briefly, freshly isolated and dissociated DRG neurons from adult male mice were treated with individual NPs (15 min at 37°C) and subjected to lysis and subsequent determination of intracellular cGMP concentration. The protein concentrations in cell lysates were determined by the BCA method (Pierce).

Immunostaining of DRG sections and cultured DRG neurons. Adult male C57BL/6 mice were killed, and L4–L6 lumbar DRG pairs were then dissected out from these animals and postfixed with 4% paraformaldehyde plus 5% picric acid [in 0.1 M phosphate buffer (PB)] overnight at 4°C before being transferred into 15% sucrose in 0.1 M PB for an additional incubation overnight at 4°C. Twenty-five-micrometer-thick sections of fixed DRG tissue were obtained using a cooled cryostat (CM3050 S; Leica Microsystems), as detailed previously (Shepherd and Mohapatra, 2012). Free-floating DRG tissue sections were washed in a 48-well tissue culture plate with 0.1 M PB, before being incubated with blocking/permeabilization solution (10% goat serum plus 0.3% Triton X-100 in TBS with 0.1% Triton X-100) at room temperature with vigorous agitation, the sections were incubated with rabbit polyclonal anti-NPR-C antibody (5 μg/ml; Abgent) along with mouse monoclonal anti-calcinonin gene-related peptide (CGRP) (1 μg/ml) or mouse monoclonal anti-neurofilament-200 (NF-200) (1 μg/ml; both from Sigma) or mouse monoclonal anti-TRPV1 (5 μg/ml; clone N221/17; NeomMab) antibody in blocking solution overnight at 4°C. After washing in blocking solution (three times for 10 min each at room temperature with vigorous agitation), the sections were incubated with goat anti-mouse IgG Alexa Fluor-555 and goat anti-rabbit Alexa Fluor-488-conjugated (1:1000 each; Invitrogen) secondary antibodies for 180 min washing in blocking solution (three times for 10 min each at room temperature protected from light. The sections were subsequently washed first with 10% goat serum in 0.1 M PB for 5 min, with 0.1 M PB for 5 min, and then with 0.05 M PB for 5 min (all at room temperature with vigorous agitation). Finally, the sections were carefully transferred onto glass slides and covered with glass coverslips with ProLong Gold antifade reagent (Invitrogen), as described previously (Shepherd and Mohapatra, 2012). Confocal fluorescence images of mounted DRG tissue sections were taken using a BX61W1 microscope equipped with the Fluoview 300 laser-scanning confocal imaging system (Olympus) with a 10× objective (NA 0.25; Olympus).

Fluorescence immunostaining of cultured mouse DRG neurons were performed following standardized procedures detailed previously (Mohapatra et al., 2008). Control and drug-treated cultured mouse DRG neurons (2–3 DIV) were washed in Dulbecco’s PBS, followed by 30 min fixation with 3% paraformaldehyde containing 4% sucrose at 4°C with 0.1% Triton X-100. Neurons were transferred onto glass slides and covered with glass coverslips with ProLong Gold antifade reagent (Invitrogen), as described above (Shepherd and Mohapatra, 2012). Confocal fluorescence images of cultured DRG neurons were taken using a BX61W1 microscope equipped with the Fluoview 300 laser-scanning confocal imaging system with a 10× objective (NA 0.25; Olympus). Quantification of cellular PKClα distribution and translocation in neurons was performed using NIH Image J software as described previously (Cesare et al., 1999; Hucho et al., 2005). From the determined PKClα signal intensity across the line on every neuron, the peak intensity in the outer 10% of the cell diameter was taken as the peripheral, and the peak intensity in the inner 90% of the cell diameter was taken as the cytoplasmic localization. Ratio of the peak peripheral versus cytoplasmic intensity in each neuron was calculated, with a higher ratio indicating more peripheral localization of PKClα. In addition, the number of individual small/medium-diameter cultured DRG neurons with PKClα immunostaining (both peripheral translocation and diffused cytoplasmic distribution) were counted for each treatment group (>500 neurons for each group) in a blinded manner. These data were quantified and plotted as percentage of neurons with peripheral PKClα translocation with different drug treatment conditions. All immunostaining experiments were repeated four times on four different batches of mouse DRG neuron cultures.

Immunoprecipitation and immunoblotting. Coimmunoprecipitation of NPR-B and NPR-C with G-protein α-subunits from cultured mouse DRG neurons (2–3 DIV) was performed following previously standardized protocols (Mohapatra et al., 2008; Schnizler et al., 2008). Briefly, cultured mouse DRG neurons were lysed with ice-cold lysis buffer consisting of 0.2 M Tris–HCl, pH 8.0, 0.15 M NaCl, 0.001 M NaF, 0.002 M EDTA, 0.001 M PMSF, 1× protease inhibitor cocktail (leupeptin, aprotinin, antipain, and benzamidine–HCl), and 1% Triton X-100, for 30 min at 4°C on a rotator, followed by centrifugation at 12,000 × g for 10 min at 4°C to pellet the debris. The lysates were then incubated with rabbit polyclonal anti-NPR-B or anti-NPR-C (both Abgent) or rabbit polyclonal anti-HA antibody-tagged recombinant protein A-Sepharose beads (Pierce) suspended in lysis buffer containing BSA (0.2 mg/ml final) at 4°C for 2 h on a rotator. The beads were then centrifuged at 3500 × g for 5 min at 4°C and washed seven times with the lysis buffer containing BSA, and the supernatant was discarded after the final wash. Immunoprecipitated proteins were released from the beads by boiling with an equal bead volume of 0.05% SDS and 2-mercaptoethanol-containing gel-loading buffer for 5 min and subsequently size fractionated on 10% SDS-PAGE gels, followed by transfer onto nitrocellulose membranes (Bio-Rad). Membranes were probed with rabbit polyclonal anti-Goα (1:200; Santa Cruz Biotechnology) or anti-Goα antibodies (1:200; Cell Signaling Technology) or mouse monoclonal anti-Goα antibody (1:200; clone N192/12; NeuroMab) and subsequently with goat anti-mouse or anti-rabbit IgG–HRP secondary antibodies (1:10,000; Antibodies Inc.). Immunoreactive proteins on membranes were developed with enhanced electrochemiluminescence-plus reagent (ECL-Plus; PerkinElmer Life and Analytical Sciences), and the signals were captured on x-ray film (Kodak–Biomas; Carestream Health).

