Diabetes mellitus is associated with one or more kinds of stimulus-evoked pain including hyperalgesia and allodynia. The mechanisms underlying painful diabetic neuropathy remain poorly understood. Previous studies demonstrate an important role of vanilloid receptor 1 (VR1) in inflammation and injury-induced pain. Here we investigated the function and expression of VR1 in dorsal root ganglion (DRG) neurons isolated from streptozotocin-induced diabetic rats between 4 and 8 weeks after onset of diabetes. DRG neurons from diabetic rats showed significant increases in capsaicin- and proton-activated inward currents. These evoked currents were completely blocked by the capsaicin antagonist capsazepine. Capsaicin-induced desensitization of VR1 was down-regulated, whereas VR1 re-sensitization was up-regulated in DRG neurons from diabetic rats. The protein kinase C (PKC) activator phorbol 12-myristate 13-acetate blunted VR1 desensitization, and this effect was reversible in the presence of the PKC inhibitor bisindolylmaleimide I. Compared with the controls, VR1 protein was decreased in DRG whole-cell homogenates from diabetic rats, but increased levels of VR1 protein were observed on plasma membranes. Of interest, the tetrameric form of VR1 increased significantly in DRGs from diabetic rats. Increased phosphorylation levels of VR1 were also observed in DRG neurons from diabetic rats. Colocalization studies demonstrated that VR1 expression was increased in large myelinated A-fiber DRG neurons, whereas it was decreased in small unmyelinated C-fiber neurons as a result of diabetes. These results suggest that painful diabetic neuropathy is associated with altered cell-specific expression of the VR1 receptor that is coupled to increased function through PKC-mediated phosphorylation, oligomerization, and targeted expression on the cell surface membrane.

Painful neuropathy is one of the most common complications in early to intermediate stages of diabetes mellitus. Diabetic patients frequently exhibit one or more kinds of stimulus-evoked pain, including increased responsiveness to noxious stimuli (hyperalgesia) as well as a hyper-responsiveness to normally innocuous stimuli (allodynia) that are often concurrent with a paradoxical loss of stimulus-evoked sensation (1, 2). The underlying mechanisms of painful diabetic neuropathy remain elusive. Similar to human painful diabetic neuropathy, animal models such as streptozotocin (STZ)-induced diabetic mice or rats demonstrate early functional and biochemical abnormalities including thermal hyperalgesia and mechanical allodynia (3–6). It has been suggested that hyperactivity of small, unmyelinated C-fibers results in hyperalgesia and allodynia in this model (6, 7). Khan et al. (4) reported that A-fiber afferents in diabetic rats developed abnormal spontaneous discharges and increased sensitivity to mechanical stimuli, suggesting a role of large A-fiber neurons in addition to nociceptive C-fibers in the development of diabetic neuropathic pain.

Capsaicin, the pungent ingredient in hot peppers, can induce action potentials, cause cation influx, and release neuropeptides from primary afferent neurons that induce hyperalgesia and inflammation (8–11). Prolonged or repeated exposure to capsaicin desensitizes nociceptors. Because of this property, capsaicin is used as an analgesic agent in the treatment of various painful disorders (12, 13). The recently cloned capsaicin receptor, vanilloid receptor 1 (VR1), also known as TRPV1, encodes a protein of 838 amino acids with a predicted molecular mass of 95 kDa that specifically localizes to small-sized, unmyelinated C-fiber (nociceptive) sensory neurons (14). VR1 is a polymodal ligand-gated cation channel that can be activated by vanilloids, noxious heat, and protons (14–16). Studies with VR1-deficient mice demonstrate that the VR1 channel is essential for selective modalities of thermal hyperalgesia induced by tissue injury and inflammation, supporting the hypothesis that VR1 is a molecular integrator of painful stimuli (17, 18). More supporting evidence comes from the study demonstrating that administration of the competitive capsaicin antagonist capsazepine reverses hyperalgesia in models of inflammatory and neuropathic pain (19). Like many ion channels, the function of the VR1 channel is modulated by phosphorylation/dephosphorylation processes. Activation of protein kinase C (PKC) induces VR1 activity in the absence of any other agonist and potentiates the response of VR1 to capsaicin (20, 21). In sensory neurons the capsaicin response is also potentiated by cAMP-dependent protein kinase that directly phosphorylates VR1 and reduces its desensitization (22). Up-regulation of VR1 protein expression has also been observed in nerve injury-induced pain (23). Nevertheless, the mechanisms underlying the modulation of VR1 expression and function are unknown in neuropathic pain associated with diabetic neuropathy.

Here we employed STZ-induced diabetic rats as a model to investigate the expression and function of the VR1 channel that is associated with thermal hyperalgesia and mechanical allodynia. We observed that VR1 protein levels were down-regulated...

The abbreviations used are: STZ, streptozotocin; PFO, perfluorooctanoic acid; VR1, vanilloid receptor 1; DRG, dorsal root ganglion; PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate; MES, 4-morpholineethanesulfonic acid; BIM, bisindolylmaleimide I; IR, immunoreactive; pF, picofarad.

