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Continuous δ-Opioid Receptor Activation Reduces Neuronal Voltage-Gated Sodium Channel ($Na_V1.7$) Levels through Activation of Protein Kinase C in Painful Diabetic Neuropathy

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The $Na_V1.7$ tetrodotoxin-sensitive voltage-gated sodium channel isoform plays a critical role in nociception. In rodent models of diabetic neuropathy, increased $Na_V1.7$ in dorsal root ganglia (DRG) neurons correlates with the emergence of pain-related behaviors characteristic of painful diabetic neuropathy (PDN). We examined the effect of transgene-mediated expression of enkephalin on pain-related behaviors and their biochemical correlates in DRG neurons. Transfection of DRG neurons by subcutaneous inoculation of a herpes simplex virus-based vector expressing proenkephalin reversed nocisponsive behavioral responses to heat, cold, and mechanical pressure characteristic of PDN. Vector-mediated enkephalin production *in vivo* prevented the increase in DRG $Na_V1.7$ observed in PDN, an effect that correlated with inhibition of phosphorylation of p38 MAPK (mitogen-activated protein kinase) and protein kinase C (PKC). Primary DRG neurons *in vitro* exposed to 45 mM glucose for 18 h also demonstrated an increase in $Na_V1.7$ and increased phosphorylation of p38 and PKC; these changes were prevented by transfection *in vitro* with the enkephalin-expressing vector. The effect of hyperglycemia on $Na_V1.7$ production *in vitro* was mimicked by exposure to PMA and blocked by the myristolated PKC inhibitor 20-28 or the p38 inhibitor SB202190 [4-(4-fluorophenyl)-2-(4-hydroxyphenyl)-5-(4-pyridyl)1*H*-imidazole]; the effect of vector-mediated enkephalin on $Na_V1.7$ levels was prevented by naltrindole. The results of these studies suggest that activation of the presynaptic δ-opioid receptor by enkephalin prevents the increase in neuronal $Na_V1.7$ in DRG through inhibition of PKC and p38. These results establish a novel interaction between the δ-opioid receptor and voltage-gated sodium channels.

Key words: **pain; diabetic neuropathy; sodium channel; gene therapy; herpes simplex; enkephalins**

Introduction

The tetrodotoxin-sensitive voltage-gated sodium channel $Na_V1.7$ expressed in nociceptive neurons of the dorsal root ganglia (DRG) plays a critical role in the perception of pain. Expression of $Na_V1.7$ is increased in rats with carrageenan-induced inflammatory pain (Black et al., 2004), and transgenic mice with a selective knock-out of $Na_V1.7$ expression in DRG neurons abolished pain behaviors evoked by a range of stimuli including formalin, carrageenan, and nerve growth factor (Nassar et al., 2004). Mutations in the *SCN9A* gene coding for $Na_V1.7$ have been identified in patients with the inherited painful neuropathy erythromelalgia (Yang et al., 2004; Dib-Hajj et al., 2005; Choi et al., 2006) and in the dominantly inherited paroxysmal extreme pain disorder (Fertleman et al., 2006). Mutations in *SCN9A* that lead to loss of $Na_V1.7$ function in nerve result in a syndrome of congenital inability to experience pain (Cox et al., 2006). Together, these results suggest that $Na_V1.7$ plays a crucial role in setting the gain on pain signaling at the level of the primary nociceptive afferent (Waxman, 2006).

Distal symmetric sensorimotor polyneuropathy is a common complication of diabetes mellitus, affecting up to 50% of patients with diabetes (Tesfaye et al., 1996). A not insubstantial fraction of patients with diabetic polyneuropathy suffer from chronic neuropathic pain, a syndrome that has been termed painful diabetic neuropathy (PDN) (Galer et al., 2000), and one that imposes a substantial burden on individuals and on society (Gore et al., 2006). Although the etiology of pain in PDN is not fully understood, an increase in the amount of $Na_V1.7$ in DRG has been described in rats with streptozotocin (STZ)-induced diabetes and lowered thresholds to mechanical and thermal stimuli (Hong et al., 2004).

We and others have previously observed that continuous expression and release of enkephalin from DRG neurons achieved by subcutaneous inoculation of a nonreplicating herpes simplex virus (HSV)-based vector containing the human proenkephalin (PE) gene can be used to reduce nocisponsive behaviors in rodent models of inflammatory (Goss et al., 2001) and neuropathic (Hao et al., 2003) pain. In the current set of studies, we investigated the

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effect of HSV-mediated enkephalin expression on nocisponsive behaviors in rats with STZ-induced diabetes. We found that continuous production of enkephalin reduced pain-related behaviors in these animals, but unexpectedly we also observed that transgene-mediated enkephalin release resulted in a reduction in the amount of $Na_V1.7$ protein in primary sensory afferents in the DRG. This previously undescribed regulation of voltage-gated sodium channels in DRG by presynaptic δ-opioid receptors (DORs) acting through protein kinase C (PKC) and p38 mitogen-activated protein kinase (MAPK) is a novel finding with important implications for the treatment of painful diabetic neuropathy.

