Nerve growth factor enhances the excitability of rat sensory neurons through activation of the atypical protein kinase C isoform, PKMζ

Y. H. Zhang, 1 J. Kays, 1 K. E. Hodgdon, 1 T. C. Sacktor, 2 and G. D. Nicol 1

1Department of Pharmacology and Toxicology, School of Medicine, Indiana University, Indianapolis, Indiana; and 2Departments of Physiology, Pharmacology, and Neurology, Robert F. Furchgott Center for Neural and Behavioral Science, State University of New York, Downstate Medical Center, Brooklyn, New York.

Submitted 12 January 2011; accepted in final form 29 September 2011

Nerve growth factor enhances the excitability of rat sensory neurons through activation of the atypical protein kinase C isoform, PKMζ. J. Neurophysiol. 107: 315–335, 2012. First published October 5, 2011; doi:10.1152/jn.00030.2011.—Our previous work showed that nerve growth factor (NGF) increased the excitability of small-diameter capsaicin-sensitive sensory neurons by activating the p75 neurotrophin receptor and releasing sphingolipid-derived second messengers. Whole cell patch-clamp recordings were used to establish the signaling pathways whereby NGF augments action potential (AP) firing (i.e., sensitization). Inhibition of MEK1/2 (PD-98059), PLC (U-73122, neomycin), or conventional/novel isoforms of PKC (bisindolylmaleimide I) had no effect on the sensitization produced by NGF. Pretreatment with a membrane-permeable, myristoylated pseudosubstrate inhibitor of atypical PKCs (aPKCs: PKMζ, PKCζ, and PKCα/λ) blocked the NGF-induced increase in AP firing. Inhibitors of phosphatidylinositol 3-kinase (PI3K) also blocked the sensitization produced by NGF. Isolated sensory neurons were also treated with small interfering RNA (siRNA) targeted to PKCζ. Both Western blots and quantitative real-time PCR established that PKMζ, but neither full-length PKCζ nor PKCα/λ, was significantly reduced after siRNA exposure. Treatment with these labeled siRNA prevented the NGF-induced enhancement of excitability. Furthermore, consistent with the high degree of catalytic homology for aPKCs, internal perfusion with active recombinant PKCζ or PKCζ augmented excitability, recapitulating the sensitization produced by NGF. Internal perfusion with recombinant PKCζ suppressed the total potassium current and enhanced the tetrodotoxin-resistant sodium current. Pretreatment with the myristoylated pseudosubstrate inhibitor blocked the increased excitability produced by ceramide or internal perfusion with recombinant PKCζ. These results demonstrate that NGF leads to the activation of PKMζ that ultimately enhances the capacity of small-diameter capsaicin-sensitive sensory neurons to fire APs through a PI3K-dependent signaling cascade.

p75 neurotrophin receptor; sensitization; action potential; neuronal firing

Nerve growth factor (NGF) plays an important role in initiation of the inflammatory response. Local inflammation can result in activation of small-diameter sensory neurons, which contributes to augmented sensitivity, vasodilatation, and plasma extravasation. The role of NGF is supported by observations that injection of NGF into a rat’s paw produces elevated sensitivity to thermal and mechanical stimulation (Lewin et al. 1993). Pretreatment with an antibody to NGF prevents thermal hyperalgesia produced by injection of complete Freund’s adjuvant into the rat paw (Lewin et al. 1994; Woolf et al. 1994). In an isolated skin-nerve-type preparation, NGF increased the firing frequency of isolated saphenous nerve in response to thermal stimulation (Rueff and Mendell 1996). More recent studies have demonstrated that NGF acts directly on sensory neurons because NGF augments capsaicin-evoked currents (Shu and Mendell 1999, 2001). In addition, our previous work demonstrated that NGF and brain-derived neurotrophic factor (BDNF) increase the number of current-evoked action potentials (APs) in capsaicin-sensitive small-diameter sensory neurons through modulation of voltage-dependent potassium currents (IK) as well as the tetrodotoxin-resistant sodium current (TTX-R INa) (Zhang et al. 2002, 2008).

The intracellular signaling cascades activated by NGF that give rise to neuronal sensitization are poorly understood. Our work has demonstrated that NGF and BDNF augment excitability through activation of the p75 neurotrophin receptor (p75NTR) and consequent downstream signaling by the sphingolipid pathway wherein the generation of ceramide appears to be a key event (Zhang et al. 2002, 2008; Zhang and Nicol 2004). Important early studies indicated that treatment with NGF led to increased neurite outgrowth in PC12 cells that was mediated by a phorbol ester-insensitive activation of an atypical PKC, PKCζ (Wooten et al. 1994, 1999). This observation raises the question as to how NGF leads to activation of PKCζ. Additional studies in a variety of cell types demonstrated that ceramide (ranging from membrane-permeant C2-ceramide to endogenous C18-ceramide) led to activation of PKCζ through a presently undefined mechanism (Bourbon et al. 2000; Lozano et al. 1994; Müller et al. 1995; Wang et al. 1999, 2005). Thus we speculate that NGF through activation of p75NTR liberates ceramide, which in turn could lead to the activation of PKCζ. In these studies, we sought to establish the intracellular signaling pathways whereby NGF enhances the excitability of small-diameter sensory neurons with focus on the idea that NGF leads to the activation of this atypical PKC.

Materials and Methods

Isolation and maintenance of adult rat sensory neurons. The procedures for primary culture of rat sensory neurons have been described previously (Lindsay 1988) with slight modification (Jiang et al. 2003). Briefly, male Sprague-Dawley rats (100–150 g) were killed by being placed in a chamber that was then filled with CO2. Dorsal root ganglia (DRG) were removed and collected in a culture dish filled with sterilized Puck’s solution. The ganglia were transferred to a conical tube filled with Puck’s solution containing 10 U/ml papain II and incubated for 12 min at 37°C. The tube was centrifuged for 50 s.
at low speed (~2,000 g), and the pellet was resuspended in Puck’s solution containing collagenase (1 mg/ml, type 1A) and dispase II (2.5 mg/ml). After a 14-min incubation at 37°C, the tube was centrifuged for 50 s before the enzyme-containing supernatant was removed. The pellet was resuspended in F-12 medium supplemented with 30 ng/ml 7S nerve growth factor (Harlan Bioproducts, Indianapolis, IN) and mechanically dissociated with fire-polished pipettes until all obvious chunks of tissues were gone. Isolated cells were plated onto plastic cover slips that had been previously coated with poly-D-lysine and laminin. The cells were maintained in F-12 medium containing NGF at 37°C and 3% CO_2 and were used within 12–24 h for electrophysiological recordings. All procedures were approved by the Animal Use and Care Committee of the Indiana University School of Medicine.

**Electrophysiology.** Recordings were made using the whole cell patch-clamp technique as previously described (Hamill et al. 1981; Zhang et al. 2008). Briefly, a cover slip with the sensory neurons was placed in a recording chamber where the neurons were bathed in normal Ringer solution of the following composition (in mM): 140 NaCl, 5 KCl, 2 CaCl_2, 1 MgCl_2, 10 HEPES, and 10 glucose, with pH adjusted to 7.4 with NaOH. Recording pipettes were pulled from borosilicate glass tubing and fire-polished. Whole cell currents or voltages were recorded with an Axopatch 200 patch-clamp amplifier (Molecular Devices, Sunnyvale, CA); the data were acquired and analyzed using pCLAMP 6.04 or pCLAMP 9.0 (Molecular Devices).

The whole cell recording configuration was established in normal Ringer solution. All drugs were applied by external superfusion of the recording chamber using a VC-8 bath perfusion system (Warner Instruments, Hamden, CT). In voltage-clamp experiments, both capacitance and series resistance compensation (typically 80%) were used. Linear leakage currents were subtracted by a P4 protocol in the recordings of TTX-R I\textsubscript{Na} but not in those of I\textsubscript{K}. To isolate I\textsubscript{K}, the Na\textsuperscript{+} in the normal Ringer solution was substituted with equimolar N-methyl glutamine (NMG). The membrane voltage was held at ~60 mV; activation of the currents was determined by voltage steps of 350 ms applied at 5-s intervals in +10-mV increments from ~80 to ~60 mV. Patch pipettes had resistances of ~2–5 M\textOmega when filled with the following solution (in mM): 140 KCl, 5 MgCl\textsubscript{2}, 4 ATP, 0.3 GTP, 2.5 CaCl\textsubscript{2}, 5 EGTA (calculated free Ca\textsuperscript{2+} concentration ~100 nM, MaxChelator), and 10 HEPES at pH 7.3 adjusted with KOH. This pipette solution was also used in the current-clamp recordings. The TTX-R I\textsubscript{Na} was isolated by superfusing cells with the following Ringer solution (in mM): 30 NaCl, 65 NGM-CI, 30 triethanolammonium (TEA), 0.1 CaCl\textsubscript{2}, 5 MgCl\textsubscript{2}, 10 HEPES, 10 glucose, 10 sucrose, and 500 mM TTX, with pH adjusted to 7.4 with TEA-OH. The recording pipette was filled with (in mM) 110 CsFl, 25 CsCl, 10 NaCl, 5 MgCl\textsubscript{2}, 4 ATP, 0.3 GTP, 1 CaCl\textsubscript{2}, 10 EGTA, 10 glucose, and 10 HEPES at pH 7.3 (maintained with CsOH). Patch pipettes had resistances of ~1–1.5 M\textOmega. Membrane voltage was held at ~60 mV; activation of the current was determined by voltage steps 30 ms in duration and applied at 5-s intervals in +5-mV increments from ~60 to ~60 mV. After the control responses were obtained, the superfuse was changed to the appropriate Ringer solution and cells were superfused continuously for the appropriate times. The currents were filtered at 5 kHz, I\textsubscript{K} was sampled at 1 kHz, and TTX-R I\textsubscript{Na} was sampled at 25 kHz.

In the current-clamp experiments, the neurons were held at their resting potentials and a depolarizing ramp (1,000 ms in duration) was applied. The amplitude of the ramp was adjusted to produce between two and five APs under control conditions, and then the same ramp was used throughout the recording period for each individual neuron. Voltages were filtered at 5 kHz and sampled at 1 kHz. For experiments involving inhibitors of different signaling molecules, a set of sensory neurons from the same tissue harvest remained untreated as a positive control to assess the effects of NGF on neuronal excitability; parallel recordings were typically obtained from two to three individual untreated neurons. In all these recordings, NGF produced a two- to threefold increase in the number of evoked APs, demonstrating that NGF sensitized these neurons. The data from these parallel neurons (n = 10) have been combined and represent the effects of NGF on neuronal excitability for the untreated condition. These results are summarized in Table 3. At the end of each recording, the neuron was exposed to 400 nM capsaicin. This neurotoxin was used to distinguish capsaicin-sensitive sensory neurons because these neurons are believed to transmit nociceptive information (Holzer 1991). However, the correlation between capsaicin sensitivity and a neuron being a nociceptor is not absolute. Some nociceptive neurons are insensitive to capsaicin, and some capsaicin-sensitive neurons are not nociceptors (see Petruska et al. 2000). Therefore, this agent was used to define a population of small-diameter sensory neurons that could serve a nociceptive function. The results reported below were obtained from capsaicin-sensitive neurons only. All experiments were performed at room temperature (~22°C).