For ERK phosphorylation assays, cultured mouse DRG neurons (2 DIV) were treated with either vehicle or 100 nM CNP, and cell lysates were prepared 30 min after treatment using the same lysis buffer and methods as mentioned above. Lysates were then run on 10% SDS-PAGE gels and transferred onto nitrocellulose membranes. Membranes were probed with either rabbit polyclonal anti-ERK1/2 (1:1000; Cell Signaling Technology) or mouse monoclonal anti-phospho-ERK1/2 (1:500; BD Biosciences) antibody, along with the mouse monoclonal anti-GRP75 antibody (1:1000; clone N252A42; NeuroMab), and subsequently with goat anti-mouse or anti-rabbit IgG–HRP secondary antibodies (1:10,000; Antibodies Inc.). Immunoreactive proteins on membranes were developed with enhanced ECL-Plus reagent (PerkinElmer Life and Analytical Sciences), and the signals were captured on x-ray film (Kodak–Biomas). All the immunoprecipitation and immunoblotting experiments were repeated three times on three different batches of mouse DRG neuron cultures.

Chemicals and reagents. Purified recombinant human/rodent ANP, BNP, and CNP, PTX, PGE2, BK, collagenase, and Pronase were purchased from EMD Chemicals and Phoenix Pharmaceuticals; the fura-2 AM was from Invitrogen; 8-Br-cGMP and 8-pCPT-cGMP were from Enzo Life Sciences; capsacin and the TRPV1 antagonist AMG9810 [(E)-(3-[(4-t-butylphenyl)-N-(2,3-dihydrobenzo[a][1,4]dioxin-6-yl)acrylamide] from Sigma; the Gβγ inhibitor gallicin was from Acros Organics; PLCβ inhibitor U73122 (1-[6]-[17β]-3-methoxyestra-1,3,5(10)-tri-en-17-yl]amino)hexyl]-1H-pyrrole-2,5-dione, PKC inhibitor bisindoylmelamine-1 (BIM), protein kinase A (PKA) inhibitor KT5720 [(159,10,12)-2,3,9,10,11,12-hexahydro-10-hydroxy-9-methyl-1-oxo-9,12-epoxy-1 H-diindolo[1,2,3-fg:3’,2’,1’-kl]pyrrolo[3,4-i][1,6]benzodiazocine-10-
Results

NPs potentiate capsaïcin responses in mouse DRG neurons

We first examined the effects of NPs on the activity of TRPV1 in small/medium-diameter cultured mouse DRG neurons by monitoring TRPV1-mediated \([Ca^{2+}]_i\) responses, as described previously (Schnizler et al., 2008). Two consecutive applications of capsaïcin (50 nM for 15 s, with 5 min washing in between) resulted in desensitization/tachyphylaxis of \([Ca^{2+}]_i\), for the second application, with a ratio of 0.35 ± 0.01 for second/first capsaïcin-induced \([Ca^{2+}]_i\) (n = 14; Fig. 1A, B). However, extracellular application of ANP or CNP (50 and 500 nM each for 5 min) to DRG neurons resulted in significant potentiation of \([Ca^{2+}]_i\) responses induced by the second capsaïcin application, with peak \([Ca^{2+}]_i\) ratios of 4.67 ± 1.17 (n = 17) and 2.22 ± 0.49 (n = 18) for 50 and 500 nM ANP, respectively, and 3.54 ± 0.8 (n = 15) and 17.2 ± 5.66 (n = 9) for 50 and 500 nM CNP, respectively (Fig. 1A, B). Conversely, BNP application (50 and 500 nM for 5 min) did not

carboxylic acid hexyl ester), PKG inhibitor KT5823 [(9S,10(R,12(R))-2,3,9, 10,11,12-hexahydro-10-methoxy-2,9-dimethyl-1-oxo-9,12-epoxy-1H-indeno[1,2,3-f:3',2',1'-kl]pyrrolo[3,4-][1,6]benzodiazocine-10-carboxylic acid methyl ester), and PKC activator phorbol 12-myristate 13-acetate (PMA) were from Tocris Bioscience. All other chemicals used in this study were purchased from Sigma, Bio-Rad, Roche Applied Science, and Thermo Fisher Scientific. All NeuroMab antibodies were purchased from the University of California, Davis/National Institutes of Health NeuroMab Facility through Antibodies Inc.