Materials and Methods

Vector construct

The recombinant replication-deficient HSV-based vector vE (also referred to as vector SHPE) has been described previously (Goss et al., 2001). The backbone of this vector is the ICP4-deleted HSV recombinant d120k-lox, with a cassette containing the human cytomegalovirus immediate early promoter (HCMV IEp), the SV40 intron, the human PE cDNA sequence, and the SV 40 polyadenylation sequence inserted into the tk locus (Goss et al., 2001). The control vector vZ (also referred to as SHZ) was identical to vE except that the inserted cassette contained the *Escherichia coli lacZ* reporter gene under the control of the HCMV IEp in the HSV tk locus.

Animal studies

Experiments were conducted on young adult male rats weighing 200–250 g at the start of the experiment, and were in compliance with approved institutional animal care and use protocols. Rats were rendered diabetic by injection of STZ (50 mg/kg, i.p.). The blood glucose were measured at 2 weeks after STZ administration, and diabetic rats with blood glucose >300 mg/dl were taken for further studies. At 2 weeks after STZ, groups of 10 rats were injected with 30 μl containing 1×10^7 plaque-forming units of the HSV-based vector expressing PE (vE), the control vector expressing *lacZ* (vZ), or vehicle alone subcutaneously into both hind feet. Ten animals that did not receive STZ served as normal controls. All analyses were performed by an observer blinded to the treatment group 4 weeks after vector inoculation (at 6 weeks of diabetes). A separate group of rats were injected in a similar manner with vE and killed by perfusion 7 d later for expression studies.

Behavioral studies

Thermal hyperalgesia. The latency to hindpaw withdrawal from a thermal stimulus was determined by exposing the plantar surface of the hindpaw to radiant heat using a modified Hargreaves thermal testing device (Hargreaves et al., 1988). Rats were placed in individual enclosures on a glass plate maintained at 30°C, and after a 30 min habituation period, the plantar surface of the paw was exposed to a beam of radiant heat applied through the glass floor. Activation of the bulb simultaneously activated a timer, and both were immediately turned off by paw withdrawal or at the 20 s cutoff time. Testing was performed by a blinded observer in triplicate at 5 min intervals.

Mechanical hyperalgesia. Mechanical nociceptive thresholds were assessed using an analgesimeter (Ugo Basile) as described by Randall and Selitto (1957). A linearly increasing pressure was applied through a cone-shaped plastic tip (diameter = 1 mm) onto the dorsal surface of the hindpaw. The tip was positioned between the third and fourth metatarsus, and force was applied until the rat attempted to withdraw its paw (paw withdrawal threshold to pressure). The pain threshold determined as the mean of three consecutive stable values, expressed in grams, was determined by a blinded observer.

Cold allodynia. Animals were placed on a mesh floor 18 inches above the table, and after 20 min of acclimatization, 0.1 ml of acetone was sprayed onto the plantar surface of the hindpaw using a 1 cc syringe. The latency of the response was measured as the delay to a withdrawal response of either flinching or licking with a cutoff limit at 40 s. A total of three responses from each animal were assessed at 5 min intervals by a blinded observer.

Cell culture

DRG from 17 d rat embryos were dissociated with 0.25% trypsin, 1 mM EDTA for 30 min at 37°C with constant shaking and then plated on poly-D-lysine-coated coverslips (10^5 cells per well in a 24-well plate) in 500 μl of defined Neurobasal media containing B27, Glutamax I, Albumax II, and penicillin/streptomycin (Invitrogen), supplemented with 100 ng/ml of 7.0S NGF per ml (Sigma). 5-Fluoro-2′-deoxyuridine (5 μM; Sigma) and uridine (5 μM; Sigma) were added on days 1 and 3 to inhibit the growth of dividing cells. At 17 d *in vitro*, the medium was changed to 45 mM glucose (20 mM glucose in addition to the basal 25 mM glucose) with Glutamax I, Albumax II, penicillin/streptomycin, and NGF, but without B27. Control cells were exposed to an identical medium without B27 but with a total of 25 mM glucose. For the transfection experiments, cells were infected with either vE or vZ at a multiplicity of infection (MOI) of 1, 24 h before exposure to high glucose. For the naltrindole experiments, 10 pM naltrindole (Sigma) was added 1 h before exposure to high glucose, and another 10 pM was added for 1 h before termination of the experiment (because of the continuous production of PE by the vector). All *in vitro* experiments were terminated by cell lysis after 18 h of hyperglycemia.