**Small interfering RNA.** To reduce expression of PKC\textsubscript{\eta}, we used a previously described small interfering RNA (siRNA) treatment protocol (Chi and Nicol, 2007). Briefly, isolated sensory neurons were maintained for 6 h in normal medium with 30 ng/ml NGF. Normal medium was replaced with OptiCell medium overnight. The Metacef-siRNA complex (200 nM) was added on day 2 in culture wherein the neurons were exposed to the siRNA or Metafectene alone for 48 h at 37°C. After 2 days (day 4 in culture), the Metacef-siRNA was washed out and the normal medium containing antibiotics and NGF was then added to the neurons and allowed to incubate for another 2 days before electrophysiological recordings, Western blots, or quantitative real-time PCR (qPCR) were performed. The siRNA targeted to PKM\textsubscript{\eta}/PKC\textsubscript{\eta} (NCBI reference sequence NM_022507.1 for PKC\textsubscript{\eta}) was a mixture of four siRNAs (siGenome SMARTpool, M-09-1560-00) obtained from Thermo Scientific (Lafayette, CO), all of which are targeted to sequences present in both PKM\textsubscript{\eta} and PKC\textsubscript{\eta} mRNAs. The sequences targeted by this siRNA pool (5’ to 3’) are as follows: siRNA1, GAAAGCAUGUAGGACCUUU (position 741–759); siRNA2, GGAACAUGACAAUAUCAAA (position 803–821); siRNA3, CGUAGCUAGUCCUCGCU (position 1218–1237); and siRNA4, CGAUGCGGAUGGACAAUA (position 1299–1310). Two additional siRNA molecules were obtained from Thermo Scientific: siRNA 638, GAAAGCTGTTTGCAATAA (5’ to 3’; position 638–656), targeted to sequence present in both species, and siRNA 407, GGGACGAAGTGCTCATCAT (5’ to 3’; position 407–425), targeted to sequence present in PKC\textsubscript{\eta} but not PKM\textsubscript{\eta}. The negative control siRNA was obtained from Ambion (SC-1; Austin, TX) and had the sequence (5’ to 3’) 5CGGCGCUUUUGAGGAUCUG. Metacef was purchased from Biontex-USA (San Diego, CA). For the electrophysiological studies, the siRNA and the negative control siRNA were labeled using the Mirus Bio Label IT* siRNA Tracker* intracellular localization kit (fluorescein) available from Fischer Scientific (Pittsburgh, PA).

**Western blot.** All procedures were conducted on ice. Isolated sensory neurons maintained in culture were scraped from the dish after addition of 150 μl of ice-cold 1× RIPA lysis buffer (no. 20-188; Millipore, Billerica, MA) containing a 100-fold dilution of Protease inhibitor cocktail set III (539134; EMD Biosciences, San Diego, CA). Cell lysates were transferred to 1.5-ml tubes and sonicated (setting 3) for 4 min at 4°C. Supernatants were centrifuged at 2,500 × g for 4 min at 4°C. Supernatants were transferred to fresh tubes. Protein concentration was measured using the Bio-Rad Protein assay dye reagent no. 500–0000 (Bio-Rad, Hercules, CA). Equivalent amounts of reduced, denatured protein (25 μg) were loaded and separated on a NuPAGE 4–12% Bis-Tris gel (NP0335BOX; Invitrogen, Carlsbad, CA) using NuPAGE MES-SDS running buffer (NP0002; Invitrogen) and then transferred to an iBlot polyvinylidene difluoride membrane (LC2005; Invitrogen). To measure expression levels, we used the following antibodies: an atypical PKC polyclonal rabbit antibody that detects PKM\textsubscript{\eta}/PKC\textsubscript{\eta} and PKCA/\alpha (sc-216, 1:500; Santa Cruz Biotechnology, Santa
PKMζ and Neuronal Excitability

Table 1. Primer sequences

<table>
<thead>
<tr>
<th>Gene</th>
<th>Ref. Sequence</th>
<th>Location</th>
<th>Size, bp</th>
</tr>
</thead>
<tbody>
<tr>
<td>PKCζ</td>
<td>NM_022507.1</td>
<td>239–258</td>
<td>178</td>
</tr>
<tr>
<td>PKMζ</td>
<td>S80195.1</td>
<td>416–398</td>
<td>88</td>
</tr>
<tr>
<td>PKCA/α</td>
<td>NM_032059.1</td>
<td>137–120</td>
<td>136</td>
</tr>
<tr>
<td>Arbp</td>
<td>NM_022402.2</td>
<td>872–852</td>
<td>58</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Gene</th>
<th>Location</th>
<th>Size, bp</th>
</tr>
</thead>
<tbody>
<tr>
<td>PKCζ</td>
<td>398–416</td>
<td>136</td>
</tr>
<tr>
<td>PKMζ</td>
<td>532–512</td>
<td>96</td>
</tr>
<tr>
<td>PKCA/α</td>
<td>460–479</td>
<td>106</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Primer sequences</th>
</tr>
</thead>
<tbody>
<tr>
<td>PKCζ F: TGCAGGGGACGGCATCAGGT T: TGGCTGTATAGACTTCCGT CC: ProQinase, MA</td>
</tr>
<tr>
<td>PKMζ F: ACCAAGAGAACGACGACGT T: TTCAAAAGCCTTGCGTGGAAA CC: ProQinase, MA</td>
</tr>
<tr>
<td>PKCA/α F: GGCTCGTACACGACGCTCC T: GTGGCCATTTCGACATTACA CC: ProQinase, MA</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Gene</th>
<th>Our Primers</th>
<th>Ghosh-Adapted Primers</th>
</tr>
</thead>
<tbody>
<tr>
<td>Brain</td>
<td>PKCζ</td>
<td>1.981</td>
</tr>
<tr>
<td></td>
<td>PKMζ</td>
<td>2.058</td>
</tr>
<tr>
<td></td>
<td>PKCA/α</td>
<td>1.975</td>
</tr>
<tr>
<td>Lung</td>
<td>PKCζ</td>
<td>2.006</td>
</tr>
<tr>
<td></td>
<td>Arbp</td>
<td>2.019</td>
</tr>
</tbody>
</table>

Adapted primers were provided by Dr. Sourav Ghosh, University of Arizona (see text). *From Hernandez et al. 2003 (forward only). †From Stretton et al. 2010.

Cruz, CA), a PKCζ-specific rabbit polyclonal antiserum (1:5,000; described in Hernandez et al. 2003), a PKCA/α-specific monoclonal mouse antibody (610207, 1:500; BD Transduction Laboratories, San Diego, CA), and for a loading control, an actin monoclonal mouse antibody (MS-1295-PABX, 1:2,500; Thermo Scientific, Waltham, MA). These antibodies were added to the blocking solution and incubated overnight at 4°C with agitation. After serial incubation with specific secondary antibodies, immunoreactive bands on the membrane were developed using enhanced chemiluminescence (ECL kit; Thermo Scientific, Rockford, IL) and visualized by exposure to Blue Lite Autorad film (F-9024–8 × 10; ISCBioExpress, Kampenhout, Belgium). The film was photographed with a Kodak DC290 charge-coupled device camera, and the density of each band was measured using Kodak ID 3.6 software (Kodak Scientific Imaging Systems, New Haven, CT). The summary data are expressed as the density of the experimental bands normalized to the density of their respective actin bands and then normalized to their respective untreated control/actin values.

Quantitative real-time PCR. DRG, brain, and lung tissue were isolated from male Sprague-Dawley rats (100–150 g). Total RNA was extracted from each tissue and from primary cultures of dissociated DRG neurons (isolated in the absence of papain) using the RNAeasy Plus minikit (Qiagen, Valencia, CA) and assessed on a NanoDrop ND-1000 spectrophotometer (Thermo Scientific, Franklin, MA) for concentration (A260) and purity by optical density (OD) ratios (A260/A280, between 2.0 and 2.2). RNA integrity was also assessed using the Experion RNA StdSens analysis kit and Experion automated electrophoresis system (Bio-Rad). RNA samples with a RNA quality indicator value >7.0 were used for this study. After treatment with DNase I (Invitrogen), 1 μg of RNA was reverse transcribed using the iScript cDNA synthesis kit (Bio-Rad) in 20-μl reactions according to the manufacturer’s instructions. To assess the specificity of these reactions, no-reverse transcription products were diluted with nuclease-free water and amplified in 10-μl reactions using Power SYBR Green PCR Master Mix (Applied Biosystems, Carlsbad, CA) and 500 nM forward and reverse primers (Invitrogen). Two sets of primers were used for each aPKC gene target: one set for PKCζ and PKMζ was designed in our laboratory using PrimerExpress software v3.0 (Applied Biosystems), whereas the primer set for PKCA/α was based on Stretton et al. 2010. The second set of sequences was kindly provided by Dr. Sourav Ghosh and his laboratory (Cellular and Molecular Medicine, University of Arizona); these were targeted to aPKC mouse transcripts wherein we modified several base pairs to match rat mRNA sequences. Acidic ribosomal protein P0 (Arbp) was selected as the reference gene for normalization. The accession number, amplicon size and position, and primer sequences for all genes targeted in this study are shown in Table 1. qPCR reactions were run in duplicate with tissue-derived cDNA or in triplicate with primary DRG cell culture cDNA on an Applied Biosystems 7500 Fast Real-Time PCR system using MicroAmp Fast 96-well reaction plates sealed with MicroAmp optical adhesive film. All reactions began with an initial cycle of 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. Efficiencies for each primer pair were determined from the fitted slope of a seven-point standard curve. The regression fit was considered to be acceptable when r² > 0.99. Calculated efficiencies for all primers are listed in Table 2. The specificity of these amplifications was verified by melt curve analysis and electrophoresis on a 2% agarose gel. The quantification cycle (Cq) was chosen to be number of cycles when the value of normalized fluorescence generated by SYBR green emission attained 0.3. For determination of the expression levels of PKCζ, PKMζ, PKCA/α, and Arbp, a calculation based on Pfaffl (2001) was used wherein the number of copies = efficiency rCq. Because the calculated efficiencies listed in Table 2 are all very close to 2, we have based the calculation of number of copies for all genes on an efficiency of 2.

Internal perfusion. Recombinant PKCζ and PKCs were aliquoted and stored according to the manufacturer’s instructions (PKCζ: Cell Signaling Technology, Beverly, MA; PKCζ and PKCs: ProQinase, Freiburg, Germany). PKCζ was dissolved in the pipette solution at a concentration of 2 ng/μl, and PKCζ at an initial concentration of 0.42 ng/μl. At these concentrations, the specific activities were similar. The tip of the patch pipette was filled with pipette solution; the pipette was then backfilled with pipette solution containing recombinant PKCζ or PKCs. A fresh batch of recombinant PKC-pipette solution was made for each recording session and kept on ice during the experiments. Control recordings were obtained as soon as possible after the whole
cell configuration was established. A concentration of 2 ng/μl was chosen based on the dose-activity relation for the recombinant PKCζ wherein 100 ng/50 μl reaction yielded maximal kinase activity (see Fig. 3, http://www.cellsignal.com/pdf/7608.pdf). Recombinant PKCζ (2 ng/μl in pipette solution) was heat inactivated by being placed in boiling water for 10 min and then immediately placed on ice for the remainder of the recording session.