Behavioral assessment of inflammatory thermal hyperalgesia. Adult male C57BL6/J mice of both TRPV1 and TRPV1 genotypes were housed as five mice per cage and maintained on a 12 h light/dark cycle (dark cycle beginning at 6:00 P.M.) with access to food and water ad libitum. Animals were acclimated to the testing environment for 2 days before testing for 30 min each and on the day of behavioral testing for 30 min by placing them in individual Plexiglas chambers situated on a glass surface maintained at thermo-neutral temperature (IITC Life Science). Nociceptive thermal sensitivity on each hindpaw was measured by focusing a high-intensity beam of light on the plantar surface, and the time required for the mouse to withdraw its hindpaw from the thermal stim-
show any significant potentiation of \([Ca^{2+}]\), responses to the second capsaicin application [ratios 0.79 ± 0.28 (n = 9) and 0.53 ± 0.1 (n = 12), respectively; Figure 1A,B]. We also compared the potentiating effects of NPs with two major inflammatory mediators, PGE2, and BK. Extracellular application of PGE2 (5 μM) and BK (50 and 500 nM) resulted in significant potentiation of \([Ca^{2+}]\), responses induced by the second capsaicin application, with peak \([Ca^{2+}]\) ratios of 4.23 ± 1.48 (n = 12), 9.52 ± 6.16 (n = 10), and 11.33 ± 6.26 (n = 9) for 5 μM PGE2, 50 nM BK, and 500 nM BK, respectively (Fig. 1B). However, application of 500 nM PGE2 did not lead to any significant potentiation of \([Ca^{2+}]\), responses to the second capsaicin application [ratio 2.12 ± 0.73 (n = 16), respectively; Fig. 1B].

Next, we performed whole-cell patch-clamp analysis to verify NP-mediated sensitization of TRPV1 channel activity suggested by our \([Ca^{2+}]\) imaging studies. Application of ANP and CNP, but not BNP (10 nM each), led to significant potentiation of capsaicin-activated (50 nM, ~5 s) inward currents (\(I_{\text{cap}}\)) in small/medium-diameter cultured mouse DRG neurons. There were 4.31 ± 1.44-fold (n = 9) and 4.87 ± 0.85-fold (n = 16) increases in the third \(I_{\text{cap}}\) for ANP and CNP treatment groups, respectively, compared with a 1.6 ± 0.53-fold (n = 10) increase in the vehicle treatment group, and a 5.89 ± 1.72- and 4.72 ± 0.67-fold increase in the fourth \(I_{\text{cap}}\) for ANP and CNP treatment groups, respectively, compared with a 1.14 ± 0.25-fold increase in the vehicle treatment group (Fig. 1C,D). We further determined the NP dose–response relationships for the potentiation of TRPV1 channel activity. Increasing concentrations of ANP and CNP (1, 3, 10, 30, and 100 nM) resulted in enhanced potentiation of \(I_{\text{cap}}\) with saturation being achieved at 30 nM, whereas BNP did not induce any significant potentiation for all concentrations (data not shown). Together, these data indicate that ANP and CNP, but not BNP, significantly potentiate TRPV1 channel activity in a dose-dependent manner.

Noncanonical NPR-C/GPCR signaling underlies the NP-induced potentiation of TRPV1 channel activity

Previous reports have shown the expression of NPR-A/B in rodent DRG neurons (Zhang et al., 2010). We also performed RT-PCR with specific primer sets for NPR-A/B/C and show that all three NPR subtypes are expressed in adult mouse DRG neurons (Fig. 2A). Activation of NPR-A by A/BNP and of NPR-B by CNP has been shown to increase intracellular cGMP production and subsequent activation of PKG (Pandey, 2005b; Potter et al., 2006). Indeed, application of ANP, BNP, or CNP (50 nM, 15 min) to mouse DRG neurons led to increased intracellular cGMP production (0.68 ± 0.5, 1.89 ± 0.26, 1.96 ± 0.29, and 2.89 ± 0.71 pmol cGMP/μg protein for untreated, ANP-, BNP-, and CNP-treated neurons, respectively), as determined by ELISA (Fig. 2B).

We next determined whether elevated levels of intracellular cGMP in response to NP treatment underlie TRPV1 channel sensitization. Direct perfusion of two membrane-permeable cGMP analogs, 8-Br-cGMP (100 μM) and 8-pCPT-cGMP (200 μM), did not significantly potentiate \(I_{\text{cap}}\) in DRG neurons (0.82 ± 0.11- and 0.78 ± 0.15-fold increase in third and fourth \(I_{\text{cap}}\), respectively, for 8-Br-cGMP treatment group (n = 6), and 1.26 ± 0.05- and 1.25 ± 0.1-fold increase in third and fourth \(I_{\text{cap}}\), respectively, for 8-pCPT-cGMP treatment group (n = 4); Fig. 2C). Furthermore, pretreatment of neurons with a PKG inhibitor (KT5823, 500 nM) did not inhibit CNP-induced potentiation of peak \(I_{\text{cap}}\) [3.83 ± 0.44- and 4.69 ± 1.21-fold increase in third and fourth \(I_{\text{cap}}\), respectively (n = 7); Fig. 2C]. These data suggest that the canonical cGMP–PKG signaling pathway is not involved in CNP-induced sensitization of TRPV1; therefore, we next tested whether other protein kinases are involved in CNP-induced potentiation of \(I_{\text{cap}}\). TRPV1 channel protein is a target for direct phosphorylation by PKA and PKC, which results in potentiation of channel activation by different agonists (Premkumar and
medium-diameter neurons. Scale bar, 50 µm. Titrations, indicating NPR-C expression in NF200-positive large-diameter neurons, as well as in CGRP- and TRPV1-positive small-diameter neurons. Scale bar, 50 µm.

CNP modulation of TRPV1 channel activity is mediated by PKA or PKC. Pretreatment of neurons with a PKC inhibitor (BIM, 1 µM), but not with a PKA inhibitor (KT5720, 400 nM), attenuated the CNP-induced potentiation of peak $I_{\text{Cap}}$ (0.89 ± 0.15- and 0.56 ± 0.19-fold increase in third and fourth $I_{\text{Cap}}$, respectively, for CNP + KT5720 treatment group ($n = 8$); Fig. 2C). These results indicate that CNP potentiates TRPV1 activity via a PKC-dependent pathway, leading us to further explore the CNP signaling through NPR-C that is coupled to the PKC activation.

