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Protein kinase C epsilon contributes to basal and sensitizing responses of TRPV1 to capsaicin in rat dorsal root ganglion neurons

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Abstract

Phosphorylation of the vanilloid receptor (TRPV1) by protein kinase C epsilon (PKCε) plays an important role in the development of chronic pain. Here, we employ a highly defective herpes simplex virus vector (vHDNP) that expresses dominant negative PKCε (DNPKCε) as a strategy to demonstrate that PKCε is essential for: (i) maintenance of basal phosphorylation and normal TRPV1 responses to capsaicin (CAPS), a TRPV1 agonist and (ii) enhancement of TRPV1 responses by phorbol esters. Phorbol esters induced translocation of endogenous PKCε to the plasma membrane and thereby enhanced CAPS currents. These results were extended to an *in-vivo* pain model in which vHDNP delivery to dorsal root ganglion neurons caused analgesia in CAPS-treated, acutely inflamed rat hind paws. These findings support the conclusion that in addition to receptor sensitization, PKCε is essential for normal TRPV1 responses *in vitro* and *in vivo*.

Keywords

capsaicin; desensitization; HSV; modulation; nociception; PKC; TRPV1

Introduction

The vanilloid receptor (TRPV1) is a pro-nociceptive cation channel that is activated by capsaicin, noxious heat, protons and endovanilloids (Pingle *et al.*, 2007). TRPV1 responses are regulated by a dynamic interplay between receptor phosphorylation and dephosphorylation (Docherty *et al.*, 1996; Koplas *et al.*, 1997; Piper *et al.*, 1999). Protein kinase C epsilon (PKCε) phosphorylates TRPV1 at residues S502 and S800 (Numazaki *et al.*, 2002) and is activated downstream of several inflammatory mediators including bradykinin (Cesare *et al.*, 1999), endothelins (Plant *et al.*, 2006) and proteases (Amadesi *et al.*, 2004). TRPV1 phosphorylation by activated PKCε causes receptor hyperactivity in response to submaximal concentrations of TRPV1-specific agonists such as capsaicin (CAPS; Mandadi *et al.*, 2006). As PKC-induced TRPV1 sensitization is associated with disorders such as chronic pain (Bhave *et al.*, 2003), bladder cystitis (Scultoreanu *et al.*,

2005b) and diabetic neuropathy (Hong & Wiley, 2005), a detailed understanding of TRPV1 modulation is essential to therapeutic interventions. The link between PKC and TRPV1 sensitization has been reported using (i) nonneuronal cells transfected with TRPV1 phosphorylation-defective mutants and (ii) TRPV1 knock-out mice in which treatment with phorbol esters that specifically activate PKC failed to induce pain responses (Bolcskei *et al.*, 2005; Mandadi *et al.*, 2006). Here we developed a novel transgene delivery system using replication-deficient herpes simplex virus (HSV) vectors to express a dominant negative PKC ϵ (DNPKC ϵ) in normal adult neurons, providing an efficient means to assess the role of PKC ϵ in TRPV1 function directly.

HSV vectors are ideally suited for this purpose as the virus is known to persist in a quiescent state in nociceptive neurons without loss of neuronal cell viability or normal cellular function. Highly defective HSV vectors that are incapable of spreading to other tissues or spinal neurons and do not alter the physiological functions of infected neurons can be used to target delivery of DNPKC ϵ to neurons of DRG ganglia by simple intra-dermal or visceral organ vector injections. This feature of targeted transgene expression makes it possible to compare functional and behavioral effects of DNPKC ϵ on TRPV1 with responses in uninfected or control vector-infected neurons of the same animal.

Infection of DRG neurons with DNPKC ϵ vector *in vitro* reduced CAPS-evoked current amplitudes, enhanced TRPV1 desensitization rates and considerably altered the ability of phorbol ester-activated PKC ϵ to sensitize the TRPV1 responses to CAPS. Taking advantage of the neurotropism of HSV and the replication defective design of our DNPKC ϵ vector, we targeted infection to DRG neurons innervating the rat hind footpad by a direct vector inoculation of the L4 and L5 dermatomes. This approach thus provided the unique opportunity to study the effects of PKC ϵ on TRPV1 function in response to CAPS-induced hyperalgesia *in vivo*. Evidence for increased paw withdrawal latency in response to heat supported the conclusion that phosphorylation by constitutively active PKC ϵ contributes to CAPS responses both in isolated neurons and in whole animals. Together, our experimental strategy confirms the role of PKC ϵ in sensitization of TRPV1 *in vitro* and *in vivo* and supports an essential function for PKC ϵ in basal TRPV1 activity.

Materials and methods

HSV-1 vector construction

The HSV-1 vectors used in this study were vHDNP (vector HCMVp: dominant negative PKC ϵ) and vHG (vector HCMVp: EGFP). These vectors are replication defective mutants, deleted for the immediate early (IE) essential genes, infected cell protein (ICP)4 and ICP27. Deletion of IE genes impairs the ability of these mutant vectors to replicate in noncomplementing cells, making *intrans* complementation of the ICP4 and ICP27 gene products a requirement in order to replicate *in vitro*. The vectors are propagated in a Vero cell line complementing for deleted viral functions (Marconi *et al.*, 1996). To construct the recombinant viral vectors, plasmids were recombined into the targeted locus of parent vectors by sequential virus infection and plasmid transfection (Srinivasan *et al.*, 2007). Screening for marker gene transfer (e.g. GFP positive plaques) was used to isolate recombinants (Goins *et al.*, 2002). Vector plaques were purified by three rounds of limiting dilution and verified by Southern blot analysis. Confirmed vector stocks were expanded in complementing cells, purified by centrifugation to remove excess proteins, titered on complementing cells, aliquoted and stored at -80°C .

The construction of vHG has been described elsewhere (Srinivasan *et al.*, 2007). For vHDNP construction, vector QOZ (ICP4-, ICP27-, β ICP22, β ICP47, ICP0p: LacZ::UL41) was recombined to contain a DNPKC ϵ cassette fused to GFP and driven by the HCMV

promoter (HDNP) at the UL41 locus using the plasmid p41HDNP. This plasmid contains the HCMVp: DNP cassette flanked by UL41 homology sequences and was constructed as follows: human PKC ϵ cDNA was isolated by RT-PCR from the total mRNA of SH-SY5Y human neuroblastoma cells, obtained using the RN easy minikit (Qiagen, Valencia, CA, USA). The PKC ϵ cDNA regulatory domain was then cloned into the *Sal* I to *Bgl* II sites of the pEGFPN1 vector (Clontech Laboratories, Inc.) by standard restriction digestion, ligation and sub-cloning techniques. The resultant plasmid contained the HCMV promoter driving transcription of the PKC ϵ regulatory domain fused to GFP. This plasmid was PCR-amplified using primers that each contained an *Mlu*I site (upper primer is 5'-GCGACGCGTGCCAAAGTACTGGCCGACCTG-3' and lower primer is 5'-GCGACGCGTGATACCGAAGTTGTGGGGCAT-3') such that parts of the cDNA coding for C2 and C1b regulatory sub-domains were deleted following cleavage of the PCR product with *Mlu*I and re-ligation to create HDNP. The HDNP construct was isolated as a *Bam*HI fragment and cloned into the *Bam*HI site of plasmid p41 to create p41HDNP. Recombinants were isolated using GFP as a marker. The UL41HDNP was PCR-amplified from viral DNA and sequenced for the transgene prior to performing experiments.

Cell culture and vector infections

U2OS cells were (American Type Culture Collection, (HTB-96), Manassas, VA, USA) grown and maintained in Dulbecco's minimum essential medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine and 100 U/mL penicillin/streptomycin (Life Technologies Inc.). Adult rat dorsal root ganglion (DRG) neurons were isolated by standard enzymatic techniques as previously described (Sculptoreanu & de Groat, 2003). Surgery was performed under deep isoflurane anesthesia. After removing the desired spinal cord segments rats were killed by cervical dislocation. Briefly, freshly dissected ganglia were minced and washed in cold, oxygenated DMEM (Sigma), followed by 10 min dissociation at 37°C in DMEM containing 0.5 mg/mL trypsin (Sigma). After a 10-min centrifugation, the medium was replaced with DMEM containing 1 mg/mL Collagenase B (Boehringer-Mannheim) and 0.5 mg/mL Trypsin Inhibitor type 1S (Sigma). Dissociation of neurons was continuously monitored and the cells were gently triturated with siliconized Pasteur pipettes every 10 min. After the ganglia dissociated into individual neurons (25–40 min), the cell suspension was centrifuged for 10 min at 112 g.