Data analysis. Data are means ± SE. The parameters of excitability described in Tables 3 and 4 were determined, in part, by differentiating the voltage trace (dV/dt) in the current-clamp recordings (sampling frequency of 500 Hz). The firing threshold and time at which the first AP was fired were taken as the point that exceeded the baseline value of dV/dt by >20-fold. The baseline value of dV/dt was determined by averaging the points between the onset of the ramp and the next 100 ms (135–235 ms). The rheobase was measured as the amount of ramp current at the firing threshold. The resistance at threshold (R_{th}) was calculated as the difference between the firing threshold and the resting membrane potential divided by the rheobase current. The voltage dependence for activation of I_{K} and TTX-R I_{Na} was fitted with the Boltzmann equation, where \( G/G_{\text{max}} = 1/[1 + \exp(V_{0.5} - V_{m}/k)], \) in which \( G \) is the conductance. Values are means ± SE; \( n = \) no. of neurons. RMP, resting membrane potential; FT, firing threshold; Rheo, rheobase; R_{th}, membrane resistance at threshold; Norm Rheo, rheobase NGF/rheobase control; Norm R_{th}, resistance NGF/resistance control. *P < 0.05, untreated + NGF vs. pretreatment + NGF (across groups, ANOVA).

Table 3. Effects of NGF on excitability parameters

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Untreated</th>
<th>PD-98059</th>
<th>U-73122</th>
<th>PKCζ inhibitor</th>
</tr>
</thead>
<tbody>
<tr>
<td>RMP, mV</td>
<td>−59.2 ± 1.6</td>
<td>−58.2 ± 2.1</td>
<td>−58.6 ± 2.5</td>
<td>−55.5 ± 2.0</td>
</tr>
<tr>
<td>FT, mV</td>
<td>−16.1 ± 3.7</td>
<td>−17.3 ± 1.3</td>
<td>−19.3 ± 2.7</td>
<td>−27.3 ± 6.9</td>
</tr>
<tr>
<td>Rheo, pA</td>
<td>440 ± 152</td>
<td>350 ± 81</td>
<td>709 ± 383</td>
<td>382 ± 201</td>
</tr>
<tr>
<td>R_{th}, MΩ</td>
<td>196 ± 48</td>
<td>161 ± 38</td>
<td>198 ± 74</td>
<td>183 ± 60</td>
</tr>
<tr>
<td>n</td>
<td>7</td>
<td>7</td>
<td>7</td>
<td>5</td>
</tr>
</tbody>
</table>

Table 4. Effects of recombinant PKCζ on excitability parameters

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Control (0 min)</th>
<th>PKCζ (6 min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>RMP, mV</td>
<td>−64.0 ± 2.5</td>
<td>−57.4 ± 1.2*</td>
</tr>
<tr>
<td>FT, mV</td>
<td>−10.5 ± 2.4</td>
<td>−19.9 ± 1.6*</td>
</tr>
<tr>
<td>Rheo, pA</td>
<td>727 ± 138</td>
<td>226 ± 84*</td>
</tr>
<tr>
<td>R_{th}, MΩ</td>
<td>100 ± 22</td>
<td>189 ± 24*</td>
</tr>
<tr>
<td>Norm Rheo</td>
<td>1.0</td>
<td>0.37 ± 0.06*‡</td>
</tr>
<tr>
<td>Norm R_{th}</td>
<td>1.0</td>
<td>2.18 ± 0.28*‡</td>
</tr>
</tbody>
</table>

Pretreatment with Myr-PKCζ inhibitor

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Control (0 min)</th>
<th>PKCζ (6 min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>RMP, mV</td>
<td>−57.0 ± 2.4</td>
<td>−57.5 ± 2.1</td>
</tr>
<tr>
<td>FT, mV</td>
<td>−29.1 ± 3.7</td>
<td>−28.3 ± 3.6</td>
</tr>
<tr>
<td>Rheo, pA</td>
<td>317 ± 270</td>
<td>306 ± 252</td>
</tr>
<tr>
<td>R_{th}, MΩ</td>
<td>651 ± 211</td>
<td>620 ± 215</td>
</tr>
<tr>
<td>Norm Rheo</td>
<td>1.0</td>
<td>1.13 ± 0.07‡</td>
</tr>
<tr>
<td>Norm R_{th}</td>
<td>1.0</td>
<td>0.96 ± 0.05*</td>
</tr>
</tbody>
</table>

NGF enhances the excitability of small-diameter capsaicin-sensitive sensory neurons. As we previously demonstrated, exposure to NGF augments the excitability of small-diameter capsaicin-sensitive neurons (Zhang et al. 2002). Bath application of NGF to different sets of untreated sensory neurons used in parallel experiments involving the inhibitors of different signaling cascades (described below) produced a significant increase in the number of evoked APs (control, 3.8 ± 0.3 APs vs. NGF, 9.5 ± 1.2 APs after 6 min, \( n = 10 \), RM ANOVA, data not shown). From these 10 neurons, the effects of NGF on different parameters of excitability were assessed. Although NGF did not alter the firing threshold, there were significant

RESULTS

NGF enhances the excitability of small-diameter capsaicin-sensitive sensory neurons. As we previously demonstrated, exposure to NGF augments the excitability of small-diameter capsaicin-sensitive neurons (Zhang et al. 2002). Bath application of NGF to different sets of untreated sensory neurons used in parallel experiments involving the inhibitors of different signaling cascades (described below) produced a significant increase in the number of evoked APs (control, 3.8 ± 0.3 APs vs. NGF, 9.5 ± 1.2 APs after 6 min, \( n = 10 \), RM ANOVA, data not shown). From these 10 neurons, the effects of NGF on different parameters of excitability were assessed. Although NGF did not alter the firing threshold, there were significant
Neither ERK nor PLC pathways are involved in NGF sensitization. In this report we sought to establish the downstream signaling pathways that contribute to the increased excitability. Therefore, to determine whether ERK/MAPK played a role in increased AP firing produced by NGF, neurons were exposed to PD-98059, a selective inhibitor of MEK1/2 (IC50 4 μM; Alessi et al. 1995; English and Cobb 2002). As shown for a representative recording (Fig. 1, A and B), bath application of NGF (100 ng/ml) to an untreated neuron greatly increased the number of APs evoked by a depolarizing ramp of current. In three untreated sensory neurons, NGF significantly increased the number of evoked APs by greater than twofold (see Fig. 1C, RM ANOVA). A parallel set of isolated neurons was pretreated with 10 μM PD-98059 for 30 min and then exposed to NGF. PD-98059 did not affect the capacity of NGF to increase the number of evoked APs in small-diameter sensory neurons (n = 7) compared with NGF alone. These results are summarized in Fig. 1C. Pretreatment with PD-98059 had no significant effects on the NGF-induced changes in the parameters of excitability, which are summarized in Table 3. Zhu and Oxford (2007) demonstrated that the NGF-induced phosphorylation of ERK (p42/p44) was greatly reduced by treatment with 10 μM PD-98059 (see their Fig. 4), indicating that this concentration was sufficient to block activation of MEK1/2. In addition, pretreatment with 10 μM PD-98059 alone for 30 min did not alter the number of evoked APs (3.3 ± 0.5 APs at 0 min vs. 3.3 ± 0.5 APs after 20 min, n = 4, RM ANOVA, data not shown) or membrane potential (−54.8 ± 2.4 at 0 min vs. −55.4 ± 2.5 mV after 20 min) over time.

To examine the role of PLC in the NGF-induced increase in AP firing, sensory neurons were exposed to U-73122, a selective inhibitor of PLC (IC50 1–2 μM; Bleasdale et al. 1990; Smith et al. 1990). U-73122 also blocks activation of PLCγ-1, including that produced by NGF (Jones et al. 2005; Sato et al. 2000; Zapf-Colby et al. 1999). As shown in Fig. 2, pretreatment with 5 μM U-73122 for 30 min did not affect the capacity of NGF to augment the number of evoked APs (n = 8) and was similar to the NGF-induced sensitization observed in a parallel set of neurons in the absence of U-73122 (n = 5, RM ANOVA). Pretreatment with U-73122 had no significant effects on the changes in the parameters of excitability produced by NGF alone, which are summarized in Table 3. Pretreatment with 5 μM U-73122 (30 min) alone did not alter the number of evoked APs (3.3 ± 0.8 APs at 0 min vs. 3.5 ± 0.9 APs after 20 min, n = 4, RM ANOVA, data not shown) or membrane potential (−53.8 ± 1.1 at 0 min vs. −51.8 ± 2.0 mV after 20 min) over time. Although U-73122 had no effect on the capacity of NGF to increase AP firing, recent work has raised questions regarding the ability of U-73122 to inhibit PLC specifically (see Horowitz et al. 2005; Klein et al. 2011). To corroborate whether PLC plays an important role in the NGF-mediated increase in excitability, sensory neurons were pretreated with 30 μM neomycin, which can be an effective inhibitor of PLC (Lipsky and Lietman 1982; Schwertz et al. 1984), for 30 min before recording. Pretreatment with neomycin also failed to block the increased AP firing produced by NGF. Under control conditions the ramp evoked 3.8 ± 0.4 APs; exposure to 100 ng/ml NGF significantly increased the number of APs to 7.6 ± 0.9, 8.2 ± 0.6, 7.6 ± 0.6, and 8.3 ± 0.3 APs after exposures of 2, 6, 10, and 20 min, respectively (n = 5 with 1 neuron lost between 10 and 20 min, ANOVA, Holm-Sidak posttest, data not shown). The NGF-induced increase in AP firing in the presence of neomycin for all time
points (2 through 20 min) was not significantly different from the increased firing obtained for the untreated control sensory neurons, pretreatment with U-73122, and pretreatment with PD-98059 ($P = 0.98$, ANOVA, all pairwise). Together, these results indicate that neither activation of ERK nor PLC contributed to the elevated excitability produced by NGF.

**NGF sensitization involves activation of an atypical PKC but not conventional or novel PKC subtypes.** To examine whether PKC played a role in increased excitability produced by NGF, neurons were treated with BIM. After 30-min pretreatment with 1 μM BIM, neurons exposed to NGF (100 ng/ml) still produced a significant increase in the number of APs (Fig. 3A, n = 5, RM ANOVA). The NGF-induced increase in AP firing after pretreatment with BIM was not different at any of the time points compared with the increases produced by NGF in the untreated neurons and the PD-98059-, U-73122-, and neomycin-pretreated neurons ($P = 0.79$, ANOVA). However, at a concentration of 1 μM, BIM inhibited only conventional (IC$_{50}$ 10–30 nM) and novel (IC$_{50}$ 100–200 nM), but not atypical (IC$_{50}$ = 6 μM), isoforms of PKC (Martin-Baron et al. 1993; Toullec et al. 1991). Because pretreatment with 1 μM BIM did not block the sensitizing actions of NGF, it is possible that NGF modulated excitability through activation of an atypical PKC.