Although NPR-C has been reported to serve as the clearance receptor for NPs, several reports have suggested that NPR-C can functionally couple to the $\alpha_i$ subunit, which in turn produces $\beta\gamma$-mediated activation of PLCβ, IP$_3$ hydrolysis, and PKC activation (Levin, 1993; Murthy and Makhlouf, 1999; Murthy et al., 2000; Anand-Srivastava, 2005). Using immunohistochemical staining of adult mouse lumbar DRGs, we found that NPR-C is expressed in most neurons, including those that express the A-fiber marker NF200 and those that express the peptidergic C-fiber marker CGRP (Fig. 3A). Almost all the TRPV1-positive neurons were also positive for NPR-C (Fig. 3A). Next, we determined the specific type of $\alpha_i$ subunits that couple to NPR-C in mouse DRG neurons. Immunoprecipitation of NPR-C, but not NPR-B, specifically coprecipitated the $\alpha_i$, but not $\alpha_q$, or $\alpha_s$ subunits in mouse DRG neurons (Fig. 3B). Activation of a number of $\alpha_i$-coupled GPCRs, including NPR-C, has been shown to activate PKC via the dissociation of $\beta\gamma$ subunits from $\alpha_i$, during receptor activation and subsequent activation of PLCβ (Murthy and Makhlouf, 1999; Murthy et al., 2000; Shi et al., 2003; Anand-Srivastava, 2005; Chen et al., 2005; Pandey, 2005b; Sabbatini et al., 2007). Activation of $\alpha_{q11}$-coupled GPCRs also leads to the activation of MAPK/ERK and PLCβ–DAG–PKC signaling cascades. Therefore, we next determined whether CNP was capable of activating these signaling cascades in DRG neurons. CNP treatment of cultured mouse DRG neurons (100 nM, 30 min) increased phosphorylation of ERK1/2, without any change in total ERK1/2 levels (Fig. 3C). Because CNP-induced potentiation of TRPV1 activity was attenuated by inhibition of PKC (Fig. 2C), we next determined whether CNP treatment could activate PKC by monitoring its translocation to the plasma membrane of DRG neurons as a readout of PKC activation (Cesare et al., 1999; Hucho et al., 2005). We focused on PKC$\varepsilon$, because this isoform is known to phosphorylate and sensitize TRPV1 upon activation by capsaicin and heat (Cesare et al., 1999; Premkumar and Ahern, 2000; Vellani et al., 2001; Numazaki et al., 2002; Bhave et al., 2003). Under control conditions, PKC$\varepsilon$ was evenly distributed among the neurons.
throughout the cytoplasm of mouse DRG neurons (Fig. 3D). CNP treatment (100 nM, 5 min) led to translocation of the bulk of PKCε immunoreactivity to the cell plasma membrane, as was similarly observed during treatment with the potent PKC activator PMA (200 nM, 2 min; Fig. 4A,C,D). Pretreatment of neurons with PTX (100 ng/ml for 24 h), a potent inhibitor of G_{i}-G_{q}/-G_{12/13} subunit dissociation during receptor activation, led to the attenuation of PKCε translocation to the cell plasma membrane in response to CNP but not to PMA treatment (Fig. 4B). These results suggest that NPR-C, but not NPR-B, couples to G_{i} subunit in mouse DRG neurons to activate MAPK and PKC via G_{i}-G_{q}/-G_{12/13}-mediated downstream signaling. Because it has been suggested that the affinities of CNP and ANP for G_{i}-coupled NPR-C are higher than that of BNP (He et al., 2001; Anand-Srivastava, 2005), it is possible that distinct NP-mediated signaling pathways prevail in different tissues/cells based on the specific expression of individual NP receptor subtypes.

**CNP potentiates acid-induced TRPV1 channel activation**

Protons (H^{+}) are well-known endogenous activators of TRPV1, and the H^{+} concentration is substantially increased at sites of tissue injury or inflammation (Dray and Read, 2007; Hucho and Levine, 2007; Basbaum et al., 2009; Gold and Gebhart, 2010; Kuner, 2010). Therefore, we examined whether CNP could potentiate proton-induced inward currents (I_{pH}) in small/medium-diameter cultured mouse DRG neurons. In these experiments, we used a protocol similar to that shown in Figure 1C, except the cells were stimulated with acidic pH extracellular buffer instead of capsaicin. Application of extracellular buffer with pH 6.4 resulted in two types of inward currents in small/medium-diameter cultured mouse DRG neurons. The first type was characterized by a rapidly activating inward current component followed by a sustained inward current (Fig. 5A, left). These neurons also exhibited capsaicin-induced inward currents that were elicited with the application of capsaicin (100 nM, 5 s) after the fourth