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Altered Expression and Function of VR1 in Diabetic Rats

EXPERIMENTAL PROCEDURES

Experimental Animals—Male Sprague-Dawley rats were housed in the animal facility of the University of Michigan Unit for Laboratory Animal Medicine, which was maintained at 22 °C, 55% relative humidity, with an automatic 12-h light/dark cycle. The animals received a standard laboratory diet and tap water ad libitum. All experiments were approved by the University of Michigan Committee on Use and Care of Animals according to the National Institutes of Health guidelines.

Diabetes Induced by STZ—Diabetes mellitus was induced by a single intraperitoneal injection of STZ (45 mg/kg) to 180–200-g rats that had been fasted overnight to maximize the effectiveness of STZ treatment. STZ solution was prepared fresh by dissolving it in 0.1 M citrate buffer, pH 5.5. Age-matched control rats were injected with citrate buffer alone. The diabetic condition was assessed by glucose levels greater than 300 mg/dl (16.7 mM). Rats meeting this criterion were used experimen-
tially 4–8 weeks after STZ induction. Our previous studies with this model demonstrated that rats with diabetes for 4–8 weeks displayed a variety of functional abnormalities, including delayed nerve conduction velocity, an increase in calcium influx, impaired inhibitory G protein function, impaired mitochondrial function, and activation of the apoptosis cascade in DRG neurons. These effects are reversible following 2 weeks of insulin-mediated euglycemia (24, 25). This model also demonstrated thermal hypersalgesia and mechanical allodynia (5).

Neuronal Dissociation—DRG neurons were prepared as described previously (5). Briefly, L1–L6 DRGs in the lumbar region of the spinal column were removed bilaterally from diabetic and control rats. The acutely dissociated ganglia were incubated for 50 min at 37 °C in 0.3% collagenase (Worthington type 2) in minimal essential medium (Invitro-
gens, Santa Cruz Biotechnology). After washing three times, cells were resuspended in M-MEM containing 10% fetal bovine serum and plated on 35-mm sterile culture dishes that were previously coated with calf collagen. Isolated neurons were incubated in 95% air + 5% CO2 at 37 °C for 2–7 h prior to electrophysiological recording. In some experiments, neurons were cultured for 24–48 h in the presence of nerve growth factor (50 ng/ml) for immunofluorescence staining.

Electrophysiology—Patch clamp recordings were performed under the whole-cell mode at room temperature (20–23 °C). Glass electrodes were filled with potassium-containing solution (140 NaCl, 5 KCl, 2 MgCl2, 2 CaCl2, 10 HEPES, 10 glucose, pH 7.4 (adjusted with NaOH). In some experiments, MES-based solution was used to adjust the pH to 5.0. The internal solution contained (in mM) 140 NaCl, 5 KCl, 2 MgCl2, 2 CaCl2, 10 HEPES, pH 7.4. All solutions were made fresh daily and filtered at 0.2 μm immediately before use. For the experiments testing the effects of pH and capsaicin, solutions were applied locally and rapidly (1–20 s duration) to the neurons of interest by using silica tips. Working capsaicin solutions were prepared fresh from a 10 mM stock solution dissolved in ethanol using extracellular solution with a negligible effect on channel function (26). Small-sized neu-
rons with cell capacitance less than 35 pF were voltage-clamped at −80 mV, and pipette resistance was compensated as necessary. Data were acquired using an Axopatch 200B amplifier (Axon Instruments, Inc.), low pass filtered at 5 kHz, digitized at 10 kHz through a Digidata 1322A 16-bit data acquisition system, and recorded with pClamp version 8.2 software (Axon Instruments, Inc.).