Our previous work showed that NGF activation of p75$^{NTR}$ enhanced neuronal excitability through the sphingomyelin/ceramide pathway (Zhang et al. 2002; Zhang and Nicol, 2004). Studies using other cellular model systems have demonstrated that ceramide can activate the ε-subtype of atypical PKC (PKC$\varepsilon$) (Bourbon et al. 2000; Lozano et al. 1994; Müller et al. 1995; Wang et al. 1999, 2005). To examine the potential role of NGF-mediated activation of PKC$\varepsilon$, neurons were exposed via the bath to a selective peptide inhibitor targeted to the pseudosubstrate domain of aPKCs (EC$_{50}$ 10–20 μM; Standaert et al. 1997). As shown in Fig. 3B, a 30-min pretreatment with 10 μM myristoylated pseudosubstrate inhibitor completely blocked the sensitization produced by NGF. Furthermore, in the presence of the PKC$\varepsilon$ inhibitor, NGF failed to alter the resting membrane potential, the rheobase, or $R_{th}$ (see Table 3). In an all pairwise comparison, the NGF-induced reduction in the normalized values for the rheobase were significantly different for untreated neurons and for pretreatment with PD-98059 and U-73122 compared with pretreatment with the pseudosubstrate inhibitor ($P = 0.004$, ANOVA, Holm-Sidak all pairwise test), although the increase for the normalized values of $R_{th}$ was not different between these groups ($P = 0.27$, ANOVA on ranks). In addition, internal perfusion of the nonmyristoylated PKC$\varepsilon$ pseudosubstrate inhibitor (although at a higher concentration, 60 μM) via the recording pipette blocked the NGF-induced increase in excitability wherein 4.0 ± 0.3 APs were evoked under control conditions compared with 4.0 ± 0.3 APs after a 20-min exposure to 100 ng/ml NGF (n = 6, $P = 0.93$, ANOVA, data not shown). In further studies, exposure of sensory neurons to 1 μM C2-ceramide produced a significant twofold increase in the number of evoked APs (Fig. 3C) and is similar to our previously reported results wherein there was a threefold increase in the number of APs generated after a 20-min exposure to 1 μM C2-ceramide (Zhang et al. 2002). In a parallel set of neurons, pretreatment with the PKC$\varepsilon$ pseudosubstrate inhibitor blocked the increased excitability produced by C2-ceramide (Fig. 3D). Consistent with the results obtained for NGF, treatment with the inhibitor prevented the ceramide-induced changes in the resting membrane potential, the decrease in normalized rheobase, and the increase in normalized $R_{th}$ (data not shown). Previous studies have indicated that myristoylation of peptides can lead to the activation of eNOS in endothelial cells through the Akt pathway (Krotova et al. doi:10.1152/jn.00030.2011 • www.jn.org
for neurons pretreated with LY-303511, the NGF-induced increase in AP firing over a 20-min period in the absence of NGF (Fig. 3E). The NGF-induced increase in AP firing after pretreatment with the PKCζ inhibitor was not different from the NGF-mediated increase in untreated sensory neurons (P = 0.40, ANOVA). As a positive control, treatment with the myristoylated pseudosubstrate inhibitor of aPKCs (60 μM for 30 min, 5 times the EC_{50} value) had no effect on the increased excitability produced by bath application of 1 μM PGE_{2} (Fig. 3F).

These results indicate that the PKCζ pseudosubstrate inhibitor lacked any nonspecific actions on the capacity of these neurons to be sensitized, since it is well established that PGE_{2} acts through the G_{s}-cyclic AMP-PKA pathway (Cui and Nicol 1995; Hingtgen et al. 1995; Taiwo et al. 1989). Thus these observations indicate that ceramide is a key upstream modulator of PKCζ activity that can alter neuronal excitability.

In addition to ceramide, previous studies have demonstrated that phosphatidylinositol 3-kinase (PI3K) is a key upstream regulator of PKCζ activity (Chou et al. 1998; Herrera-Velit et al. 1997; Mendez et al. 1997; Standaert et al. 1997). To determine whether PI3K had any role in the NGF-induced increase in excitability, sensory neurons were pretreated with 10 μM LY-294002, a selective PI3K inhibitor (IC_{50} 1.4 μM; Vlahos et al. 1994), for 30 min before recording. As shown in Fig. 4, LY-294002 suppressed the ability of NGF to augment the number of evoked APs. Similar results were obtained with exposure to 100 nM wortmannin, a structurally different PI3K inhibitor (IC_{50} 5 nM; Powis et al. 1994) (control, 3.4 ± 0.5 APs vs. 10 min of NGF, 4.0 ± 0.5 APs, n = 7, P = 0.48, RM ANOVA, data not shown). In contrast, pretreatment with 10 μM LY-303511, the inactive analog of LY-294002, did not alter the increased AP firing produced by NGF. The increase in AP firing produced by NGF in untreated neurons and in the presence of LY-303511 were not different at any of these time points (ANOVA, see Fig. 4 legend for details). For both untreated and LY-303511-pretreated neurons, exposure to NGF produced a significant depolarization of the resting membrane potential neurons (untreated control, −57.0 ± 1.5 mV vs. 10 min of NGF, −52.2 ± 2.3 mV; LY-303511 control, −60.3 ± 2.0 mV vs. 10 min of NGF, −53.7 ± 1.6 mV, RM ANOVA), whereas for those neurons pretreated with LY-294002, NGF had no effect (control, −56.3 ± 1.8 mV vs. 10 min of NGF, −56.2 ± 1.8 mV, P = 0.57, RM ANOVA).

Together, these results demonstrate that NGF via ceramide and PI3K led to the activation of PKCζ, which through presently undefined mechanisms increases the excitability of small-diameter sensory neurons.

siRNA targeted to PKMζ/PKCζ blocks NGF-induced sensitization. Because the sequences of the pseudosubstrate binding domains are the same for the aPKCs (PKCζ, PKMζ, and PKCα/γ; Moscat et al. 2006), pseudosubstrate peptide inhibition will not distinguish these isoforms. Therefore, to further establish whether one of the PKCζ isoforms was the key mediator of the enhancement of excitability produced by NGF, initial studies examined the expression of these different isoforms of aPKC in untreated naive sensory neurons and the potential effects of siRNA molecules targeted to PKMζ/PKCζ.

Isolated sensory neurons were treated with a pool of four different siRNA molecules targeted to PKCζ as well as two additional siRNA molecules targeted to PKCα (siRNA 407 and siRNA 638). The effects of these siRNAs on the expression of each subtype of aPKC (PKCζ, PKMζ, and PKCα/γ) was determined by Western blotting and quantified by densitometry. Previous studies established that the full-length isoforms of PKCζ and PKCα/γ were detected at a molecular mass of ~75 kDa (Akimoto et al. 1994; Hernandez et al. 2003; Sacktor et al. 1993; Selbie et al. 1993), whereas the PKMζ isoform was detected at 51–55 kDa (Hernandez et al. 2003; Sacktor et al. 1993). Representative Western blots are shown in Fig. 5. A–C, left. Figure 5A, left, depicts the Western blot obtained with the antibody sc-216 (Santa Cruz), which is known to have overlapping specificity for PKMζ/PKCζ and PKCα/γ. This blot indicates that for the naive untreated control conditions, there was moderate expression for full-length PKCζ or PKCα/γ (75-kDa band) and more prominent expression of PKMζ that was detected as the 49-kDa band. Treatment of sensory neurons with the siRNA pool or siRNA 638, either of which targets sequences in PKMζ and PKCζ miRNA, greatly reduced the expression of PKMζ but had little effect on the full-length isoforms. Neither siRNA 407, which specifically targets PKCζ sequences, the negative control siRNA, nor Metafectene altered expression of the 75-kDa band; however, the negative control siRNA and Metafectene appeared to have a small effect on PKMζ. Figure 5A, right, summarizes the ability of siRNAs 407, 638, and the pool to alter the expression of PKCζ/PKCα/γ or PKMζ as measured with the sc-216 antibody. The results for siRNA 407 and 638 were obtained from five different tissue harvests, whereas the results for the siRNA pool were obtained from four of those five tissue harvests. The summary results indicate that siRNA 638 and the pool significantly reduced the
expression of PKMζ by an average value of 64 ± 12% (n = 5) and 78 ± 9% (n = 4), respectively, compared with naive controls (P < 0.001, ANOVA, Holm-Sidak all pairwise test, see details in Fig. 5 legend). These treatments had no significant effect on expression of the 75-kDa band representing PKCζ/PKCζ (P = 0.23, ANOVA, average reductions of 22 ± 11% and 12 ± 10% for siRNA 638 and the pool, respectively). In these five experiments, treatments with Metafectene or negative control siRNA did not affect expression of either the 49- or the 75-kDa bands. To further establish the effects of these siRNAs, a specific PKMζ/PKCζ antibody was used (Hernandez et al. 2003). Figure 5B, left, shows a representative Western blot obtained with this antibody. Consistent with the results shown in Fig. 5A, siRNA 638 and the siRNA pool reduced the expression of the 49-kDa band but had no effect on the 75-kDa band. As summarized in Fig. 5B, right,
the 49-kDa band was significantly reduced by 64 ± 8% (n = 5) and 65 ± 13% (n = 4) by treatment with siRNA 638 and the siRNA pool, respectively, compared with the naive controls (P < 0.001, ANOVA, Holm-Sidak all pairwise test). Treatment with the PKCζ-specific siRNA 407, negative control siRNA, or Metafectene alone had no effect on the levels of the 49-kDa band. None of the treatments had a significant effect on the expression of the 75-kDa band (P = 0.15, ANOVA). To assess whether these siRNA molecules targeted to PKMζ/PKCζ influenced the expression of PKCA/λ, a selective antibody to PKCA/λ (BD Transduction) was used. Figure 5C, left, shows a representative Western blot wherein the expression of the 49- or the 75-kDa band was not altered after siRNA exposure. The summary results shown in the Fig. 5C, right, demonstrate that these different treatments had no significant effect on the expression of the 49- or the 75-kDa band (P = 0.36 and P = 0.57, respectively, ANOVA). Thus these results indicate that after exposure to the siRNA pool and siRNA 638, the expression of PKMζ was significantly and specifically reduced, whereas neither full-length PKCζ nor PKCA/λ was altered.

qPCR assessment of PKCζ, PKMζ, and PKCA/λ expression and the effects of siRNA treatment. In addition to Western blotting, qPCR with SYBR green chemistry was used to establish the expression levels of mRNA for PKCζ, PKMζ, and PKCA/λ in the DRG as well as the reduction in expression produced by the siRNA in isolated sensory neurons. Using our primer set described in Table 1, the expression of PKCζ, PKMζ, and PKCA/λ relative to the reference gene Arbp was determined in the DRG. The expression of PKCζ, PKMζ, and PKCA/λ also was determined in tissue isolated from the lung and brain to provide perspective to the levels in the DRG. These results are summarized in Table 5. In the lung, the expression of PKCA/λ relative to Arbp was ~2 times higher than that of PKCζ, whereas expression of PKMζ was ~250–500 times lower than that of PKCζ or PKCA/λ, respectively (P < 0.001, ANOVA, Holm-Sidak all pairwise test). In contrast, the levels of expression for the three aPKCs in the brain were significantly different wherein PKMζ > PKCζ/λ >> PKCζ (P < 0.001, ANOVA, Holm-Sidak all pairwise test). In the DRG, expression levels of PKMζ and PKCA/λ were similar, with PKCζ expression being significantly lower (P < 0.001, ANOVA, Holm-Sidak all pairwise test). Using another primer set adapted to rat from mouse sequences (kindly provided by Dr. Sourav Ghosh, University of Arizona), we obtained very similar results for the expression of PKCζ, PKMζ, and PKCA/λ in all three tissues (see Table 5). Thus these results are consistent with observations using Western blots, wherein the expression of PKMζ greatly exceeds that of full-length PKCζ in the DRG, and with previous results reported by Hernandez et al. (2003).