![Figure 4. CNP treatment leads to translocation of PKCε in mouse DRG neurons. Immunocytochemical analysis of PKCε translocation to the cell periphery in cultured mouse DRG neurons upon treatment with CNP (100 nM for 5 min) and PMA (200 nM for 2 min; as a positive control) compared with vehicle (DMSO) treatment. Representative photomicrographs of neurons immunostained with anti-NPR-C (red) and anti-PKCε (green) antibodies under control (A) and PTX (100 ng/ml, 24 h) (B) treatment conditions. Right panels for each image set are magnified views of cells marked with respective rectangular white boxes, and the line graphs show the PKCε distribution profile (NIH Image J) within respective cells across the drawn white lines. The high-intensity peak signals near both ends of the path described by the line denote increased PKCε translocation to the cell plasma membrane. C, Quantification of PKCε translocation to the cell periphery under different treatment conditions (for details of analysis, see Materials and Methods). Data are presented as mean ± SEM of the peak intensity ratios (peripheral/cytoplasmic; n = 25 cells for each treatment condition, from four independent cultures). D, Quantification of number of DRG neurons with PKCε translocation to the cell periphery under different treatment conditions. Data are presented as mean ± SEM of the percentage of cells with increased PKCε staining intensity at the cell periphery (n > 500 neurons for each treatment group, from 4 independent cultures). For C, D, *p < 0.05 and ***p < 0.001, significantly different compared with their respective vehicle groups; #p < 0.05 and ###p < 0.001, significantly different compared with CNP treatment in control group (one-way ANOVA with post hoc Bonferroni’s correction).
medium-diameter DRG neurons from TRPV1+/− mice and found that CNP did not potentiate peak \(I_{pH6.4}\) in these neurons [1.43 ± 0.28- and 1.65 ± 0.37-fold increase in third and fourth \(I_{pH6.4}\), respectively (n = 9); Fig. 5B,C]. Similar to CNP, application of ANP also led to the potentiation of \(I_{pH6.4}\) in small/medium-diameter DRG neurons from TRPV1+/+ but not from TRPV1−/− mice (data not shown).

We next determined the effect of CNP on the \(H^+\) dose–response relationship of TRPV1-mediated \(I_{pH}\) activation in small/medium-diameter DRG neurons that are capsaicin sensitive. CNP application led to a significant leftward shift in the \(H^+\) dose–response relationship of TRPV1-mediated \(I_{pH}\) (Fig. 5A). Data are presented as mean ± SEM of fold increase in peak \(I_{pH6.4}\) normalized to the second \(I_{pH6.4}\) and fitted with the Hill equation. The mean values and EC_{50} pH values are mentioned in the panel for vehicle and CNP treatment groups.

**Figure 5.** CNP specifically sensitizes TRPV1-mediated acidic pH-induced inward currents in mouse DRG neurons. A. Representative sets of traces of four successive proton (pH 6.4 extracellular buffer for 5 s; with 1 min interval) activated currents (inactivating inward currents (Fig. 5A). These data indicate that CNP potentiates TRPV1-mediated proton-activated inward currents in mouse DRG neurons obtained from TRPV1+/− mice, with or without continuous extracellular perfusion of CNP (10 nM) after the second \(I_{pH6.4}\). Representative sets of traces of four successive \(I_{pH6.4}\) recorded from small/medium-diameter cultured mouse DRG neurons obtained from TRPV1−/− mice, with or without continuous extracellular perfusion of CNP (10 nM) after the second \(I_{pH6.4}\). C. Quantification of \(I_{pH6.4}\) from experiments as shown in A and B. Data are presented as mean ± SEM of fold increase in peak \(I_{pH6.4}\) normalized to the second \(I_{pH6.4}\) and n values are shown in parentheses for each genotype/current/treatment group. *p < 0.05, significantly different compared with the respective \(I_{pH6.4}\) for the vehicle group (one-way ANOVA with post hoc Dunnett’s correction). B. CNP treatment (10 nM for 5 min) led to a leftward shift in the pH dose–response relationship of \(I_{pH}\) in capsaicin-sensitive small/medium-diameter cultured mouse DRG neurons. Data are presented as mean ± SEM of inward currents in response to pH 7.4, 6.8, 6.4, 6.8, 5.4, and 4.8, normalized to the peak amplitude of \(I_{pH6.4}\) and fitted with the Hill equation. The n values and EC_{50} pH values are mentioned in the panel for vehicle and CNP treatment groups.

\(I_{pH6.4}\). The other type displayed rapidly activating and rapidly inactivating inward currents (Fig. 5A, right), and these neurons did not exhibit any response to capsaicin (100 nm, 5 s). CNP application (10 nM) strongly potentiated the peak \(I_{pH6.4}\) [6.58 ± 1.07- and 9.87 ± 2.43-fold increase in third and fourth \(I_{pH6.4}\), respectively (n = 31)] in capsaicin-sensitive neurons but not in capsaicin-insensitive neurons [1.33 ± 0.05- and 1.08 ± 0.14-fold increase in third and fourth \(I_{pH6.4}\), respectively (n = 6); Fig. 5A,C]. These data indicate that CNP potentiates \(I_{pH6.4}\) only in DRG neurons that express TRPV1. To further confirm our findings, we evaluated CNP potentiation of \(I_{pH6.4}\) in cultured small/medium-diameter DRG neurons from TRPV1+/− mice. We next determined the effect of CNP on the \(H^+\) dose–response relationship of TRPV1-mediated \(I_{pH}\) activation in small/medium-diameter DRG neurons that are capsaicin sensitive. CNP application led to a significant leftward shift in the \(H^+\) dose–response relationship of TRPV1-mediated \(I_{pH}\) (Fig. 5A). Data are presented as mean ± SEM of fold increase in peak \(I_{pH6.4}\) normalized to the second \(I_{pH6.4}\) and fitted with the Hill equation. The mean values and EC_{50} pH values are mentioned in the panel for vehicle and CNP treatment groups.