The pellet was layered on 20 mL of 50% adult bovine serum (Sigma) and DMEM and centrifuged again at 75 g. The pellet was then re-suspended in DMEM containing 10% heat-inactivated horse serum and 5% FBS, and plated on collagen-coated 35-mm Petri dishes (Collaborative Research, Biocoat) or 35-mm Petri dishes with 10-mm glass bottom micro-wells (Mattek Corp., Ashland, MA, USA) for imaging experiments. Neurons were plated at low density (2000–3000 per dish) and the primary cultures were incubated in a 95% air, 5% CO₂ humidified incubator at 37°C for 3–4 days prior to infection with HSV-1 vectors.

Western blotting

Vector-infected U2OS cells (MOI 3) were harvested at 16 h post-infection (hpi) with 1× NuPAGE LDS Sample Buffer in PBS (Invitrogen, Carlsbad, CA, USA) and heated at 70°C for 10 min. Protein samples were separated by electrophoresis (4–12% Bis-Tris Gel). Following transfer, the membrane was incubated with anti-GFP goat polyclonal antibody (Abcam, Cambridge, MA, USA) at 1 : 500 dilution in PBS with 3% milk. Following washes and incubation with a donkey anti-goat secondary antibody (1 : 7000 dilution for 1 h at room temperature) (Sigma, St Louis, MO, USA), signals were detected using the Amersham ECL kit (Amersham Pharmacia Biotech, Piscataway, NJ, USA).

Immunocytochemistry and cell imaging

Adult rat DRG neurons were plated in 35-mm Petri dishes at a density of 200 cells per dish. Four days after culture, neurons were infected overnight with viral vectors (10^7 PFU per dish). The following day (16 hpi), vector-transduced neurons were incubated with $5 \text{ } \mu\text{M}$ PDBu for 2 min and immediately fixed for 20 min at room temperature in 2% paraformaldehyde. Following blocking with 10% HS in PBS (1 h), neurons were incubated with rabbit PKC ϵ antibody (1 : 300 dilution, 1 h). The PKC ϵ antibody used for this purpose is developed in rabbit using a synthetic peptide (Lys-Gly-Phe-Ser-Tyr-Phe-Gly-Glu-Asp-Leu-Met-Pro) corresponding to the C-terminal variable (V5) region (amino acids 726–737) of PKC ϵ (Sigma). Following PBS washes, a Cy3-labeled goat anti-rabbit secondary antibody (1 : 300 dilution, Sigma) secondary antibody incubation was performed. For live cell experiments, images of vector-infected U2OS cells were captured before and after $5 \text{ } \mu\text{M}$ PMA exposure in the same field. A TCS-SL confocal microscope (Leica, Dearfield, NY, USA) was used to capture images for all experiments using the appropriate filters. Images were processed using IMAGEJ software.

Whole-cell patch clamp recordings

Gigaohm-seal whole-cell recordings of CAPS-induced currents were recorded in adult DRG neurons in culture, uninfected or vector-infected (20 hpi, 107 per Petri dish for rat DRG neurons) cells using whole-cell patch clamp techniques. Patch pipettes were pulled from capillary glass tubes (Accufil 90, Clay-Adams) and fire polished. Immediately before recording, the serum-containing media was replaced with PBS. Whole-cell currents were voltage clamped using an Axopatch 200A (Axon Instruments, Foster City, CA, USA) amplifier. Pulse generation, current recording and data analysis used PCLAMP software (Axon Instruments). Currents were sampled at $200 \text{ } \mu\text{s}$ and filtered at 500 Hz. Capacitive currents and up to 80% of the series resistance were compensated. A p/4 protocol was used to subtract uncompensated capacitive currents and leak currents. The decay of TRPV1 currents in response to capsaicin and after addition of the phorbol ester, phorbol 12, 13-dibutyrate (PDBu) or the PKC inhibitor bisindolylmaleimide I HCl (BIM) in the presence of capsaicin was fitted with single exponentials using pClamp software regression analysis. Peak current amplitudes were measured with PCLAMP software. The extracellular solution was Dulbecco phosphate buffer (Sigma). The pipette (intracellular) solution contained (mM): KCl 120, K_2HPO_4 10, NaCl 10, MgCl_2 2, EGTA 1, HEPES 10, pH adjusted to 7.4 with HCl. To this solution Mg-ATP (3 mM), cAMP (0.3 mM) and Tris-GTP (0.5 mM) were added just prior to the experiments. Capsaicin (Calbiochem, San Diego, CA, USA), a TRPV1 antagonist (Neurogen, Branford, CT, USA), the phorbol ester PDBu (Research Biochemicals, Natick, MA) and the PKC inhibitor BIM (Calbiochem, San Diego, CA) were dissolved in DMSO (100 mM) and used at less than 0.01% of their stock concentration. Gö6976 was prepared in aqueous solution. At these dilutions, DMSO alone had no effect on TRPV1 responses to capsaicin. Stock solutions in 10–100 mM were stored at -20°C and diluted in the external recording solution just before use. The TRPV1 antagonist (diaryl piperazine) was a gift from Neurogen (Branford, CT, USA) and was shown previously to be a potent and selective TRPV1 inhibitor ($K_i = 7 \text{ nM}$, (Sculptoreanu *et al.*, 2005a,b). All drugs were obtained from Sigma. Micromolar dilutions for experiments were made by serial dilutions of stock solutions. Extracellularly applied drugs were pipetted from stock solutions at 10–100 times the final concentration and rapidly mixed in the recording chamber as described previously (Sculptoreanu & de Groat, 2003).

Cobalt uptake assays

The cobalt uptake assay provides a means for the quick and efficient assay of TRPV1 activity in a large number of DRG neurons in culture (Hu-Tsai *et al.*, 1992). CAPS-evoked inward currents due to activation of TRPV1 normally selective for both monovalent and

divalent cations will cause Co^{2+} ions to enter the neurons. Co^{2+} is then precipitated with ammonium polysulfide that reacts with the intracellular Co^{2+} ions in TRPV1-positive cells forming a black precipitate of cobalt sulfide (CoS). Thus, black intracellular precipitates are an indirect indicator of neurons expressing functional TRPV1 receptors. These neurons can then be counted with a light microscope and are presented as a percentage of CAPS-sensitive neurons.

Methodology for cobalt uptake assays were as previously described (Sathianathan *et al.*, 2003). Briefly, uninfected or vector-infected neurons were washed twice in buffer A (NaCl, 57.5 mM; KCl, 5 mM; MgCl_2 , 2 mM; HEPES, 10 mM; glucose, 12 mM; sucrose, 139 mM; pH 7.4). Neurons were then incubated in a cobalt uptake assay buffer (buffer A plus 5 mM CoCl_2 and 0.5 mM capsaicin) for 5 min at room temperature. Following incubation in the cobalt uptake assay buffer, 0.2% ammonium polysulfide (Sigma) was added and immediately washed off using buffer A. Following the assay, the number of black neurons was counted under bright field and was expressed as a percentage of the total number of neurons counted.

***In vivo* experiments**

All *in vivo* tests were performed on adult male Sprague–Dawley rats (200–225 g; Charles River, Boston, MA, USA). Four days prior to performing behavioral tests, the animals were subcutaneously injected with 100 L of vHG, vHDNP (10^9 pfu/mL) or PBS (sham injection) into the plantar surface of the right hind paw. Uninfected animals were included as an additional control.

Tests for thermal hyperalgesia were performed using a Hargreaves apparatus on a platform with a baseline temperature of 25°C. Each animal was placed on the platform in a 10 × 20-cm plastic container, positioned over a mirror tilted at a 45° angle. All rats were allowed to acclimatize to the plastic container and platform for 30 min prior to testing. During each evaluation of thermal hyperalgesia, a focused heat source using a light intensity of 30 lumen was aimed at the plantar surface of the foot and the time until the animal moved its foot in response to the heat was measured. Three trials per testing period were made for each animal on both the right and the left foot with 1 min rest between each measurement. The average withdrawal time per foot was used for statistical comparisons. Data are presented as a ratio of the average time taken for withdrawal of the right foot vs. the left foot for animals in each group. For capsaicin tests, 10 g capsaicin dissolved in Tween 80 and resuspended in PBS was injected intradermally into the vector-inoculated (right) footpad of rats. Thirty minutes post-capsaicin inoculation, the rats were tested for thermal hyperalgesia-induced paw withdrawal latency (PWL) in a manner similar to that described above. The protocols were approved by the Institutional Animal Care and Use Committee of the University of Pittsburgh.