Western blot

Cells from pooled primary DRG neurons in culture (three wells per sample), or pooled samples of L4–L6 DRG from each rat, were homogenized with lysis buffer (20 mM Tris, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 10% glycerol, and 1:100 dilution of protease inhibitor mixture and phosphatase inhibitor mixture (Sigma). The homogenized cells and tissues were centrifuged at 10,000 × *g* for 10 min at 4°C, and the supernatant was stored at −80°C. An aliquot of supernatant was taken for protein estimation using a protein assay kit (Bio-Rad Laboratories). Total cell extract or total protein from DRG (20 μg of protein per lane) was separated by PAGE, transferred to an Immobilon-P membrane (0.45 μm; Millipore), blocked with 5% nonfat milk, and then incubated with the primary antibody [$Na_V1.7$, 1:400 (Millipore Bioscience Research Reagents); p-p38, 1:500 (Santa Cruz Biotechnology); pPKCα/β, 1:400 (Cell Signaling Technology); DOR, 1:500 (Millipore Bioscience Research Reagents)], followed by horseradish peroxidase-conjugated anti-rabbit IgG or anti-mouse IgG (1:5000; GE Healthcare) and visualized with ECL (Pierce). The lower half of the membrane was probed with β-actin (1:2000; Sigma) as a loading control. The intensity of each band was determined by quantitative chemiluminescence using a PC-based image analysis system (ChemiDoc XRS System; Bio-Rad Laboratories). The values for each group were normalized to the respective level of β-actin.

Reverse transcription-PCR of DRG

L4–L6 DRG were removed from control animals and from diabetic animals 6 weeks after STZ injection to evaluate $Na_V1.7$ mRNA expression, total RNA isolated using a commercial kit (MicroRNA; Stratagene), and cDNA transcribed from the mRNA at 37°C using a Sensiscript Reverse Transcriptase kit (Qiagen). Reverse transcription (RT)-PCR was completed using the following primers for $Na_V1.7$: forward, 5′-CCA TCA TGA ACG TGC TTC TCG TG-3′; reverse, 5′-CAA AGC AAA GAG CAG AGT GCG GAT C-3′ for 30 cycles of PCR as follows: 94°C for 1 min, 58°C for 2 min, and 72°C for 1 min. The final PCR products were separated on 1% agarose/ethidium bromide gels, images were acquired using a PC-based image analyzer (ChemiDoc XRS System; Bio-Rad Laboratories), and relative intensity was quantitated using Quantify One software (Bio-Rad Laboratories).

Agonists and antagonists

Cells were treated with phorbol 12-myristate 13-acetate (PMA), a PKC activator, overnight. Myristolated PKC inhibitor 20-28 or the p-38 inhibitor 4-(4-fluorophenyl)-2-(4-hydroxyphenyl)-5-(4-pyridyl)1*H*-imidazole (SB202190) were added 1 h before the start of hyperglycemic treatment, and cells were collected after 18 h of hyperglycemia.

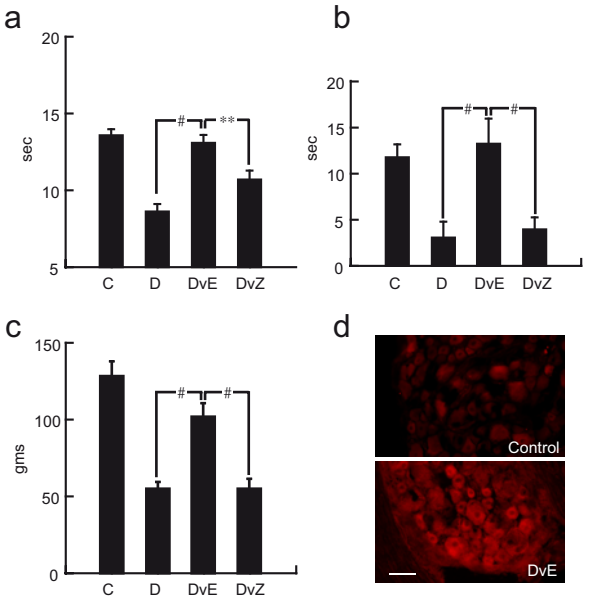

Figure 1. Enkephalin expression resulting from inoculation of vE reverses nocisponsive behaviors in PDN. ***a***, Thermal withdrawal latency (Hargreave's test) in seconds. ***b***, Cold allodynia in seconds. ***c***, Mechanical hyperalgesia (Randall–Selitto test) threshold in grams. C, Naive control; D, diabetic; DvE, diabetic inoculated with vE; DvZ, diabetic inoculated with vZ. All data are presented as mean ± SEM. $n = 6–8$ per group. $^{\#}p < 0.001$ or $^{**}p < 0.01$ versus untreated diabetic animals or DvZ. ***d***, Representative photomicrographs of enkephalin immunoreactivity 7 d after the vector inoculation. Scale bar, 20 μm.

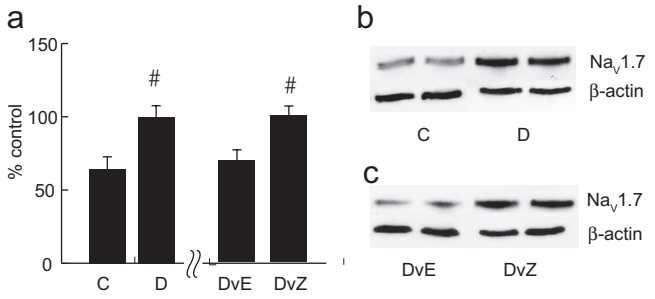

Figure 2. Enkephalin expression resulting from inoculation of vE reverses increase in $Na_V1.7$ in DRG characteristic of PDN. ***a***, Amount of $Na_V1.7$ determined by Western blot, normalized to D or DvZ. C, Control; D, diabetic; DvE, diabetic inoculated with vE; DvZ, diabetic inoculated with vZ. $^{\#}p < 0.001$; $n = 5$ animals per group. ***b***, ***c***, Representative Western blots showing two independent samples from each group.