The capacity of the siRNA molecules (siRNA pool and siRNAs 407 and 638) to knock down the expression of mRNA for PKCζ, PKMζ, and PKCA/λ was determined in isolated sensory neurons using our primer set described in Table 1. These findings are summarized in Fig. 6, where the results for siRNA 407 and 638 were obtained from four different tissue harvests; the results for the siRNA pool were obtained with three of those four tissue harvests. Figure 6A demonstrates the expression of PKCA/λ relative to Arbp (0.013 ± 0.003, Cq 23.27 ± 0.50, n = 4) was the highest of the three isoforms of aPKC in the naive untreated neurons. The expression of PKCζ/λ/Arbp for the different treatment groups was not significantly different than that for the untreated naive neurons (P = 0.07, ANOVA on ranks). In addition, the levels of the reference gene, Arbp, were not affected by any treatment compared with the naive neurons (P = 0.66, ANOVA). For example, the Cq for Arbp in the untreated neurons was 16.86 ± 0.22 (n = 4) compared with 17.04 ± 0.07 (n = 4) after treatment with siRNA 638 (data not shown). To reduce the variance in the extent of knockdown produced by the siRNA, the levels of PKCζ/λ/Arbp after treatment were normalized to their respective untreated naive values; these results are summarized in Fig. 6A2. These treatment groups were not significantly different from the untreated naive neurons (ANOVA on ranks, P = 0.10). Thus these results demonstrate that siRNA molecules targeted to PKCζ had no effect on the expression of PKCA/λ. Figure 6B2 illustrates the expression level of PKMζ relative to Arbp (0.0030 ± 0.0010, Cq 25.70 ± 0.52, n = 4) in the naive untreated neurons, which is approximately four times

### Table 5. Cq values and expression levels for PKCζ, PKMζ, PKCA/λ, and Arbp

<table>
<thead>
<tr>
<th>Tissue</th>
<th>PKCζ</th>
<th>PKMζ</th>
<th>PKCA/λ</th>
<th>Arbp</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lung Cq</td>
<td>23.91 ± 0.25</td>
<td>31.83 ± 0.14</td>
<td>22.74 ± 0.20</td>
<td>18.50 ± 0.09</td>
</tr>
<tr>
<td>Lung expression</td>
<td>0.0242 ± 0.0033*</td>
<td>0.0001 ± 0.000004*</td>
<td>0.0537 ± 0.0052*</td>
<td>24.02 ± 0.14</td>
</tr>
<tr>
<td>Brain Cq</td>
<td>29.65 ± 0.39</td>
<td>23.14 ± 0.45</td>
<td>24.02 ± 0.14</td>
<td>20.30 ± 0.16</td>
</tr>
<tr>
<td>Brain expression</td>
<td>0.0015 ± 0.0005*</td>
<td>0.1920 ± 0.0171*</td>
<td>0.0836 ± 0.0082*</td>
<td>20.20 ± 0.31</td>
</tr>
<tr>
<td>DRG Cq</td>
<td>33.96 ± 0.62</td>
<td>25.19 ± 0.44</td>
<td>24.88 ± 0.30</td>
<td>20.20 ± 0.31</td>
</tr>
<tr>
<td>DRG expression</td>
<td>0.00011 ± 0.00005*</td>
<td>0.0369 ± 0.0080</td>
<td>0.0403 ± 0.0049</td>
<td>20.20 ± 0.31</td>
</tr>
</tbody>
</table>

#### qPCR primers

- **Our primers**
  - PKCζ: 5'-TTCCTTTCCGCCTTTGCTG-3' (forward) and 5'-GCTGGTTTTCTGGAGGTG-3' (reverse)
  - PKMζ: 5'-AGGAGCTGTTGGATGGAGCTG-3' (forward) and 5'-GCTGGTTTTCTGGAGGTG-3' (reverse)
  - PKCA/λ: 5'-GGAGTGGGCTGGAGTGGT-3' (forward) and 5'-TTCCTTTCCGCCTTTGCTG-3' (reverse)
  - Arbp: 5'-CTCGGGCGCTGACCTGAG-3' (forward) and 5'-GCTGGTTTTCTGGAGGTG-3' (reverse)

- **Ghosh-adapted primers**
  - PKCζ: 5'-TTCCTTTCCGCCTTTGCTG-3' (forward) and 5'-GCTGGTTTTCTGGAGGTG-3' (reverse)
  - PKMζ: 5'-AGGAGCTGTTGGATGGAGCTG-3' (forward) and 5'-GCTGGTTTTCTGGAGGTG-3' (reverse)
  - PKCA/λ: 5'-GGAGTGGGCTGGAGTGGT-3' (forward) and 5'-TTCCTTTCCGCCTTTGCTG-3' (reverse)
  - Arbp: 5'-CTCGGGCGCTGACCTGAG-3' (forward) and 5'-GCTGGTTTTCTGGAGGTG-3' (reverse)

Quantification cycle (Cq) and expression values are means ± SE. Gene expression was determined as the no. of copies of PKCs per the no. of copies of Arbp. Results were obtained from n = 4 lung, n = 5 brain, and n = 5 dorsal root ganglion (DRG) different tissue harvests. *P < 0.001 (ANOVA). †P < 0.001, PKCζ vs. PKMζ or PKCA/λ (ANOVA). PKMζ and PKCA/λ were not different.

* J Neurophysiol • doi:10.1152/jn.00030.2011 • www.jn.org
Fig. 6. Treatment with the siRNA pool and siRNA 638 reduces the expression of mRNA PKMζ but not full-length PKCζ or PKCζ/ in sensory neurons. A1: the expression level of mRNA for PKCζ/ relative to the reference gene Arbp for the different treatment conditions. A2: the results obtained in A1 after normalization to the levels of PKCζ/Arbp obtained for the untreated naive condition. Results after treatments with siRNA 407 and 638 were obtained from 4 different tissues harvests; for the siRNA pool, results were obtained from 3 of those 4. B1 and B2: the expression of PKMζ/Arbp and the results after normalization to the untreated naive condition, respectively, for the different treatment conditions. C1 and C2: the expression of PKCζ/Arbp and the results after normalization to the untreated naive condition, respectively, for the different treatment conditions. Asterisks represent a significant reduction in the mRNA levels obtained for that treatment condition (ANOVA with a Holm-Sidak all pairwise test; see text for details).

less than that of PKCζ/Arbp. In contrast to PKCζ/ treatment with the siRNA pool, but not with siRNA 407 or 638, significantly reduced the levels of PKMζ/Arbp compared with the naive condition (ANOVA on ranks, P = 0.009, Dunn’s all pairwise test). After normalization to their respective untreated naive values (see Fig. 6B2), treatments with siRNA 638 and the pool, but not siRNA 407 or the negative control siRNA, significantly reduced the expression of PKMζ/Arbp by 86.3 ± 6.1% (n = 4) and 88.0 ± 6.7% (n = 3), respectively, (P = 0.011, ANOVA on Ranks, Dunn’s test). The levels of Arbp were not affected by any treatment compared with the naive neurons (P = 0.19, ANOVA). The Cq for Arbp in the untreated neurons was 17.07 ± 0.04 (n = 4) compared with 17.15 ± 0.22 (n = 4) after treatment with siRNA 638 (data not shown). These results indicate that siRNA 638 and the pool effectively reduced the expression of PKMζ mRNA by about 90%. Figure 6C1 shows that the expression level of PKCζ relative to Arbp was quite low (6.82 ± 0.91 × 10^{-4}/Arbp, n = 4), ~2,000 times less than PKCζ/Arbp. For PKCζ, the treatments had no significant effect on expression before (P = 0.13, ANOVA) or after normalization (Fig. 6C2, P = 0.14, ANOVA). The lack of effect of siRNA on full-length PKCζ is similar to that observed for the detection of full-length PKCζ with the sc-216 and PKCζ-specific antibodies. It is possible that the PKCζ-specific siRNA 407 is ineffective because the levels of mRNA for PKCζ (average Cq of 33.96) are quite low compared with those for PKMζ (average Cq of 25.19) under normal conditions. Thus, taken together, these results demonstrate that siRNA 638 and the siRNA pool, but not siRNA 407 or the negative control siRNA, significantly reduced the expression of PKMζ and did not alter the expression of either PKCζ or PKCζ/.

On average, immunoreactive PKMζ was reduced by 65–75% and mRNA for PKMζ was reduced by nearly 90% after treatment with siRNA; however, the critical question is whether the NGF-induced enhancement of excitability was affected by siRNA treatment. As summarized in Fig. 7A, in recordings from sensory neurons that exhibited uptake of the labeled siRNA pool, the capacity of NGF to increase the number of APs was blocked (n = 7, P = 0.55, RM ANOVA), whereas after exposure to labeled negative control siRNA, NGF significantly augmented the excitability by about twofold compared with the control (n = 5, P < 0.001, RM ANOVA). The increases in AP firing produced by NGF at 2, 6, and 10 min were not different. After treatment with the siRNA pool, NGF failed to depolarize the resting membrane potential (control, −57.3 ± 1.7 mV vs. NGF, −55.9 ± 1.6 mV after 6 min, data not shown). NGF had no effect on either rheobase or R_{th} after siRNA pool treatment. In contrast, after treatment with negative control siRNA, NGF significantly depolarized the resting membrane potential, reduced the rheobase, and increased R_{th} (data not shown); these values were similar to those obtained for NGF alone. As described for untreated sensory neurons, NGF did not alter the firing threshold for either siRNA or negative control siRNA treatments. Similar to the siRNA pool, treatment with siRNA 638 blocked the ability of NGF to increase the excitability of sensory neurons (Fig. 7B, n = 5, P = 1.0, RM ANOVA). However, after treatment with negative control siRNA, NGF significantly increased the number of APs compared with the control (n = 4, P < 0.001, RM ANOVA).
Together, these results show that either inhibition of PKCζ obtained shortly (P<0.001, ANOVA, Holm-Sidak all pairwise test) after the whole cell configuration was attained. After internal perfusion of recombinant PKCζ (2 ng/μl in the pipette) for 6 min, there was a large increase in the number of evoked APs that remained elevated up to 20 min. A scattergram (Fig. 8B) of the effects of internal perfusion with PKCζ on AP firing shows that some neurons responded with a modest increase, whereas others exhibited a robust increase in firing. One neuron (cell 7) after 10 min became spontaneously active. The time-dependent effects of internal perfusion of PKCζ for the remaining six neurons are summarized in Fig. 8C. After 6 min of perfusion, there was a significant threefold increase in the number of evoked APs (RM ANOVA). The increase in AP firing produced by PKCζ at all time points (2, 6, 10, and 20 min) was not significantly different from the NGF-induced increase in evoked APs observed in untreated neurons (P = 0.54, ANOVA). As shown in Table 4, 6 min of internal perfusion with PKCζ had effects on the parameters of excitability very similar to those of exposure to NGF. PKCζ significantly depolarized the resting membrane potential, lowered the rheobase, and increased Rth (P < 0.05, ANOVA, Holm-Sidak all pairwise test for each respective parameter group). As observed above, the value of the firing threshold was not altered compared with that of NGF after 6 min of exposure for the untreated neurons or after pretreatment with either PD-98059 or U-73122 (P = 0.51, ANOVA on ranks). To assess the specificity of the sensitizing effect, another group of sensory neurons was pretreated with the myristoylated pseudosubstrate peptide inhibitor (10 μM for 30 min) before recording. After treatment with the pseudosubstrate inhibitor, internal perfusion with recombinant PKCζ failed to increase the number of evoked APs (Fig. 8D, n = 6, P = 0.15, RM ANOVA). In addition, after treatment with the inhibitor, internally perfused PKCζ had no effect on the parameters of excitability, as observed with PKCζ alone (summarized in Table 4). The blocking effects of the pseudosubstrate inhibitor on elevated excitability produced by PKCζ are similar to the inhibitory actions on the NGF-induced modifications of excitability. In further support of the high homology in the catalytic domain of aPKCs, internal perfusion with active recombinant human PKCζ also sensitized AP firing in sensory neurons (Fig. 8E). A concentration of 0.42 ng/μl PKCζ was added to the pipette solution; this value was chosen so that the specific activity of PKCζ was equal to that of 2 ng/μl PKCζ. Internal perfusion with PKCζ produced a significant increase in the number of APs compared with control values after only 2 min (control, 3.7 ± 0.3 vs. 6.7 ± 0.9 APs, n = 3, P = 0.017, RM ANOVA, Holm-Sidak test). A higher concentration of PKCζ (2 ng/μl) produced a similar effect wherein after only 2 min, the number of APs was significantly higher compared with controls (control, 4.0 ± 0.6 APs vs. PKCs after 2 min, 7.0 ± 1.3 APs, n = 3, P = 0.018, RM ANOVA, Holm-Sidak test). Neither the values for the control APs (P = 0.64, t-test) nor those for the increase in APs produced by PKCζ at all time points (P = 0.49, ANOVA) were different for concentrations of 0.42 vs. 2 ng/μl; therefore, these values have been combined and are summarized in Fig. 8E. PKCζ produced a significant increase in AP firing at all time points compared with the control value, whereas the increases at 2, 6, 10, and 20 min were not different (P < 0.001, ANOVA, Holm-Sidak all pairwise test). In contrast, internal perfusion with 0.08 ng/μl PKCζ had no effect on AP firing over time (control, 4.5 ± 0.2 APs vs. PKCζ after 20 min, 4.2 ± 0.3 APs, n = 6, P = 0.85, ANOVA on ranks). As an additional control experiment, heat-inactivated recombinant PKCζ was internally perfused into...
sensory neurons. Under control conditions, the ramp evoked 3.2 APs (n = 5); after 10 min of internal perfusion, the number of APs remained unchanged at 3.6 APs (n = 5).