Statistical methods

Data are reported as mean ± SEM. For cobalt uptake assays, error bars are calculated based on standard deviations from a binomial distribution where a neuron with cobalt uptake is indicative of success and a neuron without uptake is indicative of failure. All images shown are representative results following multiple iterations of each experiment. Statistical testing for the data presented in Figs 3C, 4C, E and F, 5D and E, and 7A and B was carried out using a stepwise procedure depending upon the number of groups being compared. When only two means were involved in a comparison, a two-tailed *t*-test with unequal variances was used. A comparison was considered statistically significant if $P < 0.05$. When more than two means were involved, a one-way analysis of variance was first carried out in order to obtain a global test of the null hypothesis. If the global *P*-value for the test of the null hypothesis was < 0.05 , we carried out *post-hoc* comparisons between the different groups

using the Holm–Sidak test (Glantz, 2005). The results of the *post-hoc* comparisons are presented in terms of critical type 1 error rates.

Results

vHDNP transgene expression and characterization in U2OS cells

The experiments in this study exploited the use of a highly defective HSV vector (vHDNP) to target intracellular expression of a dominant negative form of PKC ϵ (DNPKC ϵ) that was previously shown to inhibit the function of the endogenous kinase (Zeidman *et al.*, 1999). Figure 1A shows the full-length PKC ϵ structure and functional domains. Figure 1B shows the dominant DNPKC ϵ gene structure which retains the pseudo-substrate region (PS), the C1a domain, the actin binding site and the variable region (V3). The construct is fused to GFP for visualization following vector expression. The mutant construct lacks the catalytic domain but remains responsive to phorbol esters. The DNPKC ϵ gene was inserted into the UL41 locus of the replication defective HSV vector to produce the recombinant vHDNP (Fig. 1C). A well-characterized reporter gene vector, vHG, was used as a control (Fig. 1C).

Expression of the DNPKC ϵ gene product was confirmed by Western blots of infected cell lysates. GFP-specific antibody detected a 47-kDa protein in vHDNP-infected U2OS cells (multiplicity of infection of 3 and 16 hpi) and a 27-kDa protein in vHG-infected U2OS cells under the same conditions (Fig. 1D). The molecular ratios were consistent with the predicted transgene product sizes and were expressed in the absence of vector replication. Treatment of vHDNP-infected cells with 5 μ M phorbol-myristate acetate (PMA) resulted in the rapid translocation of DNPKC ϵ to the plasma membrane from the perinuclear region (within 2 min), demonstrating that the remaining C1a regulatory domain was responsive to phorbol esters (Fig. 1E). Control vector (vHG)-infected cells displayed a uniform expression of GFP in the cytoplasm, the localization of which was unaffected by PMA stimulation (Fig. 1E).

Effect of DNPKC ϵ on endogenous PKC ϵ translocation in adult rat DRG neurons

Kinase translocation to the plasma membrane is a prerequisite for TRPV1 phosphorylation by PKC ϵ . As DNPKC ϵ is translocated in response to phorbol esters, we sought to determine if DNPKC ϵ translocation also occurs in adult rat DRG neurons and whether this process interferes or competes with the transport of endogenous PKC ϵ .

Control vHG-infected neurons (Fig. 2A–F) or vHDNP (Fig. 2G–L) were stimulated with 5 μ M of the PKC activator PDBu for 2 min, fixed and immunostained for endogenous PKC. PDBu was chosen as PMA has been shown to activate TRPV1 directly in addition to activating PKC ϵ (Chuang *et al.*, 2001). A red fluorescent Cy3 secondary antibody was used to visualize endogenous PKC ϵ staining. Green fluorescence visualized either transfected GFP in vHG cells or GFP-tagged DNPKC ϵ in vHDNP cells. Figure 2 shows vector-infected neurons with and without PDBu stimulation. vHDNP-infected neurons treated with PDBu demonstrated the almost complete translocation of DNPKC ϵ to the cell surface within 2 min (Fig. 2L), while the translocation of endogenous PKC ϵ was inhibited in the 2 min following PDBu stimulation (Fig. 2K and L). The merged images clearly show that in the presence of PDBu, the DNPKC ϵ and PKC ϵ products were minimally co-localized at the plasma membrane (Fig. 2L) while in the absence of drug, the products co-localized extensively in the cytoplasm (Fig. 2I). This effect was specific as in a similar experiment, vHG-infected neurons displayed a diffuse pattern of GFP localization which was unaffected by stimulation with PDBu (Fig. 2A and D) while endogenous PKC ϵ translocated to the plasma membrane nearly completely when stimulated with PDBu (Fig. 2B and E). Viral infection alone did not result in translocation of endogenous PKC ϵ (Fig. 2B). The merged images do not display co-localization of GFP and endogenous PKC ϵ (Fig. 2C and F).

vHDNP-diminished the CAPS-induced cobalt uptake in rat DRG neurons

CAPS-induced cobalt uptake assays were performed in vHDNP-infected neurons and compared with uptake in uninfected and vHG-infected neurons. Infection parameters were set such that nearly 100% of the neurons in culture were transduced with GFP or GFP-tagged DNPKCε (visualized as green fluorescing cells, Fig. 3A). Representative images of cobalt uptake by uninfected, vHG-infected and vHDNP-infected TRPV1-positive neurons are compared with images of cobalt uptake by TRPV1-negative neurons (Fig. 3B).

Figure 3C summarizes results of cobalt uptake assays. The percentage of CAPS-sensitive neurons revealed by cobalt uptake is shown for uninfected, and vHG- and vHDNP-infected neurons. The numbers of neurons counted for each group are shown in parentheses. The results show that approximately 44% of uninfected neurons were CAPS-responsive. However, only 12% of the vHDNP-infected neurons were CAPS-sensitive in contrast to 33% of vHG-infected neurons. The decrease in CAPS-responsive neurons following infection with vHDNP was significant compared with control vector-infected neurons ($P < 0.001$) and demonstrated that HSV-1 vector-expressed DNPKCε attenuates TRPV1 responses to CAPS.

HSV vector infection does not affect the electrophysiological profile of DRG neurons

Infection of CAPS-sensitive neurons with either vHG or vHDNP vectors did not alter basic properties such as cell size (C_m , membrane capacitance, Table 1), membrane resistance (R_m , Table 1), resting potential (RP, Table 1) or the firing induced by rectangular pulses 50–500 pA in intensity and 600 ms in duration. As shown in Fig. 4A, at -57 mV holding potential, long depolarizing current pulses elicited phasic firing as described in previous studies (Sculptoreanu & de Groat, 2007). The firing consisted of 1–3 action potentials in uninfected ($n = 62$), and vHG- ($n = 31$) and vHDNP- ($n = 36$) infected neurons (Table 1). Other firing parameters (voltage threshold, $dV/dt_{(max)}$) were also not altered in vHG- and vHDNP-infected cells (data not shown).