Immunocytochemistry

Rats were perfused transcardially with 0.9% NaCl followed by Zamboni's fixative (Verdú et al., 1999). The DRG were removed, postfixed with Zamboni's fixative for 2 h, and then cryoprotected with 30% sucrose in PBS overnight. All tissues were cryostat sectioned at 10 μm, collected on gelatin-coated slides, fixed with 2% paraformaldehyde for 15 min, washed with PBS, and incubated with blocking solution (PBS with 1% normal goat serum and 0.3% Triton X-100) for 1 h, then washed once. The DRG were incubated with the primary antibody anti-met enkephalin (1:400; Harlan Labs) for 2 h at room temperature and washed three times. After incubation in the secondary fluorescent antibody, Alexa Fluor 594 goat anti-rabbit IgG (1:1000; Invitrogen), for 1 h at room temperature, the specimens were washed three times and mounted in water-based Fluoromount G (Electron Microscopy Sciences).

Statistical analysis

The statistical significance of the difference between groups was determined by ANOVA (Systat 9) using Bonferroni's correction for the multiple *post hoc* analyses. All results are expressed as mean ± SEM. All the tissue culture experiments were repeated three times. The animal experiments, with 6–8 animals per group, were repeated twice.

Results

vE inoculation prevents the behavioral manifestations of PDN in diabetic rats

At 6 weeks after injection of STZ, diabetic rats demonstrated the following: thermal hyperalgesia manifested by a decrease in withdrawal latency in response to noxious thermal stimuli compared with the control animals (control, 13.5 ± 0.3 s; diabetic, 8.82 ± 0.4 s; $p < 0.001$) (Fig. 1*a*); cold allodynia manifested by a decrease in response latency to acetone (control, 11.4 ± 1.1 s; diabetic, 3.0 ± 1.2 s; $p < 0.005$) (Fig. 1*b*); and mechanical hyperalgesia measured using the Randall–Selitto method (control, 132.5 ± 3.2 g; diabetic, 55.5 ± 3.6 g; $p < 0.001$) (Fig. 1*c*). Diabetic animals inoculated with vE showed a significant reversal of each of these nocisponsive parameters compared with diabetic animals inoculated with vZ. Four weeks after inoculation of vE (6 weeks after induction of diabetes with STZ), there was a statistically significant increase in thermal latency (vE, 12.7 ± 0.3 s; vZ, 10.7 ± 0.5 s; $p < 0.001$) (Fig. 1*a*), a statistically significant reversal of cold allodynia (vE, 13.9 ± 2.8 s; vZ, 3.9 ± 1.2 s; $p < 0.001$) (Fig. 1*b*), and a significant reversal of mechanical hyperalgesia (vE, 101.8 ± 8.1 g; vZ, 55.5 ± 3.6 g; $p < 0.001$) (Fig. 1*c*) in vE-inoculated compared with vZ-inoculated diabetic animals. We have previously reported RT-PCR detection of the expression of human PE RNA sequences in DRG of vE-inoculated rats (Goss et al., 2001). In this experiment, we used immunocytochemistry to detect increased enkephalin expression in DRG neurons *in vivo* 1 week after inoculation (Fig. 1*d*).

Enkephalin expression mediated by vE prevents the increase in $Na_V1.7$ in DRG characteristic of PDN

In agreement with previously published reports (Hong et al., 2004), we found a significant increase (~50%) in $Na_V1.7$ protein in diabetic compared with control DRG (Fig. 2*a*,*b*). Animals inoculated with vE 2 weeks after induction of diabetes by STZ showed a substantial and significant reduction in the amount of $Na_V1.7$ in DRG at 4 weeks after vector inoculation compared with animals inoculated with vZ (Fig. 2*a*,*c*). The amount of $Na_V1.7$ mRNA in DRG determined by RT-PCR after 6 weeks of diabetes was not significantly different between diabetic and control animals (supplemental Fig. 1, available at www.jneurosci.org as supplemental material), suggesting that increase in $Na_V1.7$ protein represents a posttranslational process. Because of the important role played by cell signaling molecules, including PKC and p38 MAPK, in the genesis of neuropathic pain (Ji and Strichartz, 2004), we examined the phosphorylation of p38 and PKC in the DRG by Western blot. The amount of p-p38 was significantly increased (~40%) in diabetic DRG compared with control DRG; animals inoculated with vE 2 weeks after induction of diabetes by STZ showed a substantial and significant reduction in the amount of p-p38 in DRG at 4 weeks after vector inoculation compared with animals inoculated with vZ (Fig. 3*a–c*). The amount of p-PKC was also increased significantly (~50%) in diabetic DRG compared with control; in animals inoculated with vE 2 weeks after induction of diabetes by STZ, there was a substantial and significant reduction in the amount of p-PKC in DRG at 4 weeks after vector inoculation compared with animals inoculated with vZ (Fig. 3*d–f*). A separate study showed that vector vE-treated naive animals do not show any of these changes (supplemental Fig. 2, available at www.jneurosci.org as supplemental material).