It has been shown that PKC-induced potentiation of TRPV1 channel activity is governed by direct phosphorylation of the channel protein at three putative Ser/Thr residues S502, T704,
and S800 (Numazaki et al., 2002; Bhave et al., 2003). To verify whether these three PKC phosphorylation sites in TRPV1 are essential for the CNP potentiation of channel activity, we performed whole-cell voltage-clamp analysis of TRPV1 channel modulation in HEK293T cells heterologously expressing rTRPV1 and hNPR-C. CNP application (10 nM) led to the potentiation of peak $I_{\text{cap}}$ in HEK293T cells expressing rTRPV1 and NPR-C [3.08 ± 1.28- and 8.82 ± 2.8-fold increase in third and fourth $I_{\text{cap}}$, respectively ($n = 11$); Fig. 7A, B]. CNP (10 nM) also potentiated peak $I_{\text{cap}}$ in HEK293T cells expressing only rTRPV1, without the cotransfection of the plasmid containing NPR-C cDNA; however, with a time delay [1.22 ± 0.27- and 12.5 ± 4.1-fold increase in third and fourth $I_{\text{cap}}$, respectively ($n = 9$); Fig. 7A, B]. This led us to suspect that NPR-C is endogenously expressed in HEK293T cells. Indeed, RT-PCR analysis of total mRNA from HEK293T cells showed expression of all three NPRs, NPR-A/B/C, in this cell line (Fig. 6C). The delay in CNP-induced potentiation of TRPV1 currents in HEK293T cells compared with mouse DRG neurons could presumably be attributable to an inefficient $G_{\alpha_i}$ coupling to NPR-C or availability of significantly lesser pool of $G_{\alpha_i}$-coupled NPR-C in these cells. As such, we used this cell line to affirm the involvement of PKC in the potentiation of TRPV1 channel activity by CNP, by transfecting single, double, and triple Ala mutant TRPV1 channels at three putative PKC phosphorylation sites, S502, T704, and S800, in HEK293T cells. We found that CNP potentiation of $I_{\text{cap}}$ was reduced in S502A and S800A, but not T704A, single mutants and almost abolished in cells expressing TRPV1–S502A/S800A double and TRPV1–S502A/T704A/S800A triple mutants compared with cells expressing rTRPV1–WT [22.4 ± 4.6-, 9.5 ± 5.6-, 23.1 ± 12.3-, 8.6 ± 1.9-, 3.4 ± 0.7- , and 3.8 ± 1.2-fold increases in fourth $I_{\text{cap}}$ (normalized to second $I_{\text{cap}}$) for, respectively, WT ($n = 12$), S502A ($n = 10$), T704A ($n = 6$), S800A ($n = 12$), S502A/S800A ($n = 14$), and S502A/T704A/S800A ($n = 13$) channels; Fig. 7D)]. Collectively, these results suggest that CNP-induced strong potentiation of TRPV1 channel activity is mediated via PKC activation and subsequent modification of TRPV1 protein primarily at two amino acid residues: S502 and S800.

**CNP increases the frequency of AP firing in DRG neurons**

Because CNP potentiates TRPV1 channel activity, we next evaluated whether sensitization of TRPV1 by CNP alters the excitability of DRG neurons, by whole-cell current-clamp recordings in cultured mouse DRG neurons. Application of capsaicin (50 nM, ~5 s) induced AP firings; however, APs were mostly absent or at low frequency during the second capsaicin application (50 nM, ~5 s) after a 1 min wash period (Fig. 8A), likely as a result of Ca$^{2+}$-dependent desensitization of the TRPV1 channel (Koplas et al., 1997). In contrast, treatment with CNP (10 nM, 1 min) between the capsaicin applications led to a marked increase in the frequency of second capsaicin-induced AP firing (Fig. 8A). CNP application (10 nM, 1 min) also led to increased AP firing frequency during extracellular pH 6.8 application, specifically in capsaicin-sensitive, but not in capsaicin-insensitive, small-medium-diameter cultured mouse DRG neurons (Fig. 8B). Furthermore, CNP application did not lead to any increase in pH 6.8-induced AP firing frequency in small-medium-diameter DRG neurons from TRPV1$^{-/-}$ mice (Fig. 8C). Similar to CNP, application of ANP to...
small/medium-diameter DRG neurons also led to increased AP firing frequency during capsaicin and pH 6.4 applications (data not shown). These data further confirm our hypothesis that CNP-induced potentiation of TRPV1 activity leads to increased sensory neuron firing in response to mild extracellular acidic conditions.

CNP injection leads to the development of acute thermal hyperalgesia and is dependent on Gβγ–TRPV1 signaling

Given the ability of CNP to sensitize TRPV1 channel activity and strongly potentiate nociceptor firing, we next tested whether CNP administration could lead to the development of thermal hyperalgesia in mice. Intraplantar injection of different concentrations of CNP (10 μl of 500 nM and 10 μl in saline) to mice led to the development of thermal hyperalgesia ipsilateral to the injected hindpaw (quantified by a significant decrease in the PWL to a focused high-intensity beam of light on the plantar surface) compared with saline injection. CNP-induced thermal hyperalgesia was observed within 30 min of injection, which persisted for at least 6 h after the injection but subsequently recovered to baseline-level PWLs after 24 h (Fig. 9A). No significant decreases in the PWLs of contralateral paws were observed in response to CNP injection (Fig. 9A). Injection of 10 and 100 nM CNP displayed reduced PWLs, with significant decreases at the 4 h time point after 100 nM CNP injection, compared with the saline injection group (Fig. 9A). We then evaluated the role of the CNP–Gβγ–TRPV1 signaling axis in the development of acute thermal hyperalgesia. Intraperitoneal injection of gallein (100 mg/kg), a specific inhibitor of Gβγ-mediated activation of PLCβ3, 30 min before intraplantar CNP injection led to significant attenuation of CNP-induced acute thermal hyperalgesia (Fig. 9B). The magnitude of gallein–mediated attenuation of thermal hyperalgesia at 6 h after injection was abolished compared with 30 min, 2 h, and 4 h time points, possibly attributable to the rapid urinary elimination of gallein. Furthermore, intraperitoneal injection of AMG9810 (30 mg/kg), a specific inhibitor of TRPV1, 30 min before intraplantar CNP injection led to the blockade of the development of thermal hyperalgesia (Fig. 9B). No significant decreases in the PWLs of contralateral paws were observed in response to gallein and CNP or AMG9810 and CNP injections (Fig. 9B), as well as in response to gallein or AMG9810 injection alone (Fig. 9B). Furthermore, to genetically verify the key role of TRPV1 in CNP-induced development of thermal hyperalgesia, we performed intraplantar injection of CNP into TRPV1−/− mice and observed no significant decrease in the PWLs in both ipsilateral and contralateral hindpaws compared with saline-injected controls (Fig. 9C). Collectively, these results suggest that peripheral administration of CNP recruits a noncanonical Gβγ–PLCβ3–PKC signaling pathway to sensitize TRPV1 channel activity and nociceptor firing to induce the development of thermal hyperalgesia.