Effects of vHG and vHDNP vector infection on CAPS responses of DRG neurons

The cobalt uptake experiments suggested that DNPKCε diminished the ability of DRG neurons to respond to CAPS. To examine these effects, further whole-cell recordings of CAPS currents were performed in neurons infected with vHDNP and compared with the CAPS responses in vHG-infected and uninfected controls. The responses to CAPS were tested in more than 230 neurons: 140 uninfected, 42 vHG-infected and 50 vHDNP-infected neurons isolated from L4–S3 DRG. Approximately 59% of the neurons ($n = 83$, uninfected; $n = 23$, vHG-infected, $n = 30$ vHDNP-infected) exhibited inward currents in response to a CAPS concentration ($0.5 \mu\text{M}$) that is below the ED_{50} of $0.7 \mu\text{M}$ in rat DRG neurons (Koplas *et al.*, 1997; Vellani *et al.*, 2001). The CAPS-responsive cells in our experiments ranged from 20 to $40 \mu\text{m}$ in diameter. Infection with HSV vectors did not alter the proportion of neurons that responded to CAPS (Fig. 4F). Continuous application of CAPS ($0.5 \mu\text{M}$) in uninfected and vHG-infected neurons elicited currents of similar peak amplitude ranging from 50 pA to 10 nA (average -958 ± 190 pA, $n = 53$, in uninfected cells, $n = 23$; average -570 ± 220 pA in vHG cells, Fig. 4B and E). vHDNP-infected neurons exhibited CAPS-evoked currents that were significantly diminished ($P < 0.001$) in peak amplitude (average -347 ± 150 pA, $n = 30$, Fig. 4B and E) when compared with the CAPS currents in either uninfected cells or vHG-infected cells. The CAPS current densities measured as the area under the curve of currents during the first 10 min after CAPS administration were similar in control and vHG-infected cells but were significantly decreased (threefold reduction) in vHDNP-infected cells (Fig. 4C). Taken together, these data demonstrate that HSV-1 vector-expressed DNPKCε significantly attenuates CAPS-induced TRPV1 currents but does not produce any measurable changes in other electrophysiological properties of rat DRG neurons.

vHDNP alters the time course of the CAPS responses and responses to a PKC activator in DRG neurons

CAPS currents had a fast rising phase but began to decay slowly (i.e. desensitize) within 1–2 min after CAPS application (Fig. 4B and D). CAPS currents were fitted by a sum of one rising exponential time constant (τ_r) as well as a fast time constant (τ_f) and slow time constant (τ_s) exponential decay. The time constants τ_r , τ_f and τ_s were 0.22 ± 0.08 , 0.97 ± 0.09 and 7.6 ± 1.2 min in uninfected neurons ($n = 20$). In vHG-infected cells ($n = 9$), time constants τ_r and τ_f were slower (0.42 ± 0.06 min, $P < 0.001$, and 2.7 ± 1.4 min, $P < 0.05$) but τ_s was not different (4.6 ± 0.8 min, n.s.) when compared with uninfected neurons. In vHDNP-infected cells ($n = 8$; Fig. 6A and B) the rising phase was similar (0.29 ± 0.12 min, n.s.) to that seen in uninfected cells (0.22 ± 0.08 min) but both decaying components were faster (τ_f 0.32 ± 0.07 , $P < 0.001$; τ_s 2.7 ± 0.6 min, $P < 0.001$) when compared with uninfected neurons (0.97 ± 0.09 and 7.6 ± 1.2 min $P < 0.001$, Fig. 5E).

Application of PDBu alone ($0.5 \mu\text{M}$) did not induce membrane currents in uninfected neurons ($n = 5$) or after infection with either vHG ($n = 3$) or vHDNP ($n = 6$). However, in all three experimental groups ($n = 27$ uninfected cells; $n = 9$ vHG-infected cells and $n = 8$ vHDNP-infected cells, Fig. 5A, B and E) application of PDBu ($0.5 \mu\text{M}$) in the presence of CAPS enhanced the CAPS currents. These enhanced currents had slower time to peak (Fig. 5D) and desensitized more slowly than the currents induced by CAPS alone (Fig. 5E). CAPS- and PDBu-evoked currents were fully blocked by the TRPV1 antagonist diethyl piperazine (Sculptoreanu *et al.*, 2005a) in all three experimental groups (Fig. 5A and B).

The PDBu-induced increases in CAPS currents were similar in uninfected and vHG-infected neurons, but diminished ($42 \pm 5\%$ decrease, $n = 11$) in vHDNP-infected neurons (Fig. 5C). In addition, in vHDNP-infected neurons, the PDBu enhancement of CAPS currents exhibited an increased time to peak (Fig. 5D, > fourfold) and a large increase in the lag time (> sixfold) preceding the onset of the PDBu-facilitated CAPS current (Fig. 5A, inset). While the decay of currents evoked by CAPS alone was fitted by two exponentials, the decay of the PDBu-enhanced CAPS currents were better fitted by a single exponential (Fig. 5E). In vHDNP-infected neurons the time constant of decay of PDBu-enhanced CAPS currents (432 ± 60 s) was significantly greater (> 2.5-fold, $P < 0.001$) compared with that in uninfected (178 ± 16 s) and vHG-infected neurons (152 ± 34 s, Fig. 5E).

A prominent feature of the enhancement of CAPS currents by PDBu was a progressive diminution in the ability of PDBu to enhance the currents when administered at longer intervals after CAPS (Fig. 6C). The enhancement of CAPS currents by PDBu decayed exponentially with time after CAPS administration and this decay was more pronounced in vHDNP cells. The time constants for decay of responsiveness to PDBu were about five times faster in vHDNP neurons (4.5 ± 1.5 min for vHG vs. 0.85 ± 0.34 min for vHDNP neurons, $P < 0.001$). Moreover, in the initial 2–4 min after application of CAPS the administration of PDBu enhanced the currents two to fourfold in untreated and vHG neurons, while there was less than a 40% increase in the same time window in the vHDNP cells (Fig. 6A–C). At longer intervals (6–10 min) after CAPS administration, the increase in current induced by PDBu in vHDNP cells was also smaller than the PDBu enhancement in vHG cells (Fig. 6C). However, in vHG-infected neurons the fractional increase in CAPS current produced by PDBu (i.e. PDBu increase in current relative to basal current prior to PDBu application) actually increased with time whereas the opposite change occurred in vHDNP-infected cells (Fig. 6D).

Effect of PKC inhibitors (BIM, Gö6976) on CAPS responses and PDBu enhancement of CAPS responses

To test the idea that PKC ϵ may be responsible for the effect of PDBu on CAPS currents we used BIM (0.5 μ M), known for its ability to selectively inhibit conventional and atypical PKC subtypes, including PKC ϵ (Toullec *et al.*, 1991). Application of BIM (0.5 μ M) before CAPS ($n = 3$) reduced the peak CAPS-evoked responses by about 20% and decreased the time to peak and increased the desensitization rates of CAPS currents by approximately 30%. These changes resembled the effect of DNPKC ϵ on basal CAPS currents. BIM (0.5 μ M) applied after the initial rapid desensitization of CAPS currents had no measurable effect on the slow desensitization ($n = 6$).

The enhancement of CAPS currents by PDBu was rapidly reversed by BIM in all groups of cells: uninfected cells ($n = 4$), vHG-infected cells ($n = 5$) and vHDNP-infected cells ($n = 5$, Fig. 5B and E). After application of BIM, the time constants of decay of PDBu-enhanced CAPS currents fitted by a single exponential were similar in all groups of cells: uninfected cells (57 ± 1 s), vHG-infected cells (65 ± 14 s, n.s.) and vHDNP-infected cells (67 ± 1 s, n.s.; statistical comparisons between infected and uninfected cells) and only slightly larger (57 ± 1 s) than the value of the rapid decay of currents evoked by CAPS alone in uninfected cells (42 ± 12 s, $n = 57$, n.s.). These data suggest that BIM inhibits PKC equally in all cell groups and that the decline in current after BIM is due to dephosphorylation of TRPV1 by phosphatase activity that is unchanged by viral infection or the presence of DNPKC ϵ . Gö6976 (0.5 μ M), a PKC inhibitor that is selective for the conventional PKC subtypes, PKC α and PKC γ (Martiny-Baron *et al.*, 1993), had no effect on PDBu enhancement of CAPS currents in seven uninfected, three vHG-infected and four vHDNP-infected neurons (Fig. 5B). The effect of BIM in the absence of a measurable effect of Gö6976 in the same cells (Fig. 5E) constituted an internal control, which would offset differences due to an intrinsic variability in the time course of CAPS currents.

Effect of vHDNP on heat-induced PWL

Experiments in isolated neurons showed that vHDNP diminished the peak amplitudes and increased the desensitization of CAPS currents. Although these observations demonstrated that phosphorylation by PKC ϵ modulates TRPV1 activity, the question of whether dominant negative PKC ϵ performed a similar role *in vivo* remained to be explored. As the TRPV1 receptor is a known sensor of noxious heat (43°C), we carried out *in-vivo* tests to examine nociceptive responses of vector-inoculated rats following the application of intense heat. These experiments were based on previous studies using HSV gene vectors to control nociceptive behavior in rat models of pain (Glorioso & Fink, 2004; Mata *et al.*, 2004).