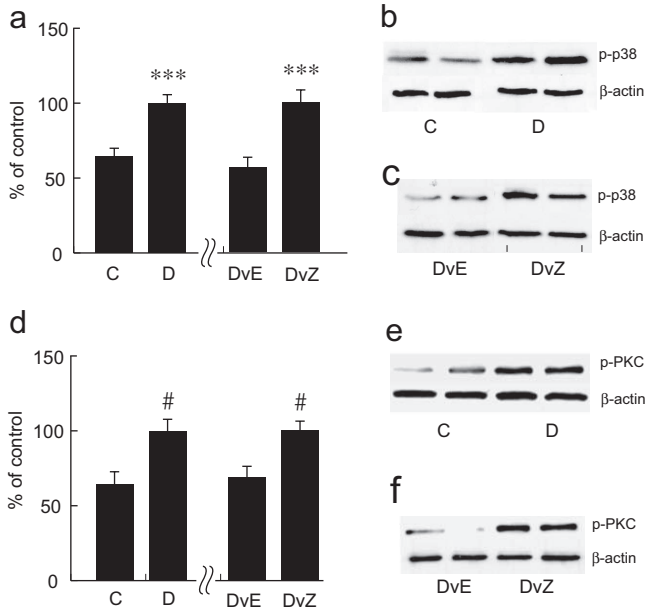

Figure 3. Inoculation of vE prevents phosphorylation of p38 and PKC in diabetic DRG *in vivo*. ***a***, Amount of p-p38 determined by Western blot, normalized to D or DvZ. ***$p < 0.005$; $n = 5$ animals per group. ***b***, ***c***, Representative Western blots showing two independent samples from each group. ***d***, Amount of p-PKC determined by Western blot, normalized to D or DvZ. #$p < 0.001$; $n = 5$ animals per group. ***e***, ***f***, Representative Western blots showing two independent samples from each group. C, Control; D, diabetic; DvE, diabetic inoculated with vE; DvZ, diabetic inoculated with vZ.

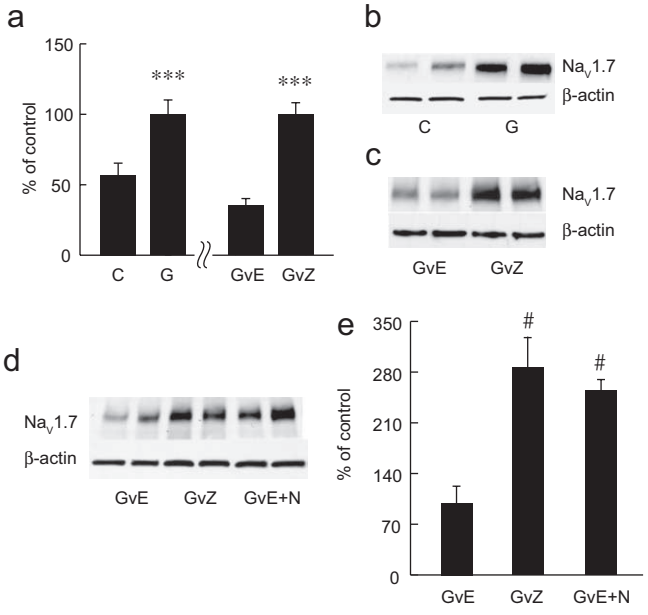

Figure 4. Transgene-mediated expression of enkephalin prevents the increase in $Na_V1.7$ resulting from exposure primary DRG neurons to hyperglycemic conditions *in vitro*. ***a***, Amount of $Na_V1.7$ determined by Western blot, normalized to G or GvZ. ***$p < 0.005$; $n = 3$ wells per group. C, Control; G, hyperglycemia; GvE, hyperglycemia, transfected with vE; GvZ, hyperglycemia, transfected with vZ. ***b–d***, Representative Western blots showing two independent samples from each group. ***d***, The effect of vE is blocked by naltrindole (GvE+N). ***e***, The amount of $Na_V1.7$ determined by Western blot, normalized to GvE. #$p < 0.001$; $n = 3$ wells per group.

Modulation of $Na_V1.7$ by glucose in primary DRG neurons *in vitro*

To study the relationship of the amount of $Na_V1.7$ in DRG neurons to hyperglycemia, we examined primary embryonic day 17 DRG neurons *in vitro*. These neurons constitutively express $Na_V1.7$. After 18 h of exposure to culture medium containing 45 mM glucose (20 mM glucose in addition to the 25 mM glucose in the usual culture medium), there was a substantial increase (~30%) in the amount of $Na_V1.7$ protein detected by Western blot (Fig. 4*a*,*b*). Transfection of cells exposed to hyperglycemia with vE (MOI of 1) blocked the increase in $Na_V1.7$ resulting from exposure to hyperglycemic conditions *in vitro* (Fig. 4*a*,*c*). The effect of transfection with vE in preventing the increase in $Na_V1.7$ was blocked by the addition of 10 pM naltrindole (Fig. 4*d*,*e*), indicating that the observed effect is mediated by the DOR.