Discussion

Our study demonstrates that functional NPR-A/B/C are expressed in mouse DRG neurons, in which CNP and ANP, but not BNP, induce sensitization of TRPV1 channel activity that is independent of classical NPR-A/B–cGMP–PKG signaling. Rather, NP sensitization of TRPV1 is mediated through a noncanonical NPR-C–Gβγ–PLCβ3–PKC signaling module, as evidenced by the attenuation of CNP-induced modulation of TRPV1 activity by specific pharmacological blockade of these signaling components. Such modulation of TRPV1 channel activity leads to
nociceptor sensitization to mild and pathophysiologically relevant acidic conditions and is absent in TRPV1−/− DRG neurons. Furthermore, intraplantar CNP injection induces thermal hyperalgesia in WT mice, but not in TRPV1−/− mice, and is significantly attenuated by systemic administration of Gβγ or TRPV1 inhibitors. Together, these findings suggest that CNP recruits a novel noncanonical Gβγ–PLCβ–PKC–TRPV1 signaling pathway to induce nociceptor sensitization and thermal hyperalgesia, which likely takes place in a variety of tissue injury and inflammatory conditions.

The influence of NPs on inflammatory pain has been suggested recently, with contrasting observations on individual NPs. Intrathecal BNP injection inhibits inflammatory thermal hyperalgesia induced by the injection of complete Freund’s adjuvant (CFA) into rat hindpaw, without any significant effect on mechanical hypersensitivity (Zhang et al., 2010). Their study also found that intrathecal BNP injection led to a reduction in formalin-induced nocifensive behaviors. Mechanistically, BNP attenuates glutamate-induced AP firing in small-diameter rat DRG neurons. Furthermore, these effects were attributed to NPR-A–cGMP–PKG signaling, leading to increased BKCa current density (Zhang et al., 2010). Interestingly, BNP alone did not have any influence on AP firing (Zhang et al., 2010). In contrast to these antinociceptive effects of BNP, direct and rapid development of mechanical allodynia was observed in rats during intrathecal injection of CNP and 8-pCPT-cGMP but not ANP (Schmidtko et al., 2008a). It was suggested that CNP-induced mechanical allodynia is mediated through cysteine-rich protein 2, a downstream effector of increased PKG activity (Schmidtko et al., 2008b). Interestingly, our study found that, in addition to NPR-A/B, mouse DRG neurons also express NPR-C, in which it couples to Gαq. Application of CNP led to PKC activation through Goq–Gβγ–PLCβ–mediated intracellular signaling. Furthermore, intraplantar injection of CNP led to the development of thermal hyperalgesia that was dependent on Gβγ-mediated signaling, leading to enhanced TRPV1 activity. To this end, TRPV1−/− mice did not exhibit CNP-induced thermal hyperalgesia. Collectively, NPs exert distinct effects on inflammatory thermal and mechanical hypersensitivities. NPR-A/B–cGMP–PKG signaling in the DRG neuron central axons projecting to the spinal cord dorsal horn contributes to the development of mechanical allodynia (Schmidtko et al., 2008a,b; Heine et al., 2011), with the exception of intrathecal BNP, which exerts an analgesic effect on CFA-induced thermal hyperalgesia (Zhang et al., 2010). Our study suggests that CNP–Gβγ–PKC–TRPV1 signaling in the peripheral afferents contributes to the development of thermal hyperalgesia. Furthermore, widespread expression of NPR-B/C in DRG neurons of all sizes and CNP-induced increases in cGMP production in neurons collectively suggest a potential involvement of CNP in sensory afferent sprouting/bifurcation in the periphery and induction of mechanical allodynia/hyperalgesia, which warrants thorough experimental verification of this idea.

Our analysis of NP modulation of TRPV1 in mouse DRG neurons revealed that both ANP and CNP significantly potentiate channel activity and subsequently enhance AP firing frequency. In contrast, BNP did not have such an effect. This is consistent with a previous report in which no direct effects of BNP on AP firing were found in DRG neurons (Zhang et al., 2010). Furthermore, our study shows no involvement of cGMP/PKG activity in NP-induced potentiation of TRPV1 currents, which rules out any modulation of channel activity by NPR-A/B-mediated signaling in DRG neurons. NPR-C has been suggested as the NP clearance receptor (Pandey, 2005a; Potter et al., 2006), yet other reports suggest that NP binding to NPR-C leads to activa-
PKC translocation assays strongly suggest the existence of a functional NPR-C–Gαi–Gβγ–PLCβ–PKC signaling cascade in mouse DRG neurons. It was shown previously that the affinities of CNP and ANP for NPR-C exceed that of BNP (He et al., 2001; Anand-Srivastava, 2005), which provides yet another explanation for the lack of BNP-induced potentiation of TRPV1 currents in our study. As such, our results suggest a novel Gαi–Gβγ–PLCβ–PKC signaling pathway for a GPCR-mediated modulation of TRPV1 and subsequent nociceptor sensitization, which otherwise have been shown to be mediated primarily through Gαq– or Gα12/13-coupled GPCRs (Bhave et al., 2009; Patapoutian et al., 2009). High concentrations of morphine were shown to directly activate and potentiate TRPV1 currents in mouse DRG neurons (Forster et al., 2009). Considering the fact that many opioid receptors induce Gαq-coupled downstream signaling (Standifer and Pasternak, 1997; Stein, 2003), it is tempting to speculate that sensitization of TRPV1 channel activity via high doses of morphine (Forster et al., 2009) could be mediated through Gαi–Gβγ–PLCβ–PKC signaling, a hypothesis that needs to be verified experimentally.