Young adult male Sprague–Dawley rats were subcutaneously injected with 100 μ L containing 108 pfu of vHG, vHDNP or PBS into the plantar surface of the right hind paw. Animals were placed on a temperature-controlled platform and tested for PWL following the application of an intense light source to the footpad. Figure 7A shows the PWL presented as ratios of the average time taken for withdrawal of the right vs. left foot for animals in each group. The PWL ratios in uninfected, PBS-injected control rats and vHG-infected rats were approximately 1, while vHDNP-infected rats displayed a small but significant increase in PWL ratio to 1.25 ($P < 0.05$, $n = 8$).

To examine the effect of vHDNP on CAPS-induced heat hyperalgesia, vector- or PBS-inoculated rats were injected with 10 g CAPS in 50 L vehicle. Thirty minutes following CAPS inoculation, PWL testing was performed on each rat. vHDNP-inoculated rats demonstrated a 2.5-fold increase in PWL as compared with PBS- or vHG-inoculated rats (P

< 0.001, Fig. 7B). These data demonstrate that the vHDNP vector effectively reduced heat hypersensitivity in a model of CAPS-induced acute inflammation in rats.

Discussion

The goals of this study were to develop a gene transfer system for DRG neurons that can be used to evaluate directly the role of PKC ϵ in the sensitization and other aspects of TRPV1 function. Our experimental approach involved the use of a defective HSV vector (vHDNP) capable of expressing a dominant negative form of PKC ϵ (DNPKC ϵ) that prevents normal PKC ϵ translocation (Zeidman *et al.*, 1999). DNPKC ϵ translocation to plasma membrane in response to phorbol esters inhibited endogenous PKC ϵ translocation. The mechanism by which the DNPKC ϵ is preferentially translocated to the plasma membrane over the wild-type PKC ϵ is unknown, but may involve binding to actin and competition with endogenous PKC ϵ for trafficking to the plasma membrane (Prekeris *et al.*, 1998; Csukai & Mochly-Rosen, 1999).

Cobalt uptake assay and whole-cell patch clamp methods were used to determine whether the inhibition of PKC ϵ translocation by DNPKC ϵ affected normal CAPS responses in DRG neurons. In the cobalt assay, vHDNP-infected neurons showed a reduced intensity of staining as well as a reduced number of cells positive for CAPS-induced cobalt uptake (15% of the vHDNP neurons vs. ~45% in uninfected and vHG infected neurons). However, the cobalt assay may have underestimated the number of positive cells in the vHDNP group if the lower intensity of staining did not reach the threshold for inclusion in the positive group.

Results from electrophysiological recordings showed that while the CAPS currents were markedly reduced in vHDNP-infected neurons, infection with vHG or vHDNP did not change the number of CAPS-responsive cells or alter the neuronal resting membrane potential, membrane resistance, membrane capacitance or the threshold and number of action potentials elicited by depolarizing current pulses. DNPKC ϵ hence did not alter neuronal excitability and did not eliminate but rather reduced by 20% the peak CAPS currents amplitude and decreased by threefold the area of the CAPS current during the first 10 min.

The dynamic interplay between receptor phosphorylation and de-phosphorylation by kinases and phosphatases is thought to modulate TRPV1 function by altering the affinity and the rate of desensitization in the continuous presence of agonists (Mandadi *et al.*, 2004). Expression of DNPKC ϵ resulted in CAPS currents that had faster time to peak and desensitized faster than currents in uninfected neurons or vHG-infected neurons. The effects of DNPKC ϵ on CAPS currents provide evidence that PKC ϵ modulates basal TRPV1 responses to CAPS. BIM and DNPKC ϵ had comparable effects on basal CAPS currents. Notably, BIM pretreatment led to faster time to peak in uninfected cells (19 ± 7 s after BIM vs. 31 ± 3 s in control neurons) comparable with the time to peak (24 ± 3 s, n.s.) in vHDNP-infected neurons. BIM pretreatment in uninfected cells also led to more rapid desensitization ($\tau_{D,fast}$: 30 ± 5 s vs. $\tau_{D,fast}$: 41 ± 3 s in control cells) as noted in vHDNP-infected cells (18 ± 1 s). Therefore, it appears that basal PKC phosphorylation maintains TRPV1 in a partially phosphorylated state and DNPKC ϵ inhibits this basal phosphorylation in a manner similar to BIM (Sculptoreanu *et al.*, 2008). In the presence of BIM or DNPKC ϵ the level of TRPV1 phosphorylation presumably declines due to dephosphorylation of TRPV1 by calcineurin, a Ca²⁺-dependent phosphatase (Docherty *et al.*, 1996). As PKC ϵ phosphorylation of TRPV1 induced by PDBu slows the onset and rate of desensitization of CAPS responses (Mandadi *et al.*, 2004), the present results showing the opposite effect with DNPKC ϵ and BIM are consistent with the idea that basal or constitutively active PKC ϵ modulates normal TRPV1 responses to CAPS.

DNPKC ϵ also altered the responses to PDBu. When PDBu was applied after partial desensitization of CAPS currents, it elicited multiple effects, including a facilitation of the CAPS currents and a slowing of desensitization. The PDBu-enhanced CAPS currents were slower in onset than currents evoked by CAPS alone and had times to peak that were more than double those of control currents (Fig. 5A, inset). It is clear that the currents evoked by PDBu were due to facilitation of TRPV1 channels because PDBu alone had no effect and the PDBu-induced currents in the presence of CAPS were blocked by a TRPV1 antagonist. Activation of PKC ϵ phosphorylation must be responsible for the effects of PDBu because they were readily reversed by a PKC ϵ inhibitor, BIM, but not Gö6976, an inhibitor of PKC α and PKC β .

The facilitating effect of PDBu might be mediated by: (i) re-sensitization and opening of partially desensitized channels or (ii) opening of silent channels that were not activated by the low concentrations of CAPS used in our experiments. The silent channels with low affinity for CAPS may only open after application of PDBu and PKC-mediated phosphorylation, which is known to increase the affinity for CAPS (Koplas *et al.*, 1997; Premkumar & Ahern, 2000). The facilitatory effect of PDBu was similar in uninfected and vHG cells, suggesting that viral infection alone, without the vHDNP construct, did not alter the ability of PDBu to activate PKC and to modulate TRPV1 behavior. However, in DNPKC ϵ -expressing neurons the PDBu-enhanced CAPS currents were delayed in onset, and decreased in amplitude. These effects are consistent with an inhibition of PKC ϵ translocation (Prekeris *et al.*, 1998) and an inhibition of TRPV1 phosphorylation. The latter effect may be due to competition between DNPKC ϵ and the translocated native PKC ϵ for endpoint localization which is responsible in part for the selectivity of PKC ϵ for TRPV1 channels. This may include binding of PKC ϵ to the anchoring protein RACK (receptors for activated protein kinase C) in that it is associated with TRPV1 in a multi-protein complex or transducisome (Csukai & Mochly-Rosen, 1999).

In vHDNP-infected neurons the PDBu-evoked TRPV1 currents were not only reduced in amplitude but they also desensitized more slowly than in uninfected neurons. This slower rate of desensitization in vHDNP-infected neurons may be related to a reduction in Ca²⁺ influx and reduced activation of calcineurin during PDBu application. Reduced calcineurin activity would in turn decrease the rate of dephosphorylation of TRPV1 channels and thereby prolong the currents.

When PDBu was applied within 1–2 min after CAPS, the amplitude of the PDBu-evoked currents was large (Fig. 6) but the currents were significantly reduced when PDBu was applied at longer intervals after CAPS. This time-dependent decline in the peak PDBu responses which occurred in both uninfected, vHG- and vHDNP-infected neurons during the CAPS desensitization may reflect a slow conversion of activated TRPV1 channels into an inactive state that cannot be reactivated by PDBu-induced phosphorylation. Activation of silent channels might also have an impact on the time-dependent decline in PDBu responses and contribute to differences in responses in vHDNP-infected and uninfected neurons. In uninfected and vHG-infected neurons the percentage change in current evoked by PDBu increased at longer intervals after CAPS, even as the absolute magnitude of the current declined (Fig. 6). This is consistent with PDBu opening silent TRPV1 channels that initially did not respond to CAPS. These channels must represent a greater proportion of the total number of channels available for opening during the later stages of CAPS desensitization. Conversely, in neurons infected with vHDNP the percentage increase in current evoked by PDBu significantly declined with time (Fig. 6), suggesting that a suppression of basal phosphorylation of TRPV1 by DNPK ϵ promotes the transition of both silent and activated TRPV1 channels to an inactive state that is unresponsive to PDBu.