After 10 min of internal perfusion with heat-inactivated PKCζ, these neurons then were exposed to 100 ng/ml NGF wherein after 5 min of NGF, the number of APs in four of the five neurons was significantly increased to 7.8 APs (n = 4, P < 0.003, ANOVA, Holm-Sidak all pairwise test); one of these five neurons became spontaneously active. Together, these results indicate that activation of the highly homologous catalytic domains of aPKCs can result in enhanced excitability and that these effects recapitulated the sensitization produced by NGF.

Recombinant PKCζ suppresses \( I_K \). To explore a possible mechanism giving rise to the increased excitability, the effects of internal perfusion with recombinant PKCζ on the amplitude and activation properties of the total outward \( I_K \) were examined. Internal perfusion with recombinant PKCζ (2 ng/\( \mu l \)) produced a significant time-dependent suppression of \( I_K \) (Fig. 9A). Figure 9A shows current traces for a representative neuron wherein the peak amplitude of \( I_K \) obtained for the step to +60 mV was reduced from a control value (left) of 6.74 to 3.40 nA after a 20-min exposure to PKCζ (middle). The PKCζ-sensitive \( I_K \) (Fig. 9A, right) was obtained by subtraction of the traces shown in the middle panel from those shown at left. The time-dependent effects of PKCζ on the current-voltage relation for \( I_K \) are summarized for seven sensory neurons in Fig. 9B. Internal perfusion with recombinant PKCζ significantly reduced the peak amplitude of \( I_K \) (at +60 mV) from a control value of 6.42 ± 0.88 to 3.84 ± 0.78 and 3.43 ± 0.76 nA (n = 7, RM ANOVA) after 10- and 20-min exposures, respectively. The current values were transformed to conductances wherein the conductance-voltage relations obtained for the different times were fitted with the Boltzmann relation and normalized to their respective values of \( G_{\text{max}} \); these results are summarized in Fig. 9C. There were small but significant increases in the values of \( G/G_{\text{max}} \) after 10 and 20 min of internal perfusion for voltages between -10 and +10 mV and at 0 mV, respectively (RM ANOVA, see Fig. 9 legend for details) and are consistent with the small but significant leftward shift in the value of \( V_{0.5} \).
PKMζ and Neuronal Excitability

Fig. 9. Internal perfusion with recombinant PKCζ suppresses the total outward potassium current (IK) in sensory neurons. A: a representative recording of the total IK obtained under control conditions (left), after internal perfusion with recombinant PKCζ for 20 min (middle), and the PKCζ-sensitive IK obtained by subtraction of the traces in the middle from those at left. The recording pipette contained recombinant PKCζ (2 ng/μl). Traces shown are for voltage steps from −80 to +60 mV in 20-mV increments. “0” lines indicate the zero-current level. B: the average current-voltage relation for the peak value of IK obtained at different times during internal perfusion with PKCζ (n = 7). The values of IK after PKCζ were significantly different from control values for voltages between −10 and +60 mV and −50 and +60 mV for the 10- and 20-min perfusion times, respectively, whereas there were no differences between the control and after 2 and 6 min (RM ANOVA). C: the conductance (G/Gmax)-voltage relations obtained from the 7 neurons for the control condition and at different times after internal perfusion with PKCζ. The values for each neuron were normalized to their respective fitted values of Gmax for each time point. The values of G/Gmax were significantly different between the control and after 10 min for voltages between −10 and +10 mV and between the control and 20 min for 0 mV (RM ANOVA). There were no significant differences between the control and after 2 and 6 min. The lines represent the fit to the data points using the Boltzmann relation. D: the current-voltage relation for the PKCζ-sensitive IK obtained by the subtraction of the current remaining after 20 min of internal perfusion from the respective controls; the peak value at 60 mV was 2.30 ± 0.41 nA (n = 7). E and F: the I/Imax- and G/Gmax-voltage relations, respectively, for the PKCζ-sensitive IK (PKCζ-sens IK) compared with the C2-ceramide-sensitive IK (C2-sens IK) obtained from our previous report (Zhang et al. 2002). The lines through the data points in F were fitted using the Boltzmann relation.

(control, −4.4 ± 1.7 mV vs. 10 min, −10.6 ± 3.4 mV and 20 min, −10.1 ± 2.2 mV, n = 7, RM ANOVA); the values at 2 and 6 min were not different from control. The value of k remained unaltered over the 20-min period (control, 12.8 ± 0.5 mV vs. 10 min, 12.2 ± 0.7 mV and 20 min, 13.1 ± 0.9 mV, n = 7, RM ANOVA). For these seven neurons, the PKCζ-sensitive IK was obtained by subtraction of the current traces obtained at 20 min from their respective controls; the current-voltage relation is summarized in Fig. 9D. The PKCζ-sensitive current begins to activate around −20 mV and thus may contribute to the increased excitability (e.g., decreased rheobase and increased RT). Recordings obtained under control conditions over a 20-min period demonstrated that IK was stable wherein the average peak IK at +60 mV at time 0 was 8.74 ± 2.55 nA compared with 8.75 ± 2.57 nA (n = 4, P = 0.96 RM ANOVA), and when normalized to their respective peak values at +60 mV, the average value after 20 min was 1.09 ± 0.04 compared with their time 0 controls (n = 4).
Previously, we demonstrated that treatment with C2-ceramide produced a time-dependent suppression of $I_K$ (Zhang et al. 2002). In addition, recent evidence suggests that ceramide directly activates PKCζ (Bourbon et al. 2000; Wang et al. 2005); together, this would suggest that currents modulated by ceramide and PKCζ should be similar. To determine whether the PKCζ-sensitive $I_K$ was similar to the C2-ceramide-sensitive $I_K$, we reexamined these previous results to determine the properties of the $I_K$ sensitive to 10 µM C2-ceramide (see Fig. 5, B and C, Zhang et al. 2002). As shown in Fig. 9, E and F, both the current-voltage and the $G_{G_{K}}$-voltage relations for the C2-ceramide-sensitive $I_K$ ($V_{0.5} = 4.5 \pm 5.6$ mV, $k = 11.5 \pm 0.9$ mV, $n = 4$) were quite similar to those for the PKCζ-sensitive $I_K$ ($V_{0.5} = 6.1 \pm 6.3$ mV, $k = 16.3 \pm 2.9$ mV, $n = 7$). Thus these results indicate that NGF, through downstream activation of ceramide and PKCζ, leads to increased excitability, in part, via the inhibition of voltage-dependent potassium currents.

**Recombinant PKCζ enhances TTX-R $I_{Na}$**

The increased excitability of small-diameter sensory neurons produced by the proinflammatory prostaglandin PGE$_2$ results, in part, from an augmentation of the TTX-R $I_{Na}$ (English et al. 1996; Gold et al. 1996). Previously, we showed that NGF increased the amplitude of TTX-R $I_{Na}$ by 1.35 ± 0.13-fold compared with the control values (0.95 ± 0.02 for the step to −10 mV, see Fig. 10 legend for details). Normalization of the PKCζ-modulated currents to their respective control peak currents (Fig. 10B, right) indicated that PKCζ significantly augmented TTX-R $I_{Na}$ by 1.35 ± 0.13-fold compared with the control values (0.95 ± 0.02 for the step to −10 mV, see Fig. 10 legend for details). Surprisingly, there was a small but significant time-dependent shift in $E_{rev}$ for TTX-R $I_{Na}$. For the control condition, $E_{rev}$ was 32.1 ± 1.9 mV ($n = 9$, the calculated $E_{rev}$ was 27.7 mV); however,

---

**Fig. 10. Internal perfusion with recombinant PKCζ enhances the tetrodotoxin-resistant sodium current (TTX-R $I_{Na}$) in sensory neurons.**

A: a representative recording of TTX-R $I_{Na}$ obtained under control conditions (left) and after 10 min of internal perfusion with recombinant PKCζ (right). The recording pipette contained recombinant PKCζ (2 ng/µl). Traces shown are for voltage steps from −60 to +40 mV in 5-mV increments. “0” lines indicate the zero-current level. B: left: the average current-voltage relation for the peak value of TTX-R $I_{Na}$ obtained for the control condition ($n = 9$) and after internal perfusion with PKCζ for 10 min ($n = 9$). The current values obtained at 2 and 6 min are not shown for clarity. The values of TTX-R $I_{Na}$ after PKCζ were significantly different from control values for voltages between −25 and +45 mV for the 10-min perfusion time and between +35 and +45 mV for the 6-min perfusion time, whereas there was no difference between the control and the 2-min perfusion time (RM ANOVA, Holm-Sidak all pairwise test). B: right: the $I_{Na}$-voltage relation for the control condition and after 10 min of internal perfusion with PKCζ. Peak currents were normalized to their respective values obtained under control conditions. The values of $I_{Na}$ after PKCζ were significantly different from control values for voltages between −30 and +45 mV for the 10-min time point, between −25 and −15 mV for the 10-min perfusion time, at −20 and −15 mV for the 2-min perfusion time (RM ANOVA, Holm-Sidak all pairwise test). The peak values of $I_{Na}$ were 1.07 ± 0.05 and 1.20 ± 0.10 ($n = 9$ neurons) for the 2- and 6-min perfusion times, respectively (data not shown). C: the $G_{G_{Na}}$-voltage relation for TTX-R $I_{Na}$ for the control condition and after 10 min of internal perfusion with PKCζ from these 9 neurons. The values of $G_{G_{Na}}$ after PKCζ were significantly different from control values for voltages at −20 and −15 mV for the 10-min time point only; there were no significant differences between the values of $G_{G_{Na}}$ for the control and at the 2- and 6-min perfusion time points (RM ANOVA, Holm-Sidak all pairwise test). The lines through the data points represent the fit using the Boltzmann relation. The values of $G_{G_{Na}}$ between 30 and 40 mV have been excluded because of their proximity to the reversal potential, which gives rise to a large variance in the data and affects the quality of the fit.
PKMζ AND NEURONAL EXCITABILITY

PKMζ is a critical effector molecule in many signaling cascades and structurally consists of two important molecular domains, the NH2-terminal regulatory domain and the COOH-terminal catalytic domain. The PKC family is made up of three subtypes: the conventional isoforms require phosphatidylserine for activation; the novel isoforms require PS and DAG for activation and are insensitive to Ca2+; and the atypical isoforms require only PS for activation (reviewed by Gould and Newton 2008; Hirai and Chida 2003; Moscat and Diaz-Meco 2000). The regulatory domain contains C1/C2 second messenger/lipid binding sites and an autoinhibitory pseudosubstrate sequence that binds to and inactivates the catalytic domain. In conventional and novel PKCs, second messenger binding to C1/C2 produces a conformational change that relieves autoinhibition by the pseudosubstrate domain, unhinging it from the catalytic domain and activating PKC. Specific synthetic pseudosubstrate peptides can bind to the active site on the catalytic domain and thus act as exogenous inhibitors of kinase activity (pseudosubstrate inhibitors). In contrast, aPKCs lack C1B/C2 so that they are neither activated by second messengers nor downregulated by phorbol esters. The aPKCs rely on PS (perhaps other lipids as well) and upstream effectors such as PI3K for activation. aPKCs have a unique PB1 domain involved in protein-protein interactions that also are critical for downstream signaling.