Activity and expression of TRPV1 in sensory afferents has been shown to undergo diverse modulations by a large number of proinflammatory mediators, cytokines, chemokines, and bioactive peptides, mainly by influencing the phosphorylation state of the channel protein. The protein kinases PKC, PKA, p38MAPK, and Src are activated downstream as a result of activation of a variety of receptors for inflammatory mediators in sensory neurons/afferents, which subsequently lead to direct phosphorylation-dependent modulation of TRPV1 channel activity and nociceptor sensitization (Bhave et al., 2002, 2003; Ji et al., 2002; Mohapatra and Nau, 2005; Hucho and Levine, 2007; Patapoutian et al., 2009). Activation of PKC and subsequent phosphorylation-dependent modulation of TRPV1 channel activity has been shown to be a convergent mechanism underlying nociceptor sensitization by several mediators (Bhave and Gereau, 2004; Hucho and Levine, 2007; Bhave et al., 2009; Patapoutian et al., 2009; Gold and Gehart, 2010; Kuner, 2010). PKC directly phosphorylates TRPV1 at amino acid residues S502, T704, S774, and S800 (Numazaki et al., 2002; Bhave et al., 2003). Indeed, our results demonstrated that substitution mutations of two of these residues, S502A and S800A, abolished CNP-induced potentiation of TRPV1 channel activity. Phosphorylation of these residues in TRPV1 by PKC has been shown to potentiate the heat activation of the channel at temperatures below body temperature and under conditions of mild acidosis. The protein kinases PKC, PKA, p38MAPK, and Src are activated downstream as a result of activation of a variety of receptors for inflammatory mediators in sensory neurons/afferents, which subsequently lead to direct phosphorylation-dependent modulation of TRPV1 channel activity and nociceptor sensitization (Bhave et al., 2002, 2003; Ji et al., 2002; Mohapatra and Nau, 2005; Hucho and Levine, 2007; Patapoutian et al., 2009). Activation of PKC and subsequent phosphorylation-dependent modulation of TRPV1 channel activity has been shown to be a convergent mechanism underlying nociceptor sensitization by several mediators (Bhave and Gereau, 2004; Hucho and Levine, 2007; Bhave et al., 2009; Patapoutian et al., 2009; Gold and Gehart, 2010; Kuner, 2010). PKC directly phosphorylates TRPV1 at amino acid residues S502, T704, S774, and S800 (Numazaki et al., 2002; Bhave et al., 2003). Indeed, our results demonstrated that substitution mutations of two of these residues, S502A and S800A, abolished CNP-induced potentiation of TRPV1 channel activity. Phosphorylation of these residues in TRPV1 by PKC has been shown to potentiate the heat activation of the channel at temperatures below body temperature and under conditions of mild acidosis.
CNP induction of thermal hyperalgesia is dependent on Gβγ signaling and TRPV1, although the involvement of other sensory ion channels, such as TRPA1, TRPV4, and Na+, channels, cannot be ruled out. Based on the role of NPR-B–cGMP–PKG signaling in neurite bifurcation (Schmidt et al., 2009), along with our observation on CNP-induced cGMP production in DRG neurons, it is plausible that CNP might also influence peripheral mechanical hypersensitivity. Our future studies will focus on a thorough investigation along these lines.

Pathological modification/activation of TRPV1 and subsequent nociceptor sensitization by mildly acidic conditions has been proposed as one of the principal mechanisms underlying the development of inflammatory thermal hyperalgesia (Huchu and Levine, 2007; Basbaum et al., 2009; Gold and Gebhart, 2010; Kuner, 2010). Our results clearly show that CNP treatment shifts the H+ dose–response relationship of TRPV1 activation, such that the channel is activated in mildly acidic pH ranges. To this end, our results provide evidence of TRPV1-dependent sensitization of nociceptor firing at pH 6.8 in response to CNP that is within a tissue acidosis range associated with injury and inflammatory conditions, including arthritic joints (Dray and Read, 2007; Huchu and Levine, 2007; Gold and Gebhart, 2010; Kuner, 2010). In view of pain associated with inflamed arthritic joints, TRPV1 modulation in sensory afferents is suggested to play a key role in both thermal and mechanical pain sensation associated with rheumatoid arthritis and osteoarthritis (Dray and Read, 2007; Premkumar and Sikand, 2008; Gold and Gebhart, 2010; Kuner, 2010). Elevated levels (approximately fivefold) of CNP have been detected in the serum of rheumatoid arthritis patients (Olewicz-Gawlik et al., 2010a), and CNP has also been shown to be secreted at elevated levels by vascular endothelial cells during inflammatory and inflammatory conditions, including arthritic joints (Dray and Read, 2007; Huchu and Levine, 2007; Kuner, 2010).

References