Although our results do not take into account the contribution of TRPV1 trafficking to the effect of vHDNP, typically TRP protein trafficking requires membrane-bound vesicles and takes place on a time scale of several minutes rather than that of tens of seconds that we report here (Lockwich *et al.*, 2000; Tsui-Pierchala *et al.*, 2002; Liu *et al.*, 2006). Therefore, while not examined in detail in our experiments, trafficking presumably contributed little or nothing to our measurements.

The association between TRPV1 function and PKC ϵ activation has been explored using PKC ϵ knockout mice (Khasar *et al.*, 1999) or intrathecal injection of the C2 domain derived peptide (V1–2; Sweitzer *et al.*, 2004). Amadesi *et al.* (2004) showed that a transient inhibition of PKC ϵ resulted in increased paw withdrawal latency in response to noxious heat, suggesting a pro-nociceptive role for TRPV1 (Bolcskei *et al.*, 2005). Although it is clear from these studies that PKC activity is correlated with nociceptive responses, a direct functional relationship between PKC ϵ and TRPV1 sensitization has not been established.

Rat PWL responses were measured following an application of noxious heat to the rat footpad. A delay of withdrawal responses was observed in vHDNP when compared with uninfected and vHG-infected rats. Vector-inoculated vHDNP rats injected subsequently with 10 g of CAPS 30 min prior to testing PWL responses exhibited edema of footpads, suggesting that in the presence of PKC ϵ inhibition, CAPS activates TRPV1 and release of pro-inflammatory substance P (SP) and calcitonin gene-related peptide (CGRP).

However, PWL times for vHDNP-inoculated rats in response to heat were significantly increased (twofold) as compared with controls. In one possible scenario, the analgesia may be mediated by an effect of the DNPKC ϵ transgene on TRPV1 at the central terminals of DRG neurons, leading to decrease in neurotransmitter release at synapses in the dorsal horn. DNPKC ϵ could be acting both at the axon terminals in the periphery and the synapses in the dorsal horn. Further studies will be required to sort out these two possibilities. In summary, our approach can be applied to studies of G-protein coupled mechanisms that modulate TRPV1 function. Moreover, recent findings (Srinivasan *et al.*, 2007) using HSV vectors indicate that the virus can be used in genomic approaches to discover other modulators of TRPV1 activity.

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Abbreviations

BIM	bisindolylmaleimide I HCl
CAPS	capsaicin
DNPKC	dominant negative PKC ϵ
DRG	dorsal root ganglion
hpi	hours post-infection
HSV	herpes simplex virus
ICP	infected cell protein

IE	immediate early
PDBu	phorbol 12, 13-dibutyrate
PKCϵ	protein kinase C epsilon
PMA	phorbolmyristate acetate
PWL	paw withdrawal latency
RP	resting potential
TRPV1	vanilloid receptor

References

- Amadesi S, Nie J, Vergnolle N, Cottrell GS, Grady EF, Trevisani M, Manni C, Geppetti P, McRoberts JA, Ennes H, Davis JB, Mayer EA, Bunnett NW. Protease-activated receptor 2 sensitizes the capsaicin receptor transient receptor potential vanilloid receptor 1 to induce hyperalgesia. *J. Neurosci.* 2004; 24:4300–4312. [PubMed: 15128844]
- Bhave G, Hu HJ, Glauner KS, Zhu W, Wang H, Brasier DJ, Oxford GS, Gereau RW iv. Protein kinase C phosphorylation sensitizes but does not activate the capsaicin receptor transient receptor potential vanilloid 1 (TRPV1). *Proc. Natl Acad. Sci. USA.* 2003; 100:12480–12485. [PubMed: 14523239]
- Bolcskei K, Helyes Z, Szabo A, Sandor K, Elekes K, Nemeth J, Almasi R, Pinter E, Petho G, Szolcsanyi J. Investigation of the role of TRPV1 receptors in acute and chronic nociceptive processes using gene-deficient mice. *Pain.* 2005; 117:368–376. [PubMed: 16150543]
- Cesare P, Dekker LV, Sardini A, Parker PJ, McNaughton PA. Specific involvement of PKC-epsilon in sensitization of the neuronal response to painful heat. *Neuron.* 1999; 23:617–624. [PubMed: 10433272]
- Chuang HH, Prescott ED, Kong H, Shields S, Jordt SE, Basbaum AI, Chao MV, Julius D. Bradykinin and nerve growth factor release the capsaicin receptor from PtdIns(4,5)P2-mediated inhibition. *Nature.* 2001; 411:957–962. [PubMed: 11418861]
- Csukai M, Mochly-Rosen D. Pharmacologic modulation of protein kinase C isozymes: the role of RACKs and subcellular localisation. *Pharmacol. Res.* 1999; 39:253–259. [PubMed: 10208754]
- Docherty RJ, Yeats JC, Bevan S, Boddeke HW. Inhibition of calcineurin inhibits the desensitization of capsaicin-evoked currents in cultured dorsal root ganglion neurones from adult rats. *Pflugers Arch.* 1996; 431:828–837. [PubMed: 8927498]
- Glantz, SA. Primer of biostatistics. New York: McGraw-Hill Medical Pub. Division; 2005.
- Glorioso JC, Fink DJ. Herpes vector-mediated gene transfer in treatment of diseases of the nervous system. *Annu. Rev. Microbiol.* 2004; 58:253–271. [PubMed: 15487938]
- Goins WF, Krisky DM, Wolfe DP, Fink DJ, Glorioso JC. Development of replication-defective herpes simplex virus vectors. *Methods Mol. Med.* 2002; 69:481–507. [PubMed: 11987796]
- Hong S, Wiley JW. Early painful diabetic neuropathy is associated with differential changes in the expression and function of vanilloid receptor 1. *J. Biol. Chem.* 2005; 280:618–627. [PubMed: 15513920]
- Hu-Tsai M, Winter J, Woolf CJ. Regional differences in the distribution of capsaicin-sensitive target-identified adult rat dorsal root ganglion neurons. *Neurosci. Lett.* 1992; 143:251–254. [PubMed: 1279477]
- Khasar SG, Lin YH, Martin A, Dadgar J, McMahon T, Wang D, Hundle B, Aley KO, Isenberg W, McCarter G, Green PG, Hodge CW, Levine JD, Messing RO. A novel nociceptor signaling pathway revealed in protein kinase C epsilon mutant mice. *Neuron.* 1999; 24:253–260. [PubMed: 10677042]
- Koplas PA, Rosenberg RL, Oxford GS. The role of calcium in the desensitization of capsaicin responses in rat dorsal root ganglion neurons. *J. Neurosci.* 1997; 17:3525–3537. [PubMed: 9133377]