Like DRG from diabetic animals *in vivo*, primary DRG neurons exposed to hyperglycemic conditions showed a substantial increase in phosphorylated of p38 MAPK (Fig. 5*a*,*b*) that was prevented by transfection with vE (MOI = 1) 24 h before hyperglycemia (Fig. 5*a*,*c*). Hyperglycemia also caused a substantial increase in p-PKC (Fig. 5*d*,*e*) that was similarly prevented by transfection with vE (Fig. 5*d*,*f*). The effect of vE on inhibiting the increase in both p-p38 and p-PKC was blocked by the addition of 10 pM naltrindole (Fig. 6*a*,*b*). Together, these results suggest that the increase in $Na_V1.7$ protein induced by hyperglycemia is mediated through activation of p38 and PKC, and that vE-produced inhibition of the increase in $Na_V1.7$ is mediated by interruption of the p38 and PKC signal transduction pathways.

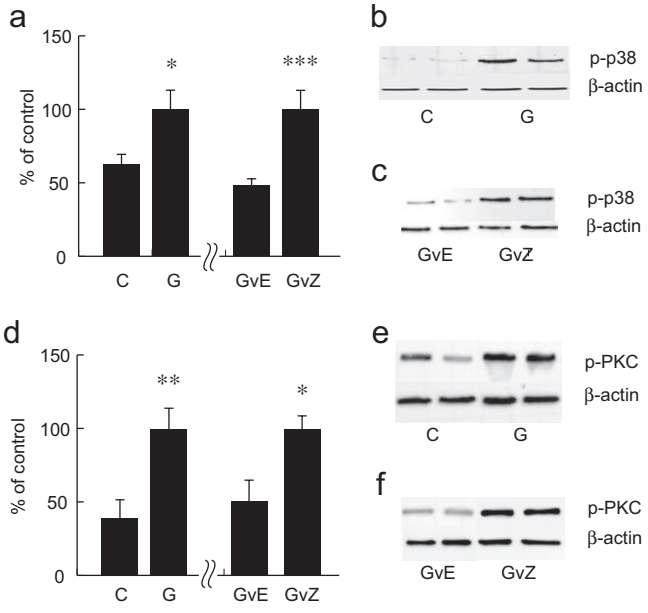

Figure 5. Transduction with vE prevents phosphorylation of p38 and PKC in DRG exposed to hyperglycemia *in vitro*. ***a***, Amount of p-p38 determined by Western blot, normalized to G or GvZ. *$p < 0.05$; ***$p < 0.005$; $n = 3$ wells per group. ***b***, ***c***, Representative Western blots showing two independent samples from each group. ***d***, Amount of p-PKC determined by Western blot, normalized to G or GvZ. *$p < 0.05$; **$p < 0.01$; $n = 3$ wells per group. ***e***, ***f***, Representative Western blots showing two independent samples from each group. C, Control; G, hyperglycemia; GvE, hyperglycemia, transfected with vE; GvZ, hyperglycemia, transfected with vZ.

Regulation of $Na_V1.7$ *in vitro*

To directly test the role of PKC in regulating $Na_V1.7$ protein levels, we exposed DRG neurons *in vitro* to the PKC activator PMA (100 nM for 18 h). The amount of $Na_V1.7$ in cells exposed to PMA was increased (~60%) compared with cells not exposed to PMA, a change that was similar in magnitude to the increase resulting from exposure to hyperglycemic conditions (Fig. 7*a*,*b*). The amount of $Na_V1.7$ mRNA in the cells was not changed by

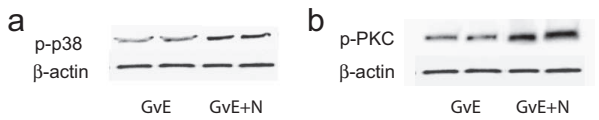

Figure 6. Naltrindole blocks the effect of vE on p38 and PKC in DRG exposed to hyperglycemia *in vitro*. ***a***, ***b***, Representative Western blots showing amount of p-p38 (***a***) and p-PKC (***b***) in primary DRG neurons exposed to hyperglycemia and transfected with vE in the absence (GvE) or presence (GvE+N) of 10 pM naltrindole. The difference between GvE and GvE+N was statistically significant for both p-p38 ($p < 0.001$) and p-PKC ($p < 0.005$).