Our understanding of the physiological actions of aPKCs is beginning to emerge. The atypical PKCζ plays an important role in cell growth and survival through its activation of the NF-κB signaling pathway (see reviews: Hirai and Chida 2003; Moscat and Diaz-Meco 2000; Moscat et al. 2006). In addition, PKCζ may have a regulatory role in the adaptive immune response. PKCζ may appear to have a significant functional role in metabolic regulation (Farese and Sajjan 2010) as well as the proliferation of cancer cells (Win and Acevedo-Duncan 2009). Generally speaking, the role of aPKCs in neurophysiological function is poorly understood. However, PKMζ has received much attention for its critical role in the maintenance of both late-long-term potentiation (LTP) and memory retention (reviewed by Sacktor 2008, 2011). Sacktor’s laboratory has shown that tetanization stimulation of the CA1 region of the hippocampus produced a dramatic increase in the level of PKMζ that proved to be necessary and sufficient for the maintenance of LTP (Ling et al. 2002; Sacktor 2011; Sacktor et al. 1993). Additional studies showed that PKMζ was a 51- to 55-kDa protein expressed only in the brain and that an internal promoter within the PKCζ gene generates PKMζ mRNA that codes for only the catalytic domain (Hernandez et al. 2003; Sacktor 2008, 2011). Because there is no regulatory domain and no autoinhibition, PKMζ is constitutively active. However, translation of PKMζ mRNA is repressed; in this state, inactive mRNA is transported to neuronal dendrites. Upon synaptic activation, repression is released by local kinases such as PI3K (Kelly et al. 2007) and PKMζ is synthesized (essentially translation on demand). At this point, PKMζ has low activity; however, phosphoinositide-dependent kinase-1 (PDK1) phosphorylation of the activation loop (necessary for activation of all aPKCs) shifts PKMζ to high persistent activity wherein phosphorylation of specific targets is believed to maintain LTP. The key role of PKMζ is supported by in vivo observations where pseudosubstrate inhibition of PKMζ prevented the retention of cortical long-term memory and overexpression of PKMζ enhanced this retention (Shema et al. 2007, 2011). Specific targets whereby PKMζ promotes LTP maintenance are unknown, although recent work suggests that PKMζ may regulate the trafficking of AMPA receptors (Migues et al. 2010). A physiological role for PKMζ in the regulation of neuronal function in the peripheral nervous system remains to be defined.
PKMζ AND NEURONAL EXCITABILITY

(Li et al. 2010). Also, injection of the αPKC pseudosubstrate peptide inhibitor into the anterior cingulate cortex prevented the mechanical allodynia that resulted from the injury. Interestingly, these authors reported no change in PKMζ expression in the spinal cord after injury. In another study, PKMζ appeared to maintain the persistent sensitization of mechanical responses after a priming injection of IL-6, because intrathecal injection of the pseudosubstrate inhibitor reversed this sensitization (Asiedu et al. 2011). Expression of constitutively active PKCζ in the dorsal horn of the spinal cord also enhanced mechanical sensitivity, which persisted for at least 12 days. Additional studies are necessary to establish the exact functional role of PKMζ in regulating the nociceptive sensitivity in the spinal cord. These reports suggest that PKMζ mediates enhanced nociceptive sensitivity; however, these interpretations must be tempered by the fact that the phosphospecific antibodies and the pseudosubstrate inhibitor are not selective for PKMζ but target all αPKCs. This suggests that PKCζ and/or PKCα/δ could be equally important. However, our results for the expression of the different αPKCs as well as the siRNA-induced specific reduction obtained in isolated sensory neurons clearly define PKMζ as a key effector of sensitization. Nevertheless, these results indicate that αPKCs play a significant role in regulating the sensitivity of nociceptive neurons after injury or inflammatory conditions.

NGF, PKMζ, and sensory neuron excitability. In this report, we show that NGF enhanced the excitability of small-diameter capsaicin-sensitive sensory neurons through a depolarization of the resting membrane potential, a decrease in the rheobase, and an increase in the resistance at threshold; however, NGF did not affect the firing threshold. In experiments to establish the signaling pathways whereby NGF augments excitability, we found that inhibition of MEK1/2 (PD-98059), PLC (U-73122 and neomycin), or conventional/novel isoforms of PKC (BIM) had no effect on the sensitization produced by NGF. In contrast, studies with a pseudosubstrate inhibitor of αPKCs and specific knockdown by siRNA indicated that NGF somehow leads to the activation/synthesis of the atypical PKC, PKMζ, which plays a critical role in enhancing neuronal excitability. Both Western blots using selective antibodies and qPCR using gene-specific primers demonstrated that neither full-length PKCζ nor PKCα/δ were altered by siRNA treatment. Importantly, inhibitors of PI3K suppressed the capacity of NGF to increase AP firing. This PI3K-dependent inhibition is consistent with a role of PKMζ in neuronal sensitization wherein the enhanced expression of PKMζ in the hippocampus after teta-nizing stimulation was blocked by inhibition of PI3K (400 nM wortmannin; Kelly et al. 2007). In addition, PI3K and ceramide are thought to be critical upstream regulators of αPKC activity, although to our knowledge, the actions of ceramide on PKMζ have not been examined. It is well established that the catalytic domains of αPKCs are highly homologous. Based on this similarity, internal perfusion with active recombinant PKCζ or PKCζ-augmented neuronal excitability and recapitulated the sensitization produced by NGF. Together, these findings demonstrate that the activation/synthesis of PKMζ by NGF is a critical factor in the regulation of neuronal excitability.

Our current observations demonstrate that internal perfusion with recombinant PKCζ plays a key role in regulating the activity of potassium channels wherein PKCζ led to a large suppression of \( I_K \) (≈40%) with a small but significant leftward shift in the \( V_{0.5} \) of activation. Our previous work showed that NGF could suppress \( I_K \) in a similar manner. Also, 10 μM C2-ceramide inhibited \( I_K \) by ∼60% and produced a leftward shift in \( V_{0.5} \) from a control value of ∼0.3 ± 5.2 to ∼11.1 ± 1.3 mV after a 20-min exposure to ceramide (Zhang et al. 2002). The idea that PKMζ modulation of \( I_K \) gives rise to enhanced excitability is consistent with the observation that overexpression of constitutively active PKCζ in the dorsal horn produced a persistent sensitization to mechanical stimulation (Asiedu et al. 2011). In a variety of cell types, agonist-mediated activation of PKCζ led to a depolarization that resulted from a decrease in potassium current; these depolarizations were blocked by the pseudosubstrate inhibitor of PKCζ (Cogolludo et al. 2003; Ramström et al. 2004). These studies also raise questions as to the possible actions of αPKCs on other types of ion channels that regulate excitability. Our earlier study showed that NGF and ceramide enhanced TTX-R \( I_{Na} \) in small-diameter sensory neurons (Zhang et al. 2002). In this report, we now demonstrate that recombinant PKCζ also augments TTX-R \( I_{Na} \). To our knowledge, nothing is known about the capacity of PKMζ to modulate voltage-dependent ion channel activity. However, previous studies have established that both conventional and novel isoforms of PKC can modulate \( I_{Na} \). For example, phorbol ester activation of PKC augmented the amplitude of TTX-R \( I_{Na} \) in both sensory neurons (Gold et al. 1998) and astrocytes isolated from spinal cord (Thio and Sontheimer 1993). In addition, the epinephrine-induced increase in TTX-R \( I_{Na} \) was partially suppressed by the conventional/novel PKC inhibitor BIM (Khasar et al. 1999b) and the more selective peptide inhibitor of PKCe, eV1-2 (Khasar et al. 1999a). Understanding the cellular mechanisms whereby PKMζ and potentially other αPKCs modulate ion channel activity and ultimately neuronal excitability are important studies for the future.

To our knowledge, there has been only one study (Martiny-Baron et al. 1993) that has examined the concentration dependence for the inhibition of the three classes of PKC by BIM. However, Touleec et al. (1991) examined the effects of BIM on the conventional and novel isoforms and reported IC_{50} values similar to those of Martiny-Baron et al. Thus this is partly a problem in not having a greater understanding of the specific actions of BIM on the three classes of PKC. Nevertheless, there is an order of magnitude between the IC_{50} values for BIM between the three classes: conventional isoforms at 10–20 nM, novel isoforms at 100–200 nM, and the atypical PKCζ at 5.8 μM. The exact IC_{50} depends on the specific isoform of PKC. In support of the effectiveness of BIM, Bonnington and McNaughton (2003) demonstrated that under normal conditions, 500 nM BIM blocked the NGF-induced enhancement of capsaicin-evoked increases in intracellular Ca^{2+}. McNaughton’s group provided clear evidence that the novel PKC isoform PKCe is involved in the enhancement of the transient receptor potential (TRPV1) current (Cesare et al. 1999; Vellani et al. 2001), showing that the functionally effective concentration of BIM is only 2.5–5 times the IC_{50} value for the novel isoforms. Furthermore, Cogolludo et al. (2003) found that BIM (1 μM) failed to suppress the U46619-induced (a thromboxane A2 agonist) contraction of isolated pulmonary arteries, whereas the contraction was blocked by Gö-6983 (10 nM), which is an inhibitor of conventional PKCs, some novel isoforms, and PKCζ (see Way et al. 2000). Because contractions were insensitive to BIM but suppressed by Gö-6983, these authors sug-
gest that U46619 may act through PKCζ. Indeed, they found that treatment with the pseudosubstrate inhibitor of PKCζ (10 μM) blocked U46619-mediated contractions (Cogolludo et al. 2003). In this report, we show that with 1 μM BIM, the sensitizing effects of NGF are not affected. This concentration is 50–100 times the IC₅₀ for the conventional isoforms and 5–10 times the IC₅₀ for the novel isoforms. If either the conventional or novel isoforms contribute to the increased excitability produced by NGF, then at 1 μM BIM, we would expect to measure some amount of diminution of the increase in AP firing. However, this is clearly not the case given that the NGF-induced increase in firing was not significantly different between the untreated controls and in the presence of BIM. In contrast, inhibition produced by the atypical pseudosubstrate inhibitor is complete, demonstrating that the atypical PKCs play a critical role in the NGF-mediated enhancement of neuronal excitability.