- Liu M, Huang W, Wu D, Priestley JV. TRPV1, but not P2X, requires cholesterol for its function and membrane expression in rat nociceptors. *Eur. J. Neurosci.* 2006; 24:1–6. [PubMed: 16800863]
- Lockwich TP, Liu X, Singh BB, Jadowiec J, Weiland S, Ambudkar IS. Assembly of Trp1 in a signaling complex associated with caveolin-scaffolding lipid raft domains. *J. Biol. Chem.* 2000; 275:11934–11942. [PubMed: 10766822]
- Mandadi S, Numazaki M, Tominaga M, Bhat MB, Armati PJ, Roufogalis BD. Activation of protein kinase C reverses capsaicin-induced calcium-dependent desensitization of TRPV1 ion channels. *Cell Calcium.* 2004; 35:471–478. [PubMed: 15003856]
- Mandadi S, Tominaga T, Numazaki M, Murayama N, Saito N, Armati PJ, Roufogalis BD, Tominaga M. Increased sensitivity of desensitized TRPV1 by PMA occurs through PKCepsilon-mediated phosphorylation at S800. *Pain.* 2006; 123:106–116. [PubMed: 16564619]
- Marconi P, Krisky D, Oligino T, Poliani PL, Ramakrishnan R, Goins WF, Fink DJ, Glorioso JC. Replication-defective herpes simplex virus vectors for gene transfer *in vivo*. *Proc. Natl Acad. Sci. USA.* 1996; 93:11319–11320. [PubMed: 8876133]
- Martiny-Baron G, Kazanietz MG, Mischak H, Blumberg PM, Kochs G, Hug H, Marme D, Schachtele C. Selective inhibition of protein kinase C isozymes by the indolocarbazole Go 6976. *J. Biol. Chem.* 1993; 268:9194–9197. [PubMed: 8486620]
- Mata M, Glorioso JC, Fink DJ. Gene therapy: novel treatments for polyneuropathy and chronic pain. *Curr. Neurol. Neurosci. Rep.* 2004; 4:1–2. [PubMed: 14683620]
- Numazaki M, Tominaga T, Toyooka H, Tominaga M. Direct phosphorylation of capsaicin receptor VR1 by protein kinase Cepsilon and identification of two target serine residues. *J. Biol. Chem.* 2002; 277:13375–13378. [PubMed: 11884385]
- Pingle SC, Matta JA, Ahern GP. Capsaicin receptor: TRPV1 a promiscuous TRP channel. *Handb. Exp. Pharmacol.* 2007; 179:155–171. [PubMed: 17217056]
- Piper AS, Yeats JC, Bevan S, Docherty RJ. A study of the voltage dependence of capsaicin-activated membrane currents in rat sensory neurones before and after acute desensitization. *J. Physiol.* 1999; 518:721–733. [PubMed: 10420009]
- Plant TD, Zollner C, Mousa SA, Oksche A. Endothelin-1 potentiates capsaicin-induced TRPV1 currents via the endothelin A receptor. *Exp. Biol. Med.* 2006; 231:1161–1164.
- Prekeris R, Hernandez RM, Mayhew MW, White MK, Terrian DM. Molecular analysis of the interactions between protein kinase C-epsilon and filamentous actin. *J. Biol. Chem.* 1998; 273:26790–26798. [PubMed: 9756923]
- Premkumar L, Ahern G. Induction of vanilloid receptor channel activity by protein kinase C. *Nature.* 2000; 408:985–990. [PubMed: 11140687]
- Sathianathan V, Avelino A, Charrua A, Santha P, Matesz K, Cruz F, Nagy I. Insulin induces cobalt uptake in a subpopulation of rat cultured primary sensory neurons. *Eur. J. Neurosci.* 2003; 18:2477–2486. [PubMed: 14622148]
- Sculptoreanu A, de Groat WC. Protein kinase C is involved in neurokinin receptor modulation of N- and L-type Ca²⁺ channels in DRG neurons of the adult rat. *J. Neurophysiol.* 2003; 90:21–31. [PubMed: 12660348]
- Sculptoreanu A, de Groat WC. Neurokinins enhance excitability in capsaicin-responsive DRG neurons. *Exp. Neurol.* 2007; 205:92–100. [PubMed: 17362934]
- Sculptoreanu A, de Groat WC, Buffington CA, Birder LA. Abnormal excitability in capsaicin-responsive DRG neurons from cats with feline interstitial cystitis. *Exp. Neurol.* 2005a; 193:437–443. [PubMed: 15869946]
- Sculptoreanu A, de Groat WC, Buffington CA, Birder LA. Protein kinase C contributes to abnormal capsaicin responses in DRG neurons from cats with feline interstitial cystitis. *Neurosci. Lett.* 2005b; 381:42–46. [PubMed: 15882787]
- Sculptoreanu A, Kullmann FA, de Groat WC. Neurokinin 2 receptor mediated activation of protein kinase C modulates capsaicin responses in DRG neurons from adult rats. *Eur. J. Neurosci.* 2008; 27:3171–3181. [PubMed: 18598261]
- Srinivasan R, Huang S, Chaudhry S, Sculptoreanu A, Krisky D, Cascio M, Friedman PA, de Groat WC, Wolfe D, Glorioso JC. An HSV vector system for selection of ligand-gated ion channel modulators. *Nat. Methods.* 2007; 4:733–739. [PubMed: 17676048]

- Sweitzer SM, Wong SM, Peters MC, Mochly-Rosen D, Yeomans DC, Kendig JJ. Protein kinase C epsilon and gamma: involvement in formalin-induced nociception in neonatal rats. *J. Pharmacol. Exp. Ther.* 2004; 309:616–625. [PubMed: 14762097]
- Toullec D, Pianetti P, Coste H, Bellevergue P, Grand-Perret T, Ajakane M, Baudet V, Boissin P, Boursier E, Loriolle F, Duhamell L, Charon D, Kirilovsky J. The bisindolylmaleimide GF 109203X is a potent and selective inhibitor of protein kinase C. *J. Biol. Chem.* 1991; 266:15771–15781. [PubMed: 1874734]
- Tsui-Pierchala BA, Encinas M, Milbrandt J, Johnson EM Jr. Lipid rafts in neuronal signaling and function. *Trends Neurosci.* 2002; 25:412–417. [PubMed: 12127758]
- Vellani V, Mapplebeck S, Moriondo A, Davis JB, McNaughton PA. Protein kinase C activation potentiates gating of the vanilloid receptor VR1 by capsaicin, protons, heat and anandamide. *J. Physiol.* 2001; 534:813–825. [PubMed: 11483711]
- Zeidman R, Lofgren B, Pahlman S, Larsson C. PKC ϵ , via its regulatory domain and independently of its catalytic domain, induces neurite-like processes in neuroblastoma cells. *J. Cell Biol.* 1999; 145:713–726. [PubMed: 10330401]

FIG. 1.

Construction and characterization of dominant-negative PKC ϵ -expressing vector. (A) Full-length PKC ϵ structure and functional domains. (B) DNPKE retains the pseudo-substrate region (PS) and the C1a domain that contains the actin binding site and the variable region (V3); to this GFP was fused to allow identification of DNPKE-expressing cells. (C) The DNPKE gene was inserted into the UL41 locus of the replication defective HSV vector to produce the recombinant vHDNP and a reporter gene vector vHG expressing GFP from the same promoter that was used as a control for all experiments. (D) Western blot using a GFP-specific antibody that detected a 47-kDa protein in vHDNP-infected U2OS cells (multiplicity of infection of 3 at 16 hpi) and a 27-kDa protein in vHG-infected U2OS cells. (E) In vHDNP-infected cells, the DNPKE product rapidly translocated to the plasma membrane from the perinuclear region in response to phorbolmyristate acetate (PMA). Control vector (vHG)-infected cells displayed a uniform expression of GFP in the cytoplasm and its localization was unaffected by PMA treatment. Scale bars as shown in E.

FIG. 2.

DNPKCε inhibits endogenous PKCε translocation in rat DRG neurons. GFP was used to identify neurons infected with either a vHG construct (A–F) or a vHDNP construct (G–L). A red fluorescent Cy3 secondary antibody was used to visualize endogenous PKCε. vHG-transduced neurons (A–F) displayed a diffuse pattern of GFP localization which was unaffected by stimulation with PDBu (D–F). Endogenous PKCε translocated to the plasma membrane when stimulated with PDBu (B and E). The merged images do not display co-localization of GFP and endogenous PKCε (C and F). (G–L) After PDBu treatment in vHDNP-infected neurons, DNPKCε translocated to the plasma membrane to a greater extent than endogenous PKCε (H and K). (L) The merged images show that after PDBu, the DNPKCε and PKCε products were also co-localized at plasma membrane to a lesser extent

than the co-localization of the two proteins in the cytoplasm in the absence of PDBu, when they co-localized extensively (I). Scale bars as shown in C, F, I and L.

FIG. 3.

Cobalt uptake in response to CAPS was used to determine the number of CAPS-responsive neurons in DRG cultures infected with vHDNP construct, with vHG construct and in noninfected neurons. (A) The infection parameters were set to give nearly 100% efficiency. (B) Representative micrographs of a TRPV1-negative neuron with no cobalt uptake and of noninfected, vHG-infected and vHDNP-infected CAPS-responsive neurons that turn various shades of gray to black after cobalt uptake. (C) The percentage of CAPS-sensitive neurons revealed by cobalt uptake is shown for uninfected, vHG- and vHDNP-infected neurons. The numbers of neurons (N) are shown in parentheses. Approximately 44% of noninfected neurons were CAPS-responsive, while 33% of vHG-infected neurons demonstrated CAPS

sensitivity. By contrast, only 12% of the vHDNP-infected neurons were CAPS-sensitive. This was a significant reduction when compared with the number of CAPS-sensitive vHG-infected neurons ($P < 0.001$). Scale bars, 20 μm in all panels on A and B.