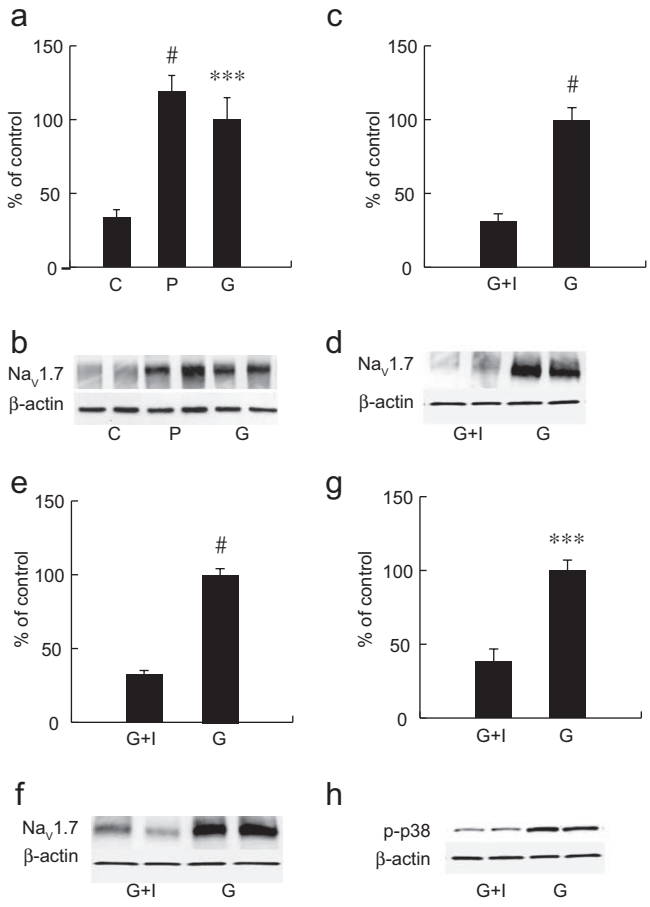

Figure 7. The amount of $Na_V1.7$ in DRG is regulated by PKC acting through p38. ***a***, The amount of $Na_V1.7$ in control (C) DRG neurons *in vitro* and in DRG neurons exposed to PMA in 25 mM glucose-containing medium (P) or to 45 mM glucose (G). $^{\#}p < 0.001$; $^{***}p < 0.005$; $n = 3$ wells per group. ***b***, Representative Western blots. ***c***, The amount of $Na_V1.7$ in DRG neurons exposed to 45 mM glucose *in vitro* treated with the myristolated PKC inhibitor 20-28 (G+I) normalized to the amount of $Na_V1.7$ in cells exposed to 45 mM glucose (G). $^{\#}p < 0.001$. ***d***, Representative Western blots. ***e***, The amount of $Na_V1.7$ in DRG neurons exposed to 45 mM glucose *in vitro* treated with the p38 inhibitor SB202190 (G+I) normalized to the amount of $Na_V1.7$ in cells exposed to 45 mM glucose (G). $^{\#}p < 0.001$. ***f***, Representative Western blots. ***g***, The amount of phosphorylated p38 in DRG neurons exposed to 45 mM glucose *in vitro* treated with the myristolated PKC inhibitor 20-28 (G+I) normalized to the amount of phosphorylated p38 in cells exposed to 45 mM glucose (G). $^{***}p < 0.005$; $n = 3$ wells per group. ***h***, Representative Western blots.

exposure to hyperglycemia (supplemental Fig. 1, available at www.jneurosci.org as supplemental material). Inhibition of PKC activation by addition of 10 μM of the myristolated PKC inhibitor 20-28 blocked the increase in $Na_V1.7$ (Fig. 7*c*,*d*; supplemental Fig. 3*a*,*c*, available at www.jneurosci.org as supplemental material), as did addition of 10 μM of the p38 inhibitor SB202190 (Fig. 7*e*,*f*; supplemental Fig. 3*b*,*d*, available at www.jneurosci.org as supplemental material). Inhibition of PKC activation by addition of 10 μM of the myristolated PKC inhibitor 20-28 blocked the phosphorylation of p38 (Fig. 7*g*,*h*), suggesting that the PKC lies upstream of p38 in the pathway leading to increases in $Na_V1.7$.

Discussion

There are several novel observations from these studies with important implications. The first is that vector-mediated expression of enkephalin in DRG neurons, achieved by HSV-mediated gene transfer, reduces several manifestations of pain-related behaviors in the rat model of painful diabetic neuropathy. STZ-induced diabetes results in alterations of spontaneous and inflammation-related pain thresholds (Courteix et al., 1993; Calcutt et al., 1996) that have been used as surrogate markers for the pain in painful diabetic neuropathy, and in agreement with previous reports, the diabetic animals in this study demonstrated thermal hyperalgesia, cold allodynia, and mechanical hyperalgesia similar to that reported previously (Courteix et al., 1993). We and others have demonstrated that HSV-mediated gene transfer to the DRG can be used to reduce nocisponsive behaviors in rodent models of inflammatory, neuropathic, and cancer-related pain (Wilson et al., 1999; Braz et al., 2001; Goss et al., 2001, 2002; Hao et al., 2003). In those studies, it has been established that the vector-mediated enkephalin effect is reversed by opioid receptor antagonists (naloxone or naltrindole) and that the effect of release of the vector-encoded peptide is continuous, both over a scale of hours within a single day and over a scale of days over the course of the several weeks of transgene expression (Hao et al., 2003). The effect of transgene-mediated enkephalin release on pain-related behaviors is not entirely unanticipated. Although early studies in animals suggested that morphine was not effective in reducing pain-related behaviors in diabetic animals (Raz et al., 1988), confirming a widely held clinical impression that opiate drugs are relatively ineffective in this condition, more recent randomized controlled human trials have confirmed that opiates are indeed effective in the treatment of painful diabetic neuropathy (Watson et al., 2003; Gilron et al., 2005), although not without substantial off-target adverse effects of the systemically administered drugs.