Our previous work demonstrated that NGF and membrane-permeant C2-ceramide (N-acetyl sphingosine) enhanced the excitability of small-diameter sensory neurons (Zhang et al. 2002). The sensitization produced by NGF was blocked by an inhibitor of neutral sphingomyelinase (SMase), suggesting that NGF led to the liberation of an endogenous ceramide(s). Other studies have indicated that short-chain ceramides, such as C2-ceramide, do a poor job of mimicking the actions of the natural longer chain ceramides, e.g., C16-ceramide (see Hashizume et al. 1998; Simon and Gear 1998; Takeda et al. 2006; Upham et al. 2003). For example, C2-ceramide inhibits aggregation of platelets, whereas C6-ceramide, C8-ceramide, and SMase augment aggregation, suggesting that these ceramides can have different actions (Hashizume et al. 1998). In sensory neurons, our results indicate that C2-ceramide does effectively recapitulate the actions of native ceramides. This is based on the following observations. NGF, the activating ligand of p75NTR, and C2-ceramide produce very similar increases in AP firing over time, and both are associated with quite similar changes in different parameters of excitability. NGF and C2-ceramide have nearly identical effects in augmenting TTX-R I₅Na and suppressing Iₖ in sensory neurons. Treatment with a bacterial SMase, which should liberate endogenous ceramides, increased AP firing in a manner that was very similar to that produced by NGF and exogenous C2-ceramide (Zhang et al. 2002). The increased AP firing produced by NGF and C2-ceramide was blocked by pretreatment with 20 μM dimethylsphingosine, an inhibitor of sphingosine kinase, suggesting that C2-ceramide was effective in stimulating downstream signaling (Zhang et al. 2006). In the present report, the sensitization produced by both NGF and C2-ceramide was blocked by the pseudosubstrate inhibitor of PKCζ, again supporting the idea that C2-ceramide is capable of activating downstream cascades that modulate AP firing. In additional support of our contention that C2-ceramide effectively activates downstream signaling, intradermal injection of C2-ceramide (10 μg) into the hind paw of a rat produced mechanical and thermal hyperalgesia (Doyle et al. 2011; Joseph and Levine, 2004), whereas dihydro-C2-ceramide, an inactive analog, had no effect (Doyle et al. 2011). In addition, injection of TNF-α produced a mechanical hyperalgesia that was suppressed by GW4869, a selective inhibitor of neutral SMase (Joseph and Levine 2004). Early studies demonstrated that C2-ceramide produced a dose-dependent activation of a ceramide-activated phosphatase that was very similar to the dose dependency produced by either C6-ceramide or native ceramides isolated from bovine brain (Dobrowsky and Hannun 1992). C2- and C6-ceramide were capable of inducing DNA synthesis in Swiss 3T3 cells in a manner that was analogous to that produced by bacterial SMase (Olivera et al. 1992). Additional studies have indicated that these short-chain ceramides can be converted into longer chain ceramides and mimic the biosynthetic actions of the longer chain ceramides (Ardail et al. 2002; Ridgway and Merriam 1995; Venkataraman and Futerman 2001). Particularly relevant to our findings are two observations. First, treatment with C2-ceramide was capable of activating PKCζ purified from rat brain (Wang et al. 1999), and C2-ceramide and SMase caused translocation of PKCζ to the particulate fraction isolated from rat astrocytes (Galve-Roperh et al. 1997). Second, application of C2-ceramide led to significant activation of PI3K in both alveolar macrophages (500 nM or 10 μM C2-ceramide, Monick et al. 2001) and rat2 fibroblasts (40 μM C2-ceramide, Hanna et al. 1999). These observations support the notion that NGF via ceramide and PI3K could lead to the activation of PKMζ and thereby enhance neuronal excitability. Together, these results indicate that C2-ceramide effectively parallels the actions of NGF in sensory neurons; however, caution must be taken, because the efficacy of these short-chain ceramides in signaling cascades appears to be dependent on cell type.

Ngf signaling and sensitization. Activation of multiple signaling cascades by either TrkA or p75NTR could function as the intracellular pathways mediating NGF-induced peripheral sensitization. On the basis of the existing literature that has focused on small-diameter sensory neurons, it appears that NGF augments the current conducted by TRPV1 via activation of TrkA receptors, whereas NGF enhances the capacity of these neurons to fire APs by activation of p75NTR. This idea is based on observations wherein an inhibitor of tyrosine kinase receptors, K-252a (Knüsel and Hefti 1992, but see Kase et al. 1987), blocked NGF-induced sensitization of TRPV1 (100 nM, Shu and Mendell 1999: 100 nM, Shu and Oxford 2007), whereas 400 nM K-252a (or the structurally different inhibitor AG-879, 30 μM) had no effect on increased AP firing produced by either NGF or BDNF (Zhang et al. 2008). Furthermore, the enhanced excitability produced by NGF or BDNF was prevented by pretreatment with the p75NTR blocking antibody (Zhang and Nicol 2004; Zhang et al. 2008).

Despite similar neuronal preparations and experimental designs, the results regarding the nature of the signaling pathways that are causal in NGF-mediated sensitization have been surprisingly inconsistent, and this remains an unanswered question (see review by Nicol and Vasko 2007). The role of PLC in NGF-induced sensitization is controversial. In human embryonic kidney (HEK) cells, a signaling complex comprising TrkA, TRPV1, and PLCγ is thought to form wherein NGF leads to the enhancement of TRPV1 activity (Chuang et al. 2001). These authors demonstrated that NGF activation of a mutant TrkA incapable of coupling to PLCγ did not augment capsaicin-evoked current. In another study, treatment with 5 μM U-73122, a PLC inhibitor, suppressed the NGF-induced sensitization of the heat response in sensory neurons (Galoyan et al. 2003). Similarly, inhibition of PLC by 10 μM neomycin reduced enhancement of the capsaicin-evoked increase in intracellular Ca²⁺ levels produced by NGF (Bonnington and McNaughton 2003). In contrast, other studies indicated that PLC inhibition by U-73122 had no effect on NGF
enhancement of the capsaicin-evoked current (20 μM, Zhu and Oxford 2007; 10 μM, Zhuang et al. 2004). In many different cell systems, NGF activates Erk with consequent activation of Erk. The MEK inhibitor PD-98059 reduced the ability of NGF to augment capsaicin-evoked currents (10 μM, Zhu and Oxford 2007; 20 μM, Zhuang et al. 2004). Another MEK inhibitor, U0126 (10 μM), similarly diminished the NGF-induced enhancement of the capsaicin-gated current recorded from adult sensory neurons (Shu and Mendell 2001). In contrast, inhibition of PKA by 200 nM KT-5720 had no effect on the capacity of NGF to augment the capsaicin-evoked increase in intracellular Ca$^{2+}$ in neonatal sensory neurons (Bonnington and McNaughton 2003). Finally, novel findings in a heterologous expression system indicated that NGF produced a rapid insertion of additional TRPV1 channels into the plasma membrane, which could account for the increased capsaicin-evoked influx of Ca$^{2+}$ (Zhang et al. 2005). This sensitization was blocked by PP2, an inhibitor of Src kinase. Additional experiments showed that Src kinase phosphorylation of Tyr-200 on TRPV1 was critical for membrane insertion of TRPV1. One must also consider the fact that among these signaling pathways activated by NGF, there is likely to be significant cross talk between them. Establishing the exact sequence of events has been limited by the paucity of specific inhibitors to block targeted effector molecules in these pathways. There does, however, appear to be a point of commonality between TrkA and p75NTR signaling in that both the NGF-mediated sensitization of TRPV1 and excitability are blocked by inhibition of PI3K (Bonnington and McNaughton 2003; Zhu and Oxford 2007; Zhuang et al. 2004; present study). NGF via TrkA activates PI3K (reviews by Huang and Reichardt 2003; Reichardt 2006). NGF via p75NTR also activates PI3K through interaction with specific adaptor proteins (Roux et al. 2001). It is downstream of PI3K that the signaling cascades activated by NGF appear to diverge. As an example, consider the role of the PKC pathway. BIM, a PKC inhibitor that is more selective for conventional and novel isoforms, blocks sensitization of TRPV1 currents by NGF (500 nM, Bonnington and McNaughton 2003; 1 μM, Zhu and Oxford 2007), whereas in other studies BIM (500 nM, Shu and Mendell 2001) or staurosporine (a conventional/novel PKC inhibitor, Ling et al. 2002) had no effect on NGF sensitization of TRPV1 (Chuang et al. 2001). These differences remain to be resolved. In our studies, treatment with BIM had no effect on increased AP firing produced by NGF; however, inhibition of αPKC suppressed this enhanced excitability. These findings indicate that although NGF can enhance the sensitivity of small-diameter sensory neurons, this sensitization is accomplished through activation of different intracellular signaling pathways. These different isoforms of PKC may act on different targets, i.e., the conventional/novel isoforms may target TRPV1, whereas the atypical PKMζ may target voltage-dependent channels. Recent studies have demonstrated that activation of individual isoforms of PKC can produce differential outcomes. For example, different isoforms of PKC give rise to differential phosphorylation of distinct regions of the α-subunit of Cav1.2, which can then regulate activity (Yang et al. 2005, 2009). In addition, activation of different isoforms can alter the outcomes of downstream activation patterns. In bovine aortic endothelial cells, siRNA knockdown of novel PKCe blocked the VEGF-stimulated phosphorylation of Akt, whereas knockdown of conventional PKCζ enhanced VEGF-induced Akt phosphorylation (Rask-Madsen and King 2008). Thus these observations support the idea that receptor-mediated activation of particular isoforms of PKC can modulate specific downstream targets that alter cellular activities.

**NGF and PKMζ activation.** Our results indicate that NGF via a PI3K- and PKMζ-dependent pathway leads to the increased excitability. On the basis of current literature, one could speculate that NGF somehow initiates the synthesis of PKMζ from repressed mRNA localized in the neuronal cell body. Consistent with this idea are recent findings showing that after a 15-min treatment with 10 ng/ml NGF, there was significant activation of eukaryotic initiation factors, i.e., the translation machinery, in isolated adult sensory neurons (Melemedjian et al. 2010). If, in fact, this is the mechanism of action, then our results would suggest the translational events can occur rapidly given that significant increases in AP firing were observed within 2 min after exposure to NGF or BDNF (Figs. 1–4 in the present study; Zhang et al. 2008). Alternatively, NGF by a presently undefined pathway leads to the activation of existing PKMζ in the neuronal cell body and does not require protein synthesis. Recent evidence suggests that tetanus-induced long-term LTP in the presence of BDNF leads to increased expression of PKMζ in the absence of protein synthesis; however, this occurred over a much longer time scale (3 h) than our present studies (Mei et al. 2011). These findings raise critical questions regarding the specific cellular mechanisms whereby NGF activates or generates PKMζ in sensory neurons and what are the potential targets of PKMζ modulation. Thus our results demonstrate that NGF increases the excitability of small-diameter capsaicin-sensitive sensory neurons through a PI3K-mediated and perhaps a ceramide-dependent activation/synthesis of the atypical PKC, PKMζ. Important future studies need to focus on whether the increased AP firing mediated by NGF requires protein synthesis and what other effectors molecules may be necessary, e.g., what role ceramide has in the activation of generation of PKMζ.

**GRANTS**

This investigation was conducted in a facility constructed with support from National Institutes of Health (NIH) Research Facilities Improvement Program Grant C06 RR015481-01. This work was supported by NIH Grant NS46084 and in part by funding from the Ralph W. and Grace M. Showalter Trust (to G. D. Nicol) and NIH Grants R01 MH53576 and MH 57068 (to T. C. Sacktor).

**DISCLOSURES**

No conflicts of interest, financial or otherwise, are declared by the author(s).

**AUTHOR CONTRIBUTIONS**


**REFERENCES**

Akimoto K, Mizuno K, Osada S, Hirai S, Tanuma S, Suzuki K, Ohno S. A new member of the third class in the protein kinase C family, PKC...


PKMζ and neuronal excitability