FIG. 4.

Infection of DRG neurons with a virus containing the DNPKC ϵ construct (vHDNP) reduces the amplitude and increases the rate of desensitization of CAPS-induced currents but does not alter depolarization-evoked firing. (A) Firing in response to short (5 ms, first action potential in the sequence) or long (600 ms, second action potential in the sequence) depolarizing pulses is not altered by infection with either vHG or vHDNP. (B) Time course of average CAPS (0.5 μ M) currents expressed as current densities (pA/pF) in 53 uninfected (control), 23 vHG-infected and 30 vHDNP-infected neurons. CAPS application indicated by the horizontal line above the records was present throughout the duration of the experiment. Dotted lines are best fits of average current densities with a sum of one rising and two

decaying exponentials as shown above control curve (see Methods). Note that in vHG cells currents desensitize more slowly than in control (con) cells while in vHDNP neurons the currents are significantly reduced in amplitude and desensitize more rapidly. Number of cells in each group shown in parentheses. (C) Area of current densities during the first 10 min of records in B in control, vHG and vHDNP cells. Note that the area under the vHG curve increases despite the apparent reduction of peak amplitudes, whereas the area under the vHDNP curve is significantly decreased (threefold reduction). (D) Summary of fitted time course of CAPS currents shown in B, on an expanded time scale and scaled to nearly match the amplitude of control current densities for ease of comparison. Solid line above recordings indicates that CAPS was present throughout the records. Note the slow desensitization in vHG cells and more rapid desensitization in vHDNP neurons. (E) Average peak current density in uninfected cells (control) and vHG-infected cells was reduced in vHDNP-infected cells. (F) The percentage of CAPS-responsive cells was not altered by infection with either vHG or vHDNP. Data in E and F are averages $\pm$ SEM, for the number of cells shown in parentheses. Comparison was considered statistically significant if $P \leq 0.05$ (*); a one-way analysis of variance was first carried out followed by a post-hoc comparisons between the different groups using the Holm-Sidak test as described in the Methods (for $P < 0.05$, a two-tailed t -test, unequal variance was used, $P < 0.001$).

FIG. 5.

Comparison of CAPS-evoked currents and enhancement of CAPS currents by PDBu in vHG- and vHDNP-infected DRG neurons. (A) Infection of DRG neurons with vHDNP-construct led to a CAPS response that reached maximum faster, was considerably smaller in amplitude and desensitized faster than response to CAPS in vHG cells. PDBu enhanced the partially desensitized CAPS currents in both types of cells but in DNPKC ϵ -expressing cells, the lag of the response to PDBu (inset) was larger and the time to peak considerably slower than in vHG-infected cells. TRPV1 antagonist (5 μ M) blocked the PDBu-enhanced CAPS currents in both cell types. Solid line above recordings indicates that CAPS was present throughout the recordings. (B) BIM, a broad-range PKC inhibitor, suppressed the PDBu effect in both vHG and vHDNP cells. However, Gö6976, a PKC inhibitor selective for the conventional PKC subtypes but with little effect on PKC ϵ , did not alter the amplitude or the rate of desensitization of the PDBu-enhanced CAPS currents in all the cell types tested. Current trace in vHDNP cells shows lack of Gö6976 effect. Solid line above recordings indicates that CAPS was present throughout the records. Drug concentrations (in μ M): CAPS, 0.5, PDBu, 0.5; BIM, 0.5; Gö6976, 0.5; TRPV1Ant, 5. (C) PDBu administered in the

presence of CAPS, 1–16 min after CAPS, markedly enhanced the currents in uninfected ($168 \pm 84\%$ increase, $n = 11$) and vHG-infected ($144 \pm 84\%$ increase, $n = 12$) neurons. However, vHDNP infection significantly diminished the effect of PDBu on CAPS-evoked currents ($97 \pm 79\%$ increase, $n = 23$). (D) Infection with vHG or vHDNP had no measurable effect on the time to peak of CAPS-evoked currents. However, vHDNP slowed the time to peak of PDBu-induced currents in the presence of CAPS. (E) Compared with uninfected (con) cells, infection with vHG construct slowed the rapid desensitization (τ_{fast}) and accelerated the slow desensitization (τ_{slow}) of CAPS currents. Infection with vHDNP construct accelerated both the rapid and slow desensitization of CAPS-evoked currents. Infection with vHG had no effect on the desensitization of PDBu-induced currents in the presence of CAPS; however, vHDNP significantly increased the time constant of desensitization of PDBu-induced currents in the presence of CAPS. Data in D and E are averages $\pm$ SEM, for the number of cells shown in parentheses. Comparison was considered statistically significant if $P \leq 0.05$ (*); a one-way analysis of variance was first carried out followed by post-hoc comparisons between the different groups using the Holm-Sidak test as described in the Methods (for $P < 0.05$, a two-tailed t -test, unequal variance was used, ** $P < 0.001$).

FIG. 6.

The responsiveness of CAPS-activated currents to the facilitatory effect of PDBu decays during the desensitization of CAPS-evoked currents. CAPS currents were normalized to the peak and averaged in nine vHG (A) cells and eight vHDNP (B) cells (cross hairs, x). Dotted lines were fitted to the average normalized currents by a sum of one rising and two decaying exponentials as shown above traces. (C) Graphs showing the decline in the responsiveness to PDBu measured as a decrease in the peak PDBu-evoked current (normalized to peak CAPS response) when PDBu was applied at various times after the start of CAPS application and during the desensitization of CAPS currents in the continuous presence of CAPS, as illustrated in Fig. 5. Data are fitted by a single exponential curve as shown above graphs using nonlinear regression analysis available in Sigma Plot. The time constants for decay were 4.5 ± 1.5 min for vHG cells and 0.85 ± 0.34 min for vHDNP neurons. (D) Percentage increase in CAPS current by PDBu relative to the amplitude of CAPS-evoked

current at the time of PDBu application plotted against peak time of response for the initial three blocks of pooled data shown as symbols in A and B. Drug concentrations (in μM): CAPS, 0.5; PDBu, 0.5.

FIG. 7.

Effect of vHDNP on paw withdrawal latencies (PWL). PWL is presented as ratios of the average time taken for withdrawal of the right vs. left foot for animals in each group. (A) The PWL ratios in uninfected (PBS injected) control rats and vHG-infected rats were approximately 1, while vHDNP-infected rats displayed a small but significant increase in PWL ratio to 1.25 ($*P < 0.05$, $n = 8$). (B) Effect of vHDNP on CAPS-induced heat hyperalgesia. vHDNP-inoculated rats had a 2.5-fold increase in PWL as compared with PBS- or vHG-infected rats ($*P < 0.001$).

TABLE 1

Passive membrane properties and firing parameters in phasic firing neurons

Parameter	Uninfected (<i>n</i>)	vHG-infected (<i>n</i>)	vHDNP-infected (<i>n</i>)
Cm (pF)	53.5 ± 2.3* (140)	54.9 ± 2.6 ^{NS} (43)	56.9 ± 3.3 ^{NS} (50)
Rm (MΩ)	219.1 ± 12.1* (135)	166.1 ± 14.1 ^{NS} (23)	192.2 ± 21.9 ^{NS} (31)
RP (mV)	−53.6 ± 0.9* (65)	−50.9 ± 1.1 ^{NS} (23)	−50.8 ± 1.1 ^{NS} (31)
APs/600 ms	2.9 ± 0.3* (62)	2.8 ± 0.3 ^{NS} (31)	3.2 ± 0.6 ^{NS} (36)

Data are presented as means ± SEM. Cm, membrane capacitance; Rm, membrane resistance; RP, resting potential; NS, not significant. The number of action potentials (AP) was determined for stimulus intensities 50–200 pA in intensity and 600 ms long that gave maximum firing.

* $P < 0.05$, a one-way analysis of variance was first carried out followed by a *post hoc* comparisons between the different groups using the Holm-Sidak test as described in the Methods.