The pathogenesis of pain in the setting of diabetes is complex. Previous studies have implicated involvement alterations in the peripheral kinin system (Gabra et al., 2006) and in peripheral vanilloid receptors (Hong and Wiley, 2005), in addition to described spinal and supraspinal mechanisms (Calcutt, 2002; Ramos et al., 2007) in the development of pain. The first study of sodium channels in peripheral nerve in PDN identified increases in RNA and protein of several voltage-gated sodium channel α subunit isoforms, although in that study the investigators did not observe an increase in $Na_V1.7$ RNA or protein (Craner et al., 2002). Our results conform closely to those of Hong et al. (2004), who reported an increase in $Na_V1.7$ protein with corresponding alterations in the electrophysiology of DRG neurons in rats with PDN. Among sodium channel isoforms, $Na_V1.7$ is of particular interest because of emerging evidence from genetic studies that correlate alterations in $Na_V1.7$ with human syndromes characterized by spontaneous pain (Yang et al., 2004; Choi et al., 2006; Fertleman et al., 2006). Mutations in the coding sequence of $Na_V1.7$ that result in channels that are electrically hyperexcitable produce a painful condition (Harty et al., 2006), and it has been shown that an increase in the number of channels present in the membrane of DRG neurons can lead to a similar state of hyperexcitability of the primary nociceptive afferent (Devor, 2006).

The role of PKC in modulating Na_V protein levels in DRG has

not been reported previously. PKCβ activity is increased in the DRG of diabetic compared with control mice (Uehara et al., 2004), phorbol esters that activate PKC increase nociceptive responsiveness in inflammatory pain (Coderre, 1992) and enhance thermal hyperalgesia in diabetic rats (Ohsawa and Kamei, 1999), and inhibition of PKC decreases hyperalgesia in STZ diabetic rats (Ahlgren and Levine, 1994), attenuates thermal hyperalgesia induced by partial sciatic nerve ligation, and attenuates opiate-induced antinociception in diabetic mice (Ohsawa and Kamei, 1997). Previous studies have suggested that PKC activation may modulate the electrophysiologic properties of tetrodotoxin (TTX)-sensitive and TTX-resistant voltage-gated sodium channels (Dascal and Lotan, 1991; Numann et al., 1991; Thio and Sontheimer, 1993), but this is the first report to demonstrate PKC-mediated regulation of voltage-gated sodium channel protein levels in primary sensory neurons *in vitro* and *in vivo*.

Phosphorylated (activated) p38 is increased in DRG in a variety of painful conditions (Obata and Noguchi, 2004; Obata et al., 2004; Xu et al., 2007), and activation of p38 has been implicated in the upregulation of TTX-resistant Na channels in DRG (Jin and Gereau, 2006). In the current study, we found that in diabetic neuropathy an increase in p-PKC and p-p38 correlated with the increase in amount of the TTX-sensitive Na_V isoform $Na_V1.7$ in DRG neurons in rats after 6 weeks of diabetes, and in DRG neurons *in vitro* after 18 h of exposure to hyperglycemia. The increase in $Na_V1.7$ was reproduced by activation of PKC by PMA, and that effect was blocked by the application of a PKC inhibitor *in vitro*. Together, these results imply that hyperglycemia *in vitro* and diabetes *in vivo* results in activation of PKC, activation of p38, and an increase in $Na_V1.7$ in DRG neurons.

δ-Opioid receptors are widely expressed in DRG neurons (Zhang et al., 1998; Wang and Wessendorf, 2001), and using double-label immunocytochemistry we confirmed coexpression of δ-opioid receptor and $Na_V1.7$ in DRG neurons *in vivo* (supplemental Fig. 4, available at www.jneurosci.org as supplemental material). The molecular mechanisms of δ-opioid receptor signaling are complex, varying according to the receptor and the identity, location, and concentration of interacting protein partners within membrane microdomains and the local cytoskeletal network (Law et al., 2000). There are no previous reports regarding the effects of δ-opioid receptor activation on PKC or p38 in primary DRG neurons, but vector-mediated transduction to release enkephalin *in vitro* or *in vivo* resulted in a reduction of p-PKC and concomitant reduction in p-p38, with a resultant reduction of $Na_V1.7$ protein in DRG *in vivo* and *in vitro*. This novel finding, implicating presynaptic receptors of primary sensory afferents in modulating the amount of voltage-gated channels in those cells through a pathway that has not previously been described, has important implications for the treatment of painful diabetic neuropathy.

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