Immunoprecipitation and Western Blotting—Immunoprecipitation was performed previously (27). Briefly, L1–L6 DRGs from control and diabetic rats were isolated and homogenized in ice-cold lysis buffer containing 50 mM Tris, pH 8.0, 150 mM NaCl, 1 mM EGTA, 50 mM NaF, 1.5 mM MgCl2, 10% v/v glycerol, 1% v/v Triton X-100, 1 mM phenylmethylsulfonyl fluoride, 1 mM Na3VO4, and “Complete” protease inhibitor mixture (Roche Diagnostics). Immunoprecipitation was per-
formed using rabbit anti-VR1 antibodies (1:50, Neuronics) or anti-
phosphoserine antibodies (1:40, Chemicon). The immunoprecipitated proteins were separated on 4–15% Tris-HCl gels and transblotted to polyvinylidene difluoride membranes (Amersham Biosciences). In some experiments, crude DRG homogenates and enriched plasma membrane preparations were separated by electrophoresis on 10% polyacrylamide gels, followed by Western blot analysis. The following antibodies were used: C-terminal VR1 (1:500, Santa Cruz Biotechnology), rabbit anti-VR1 (carboxyl-terminal; 1:1,000, Neuronics); rabbit anti-VR1L1 (1:200, Chemicon); mouse anti-peripherin (1:200, Chemicon); mouse anti-NF200 (1:500, Sigma). In some experiments, crude DRG homogenates and enriched plasma membrane preparations were mixed with 2× sample buffer (100 mM Tris, 6% NP40, 20% glycerol, 2% β-mercaptoethanol, and 0.1% bromphenol blue, pH 6.8). After incubation at room temperature for 30 min, the samples were centrifuged at 15,000 × g for 5 min to remove insoluble debris before loading onto gels for SDS-PAGE. Western blots were visualized using horseradish peroxidase-conjugated secondary antibodies (Amer-
sham Biosciences) and enhanced chemiluminescence reagents (Fisher). The images were captured using a Fluorchem 8000 Chemidoc system (Fisher). The membranes were rehybridized with horseradish peroxidase-conjugated secondary antibodies (Amersham Biosciences) for 1 h at room temperature and developed using the Enhanced Chemiluminescence Western Blotting Detection System (Amersham Biosciences). The following reagents were used: Crystal Chem BioMax Western Chemiluminescent Detection Reagent (Supersignal West Dura; Pierce; Waltham, MA), SuperSignal West Pico Chemiluminescent Substrate (Pierce; Waltham, MA), CDP-star (Roche Diagnostics; Indianapolis, IN), and Pierce SuperSignal West Femto Chemiluminescent Substrate (Pierce; Waltham, MA). The following antibodies were used: rabbit anti-VR1L1 (1:200, Chemicon); rabbit anti-VR1 (1:200, Chemicon); mouse anti-peripherin (1:200, Chemicon); and rabbit anti-NF200 (1:500, Sigma). The sections were then washed three times, mounted serially onto Superfloures plus medium (Fisher) for staining as described previously (5). After incubating in 0.3% Triton X-100 in phosphate-buffered saline (PBST) for 2 h, the sections were blocked in 10% goat serum in PBST for at least 4 h, followed by incubation with primary antibody overnight at 4 °C. Primary antibodies were used at the following dilutions: goat anti-VR1 (carboxyl-terminal; 1:500, Santa Cruz Biotechnology); rabbit anti-VR1 (carboxyl-terminal; 1:1,000, Neuronics); rabbit anti-VR1L1 (1:200, Chemicon); goat anti-peri-
pherin (1:200, Chemicon); mouse anti-NF200 (1:500, Sigma). In some experiments, antibodies were used at different concentrations: goat anti-VR1 (carboxyl-terminal; 1:100, Neuronics); rabbit anti-VR1 (1:200, Chemicon). The following reagents were used: mouse anti-VR1L1 (1:200, Chemicon); rabbit anti-VR1 (1:200, Chemicon); rabbit anti-VR1L1 (1:200, Chemicon); mouse anti-NF200 (1:500, Sigma). In some experiments, the experiments utilizing goat-derived primary antibodies, the sections were blocked in 10% donkey serum in PBST. After washing three times with PBST, the sections were incubated with secondary antibodies (Molecular Probes) at the following dilutions: goat anti-mouse Alexa Fluor 488 (1:400); goat anti-rabbit Alexa Fluor 594 (1:500); or donkey anti-goat Alexa Fluor 594 (1:400). The sections were then washed three times, mounted using an anti-fade fluorescence mounting medium, and analyzed by confocal microscopy (Zeiss LSM510) or a fluorescence microscope.

Data Analysis—For each series of experiments, immunostained DRG sections were viewed under a Zeiss Axioplan fluorescence microscope, and digitized images were acquired covering the entire DRG in one field under low magnification. Images from 4 to 8 different experimental series were pooled for counting in each group. Neurons were judged to be positive if they had mean brightness values greater than the corresponding control value stained with the secondary antibody alone. The average staining intensity was determined for each cell body. Signifi-
cance was analyzed by Student’s t test.

RESULTS

Diabetic Neuropathy Is Associated with Increased Capsaicin-induced Current—Two days after injection of STZ, 75% of the rats developed high levels of blood glucose (mean level = 475 ±


16 mg/dl), whereas untreated rats had normal levels (mean level = 83 ± 7 mg/dl). The elevated level of blood glucose in STZ-injected rats was maintained during the entire experimental period. These results are very similar to our previous studies (5, 30). As reported previously (5), diabetic rats demonstrated a significant reduction in the threshold for escape from innocuous mechanical pressure (alldynia) and a reduction in the latency to withdrawal from a noxious thermal stimulus (hyperalgesia).

To investigate the function of VR1 receptors in diabetic rats, whole-cell currents activated by capsaicin were recorded in whole-cell mode using acutely dissociated DRG neurons. The average cell membrane capacitance was 25.3 ± 2.2 pF (n = 42) in control rats and 25.9 ± 3.0 pF (n = 50) in the diabetic group. Provided that the cells have a spherical shape, the mean capacitance corresponds to a cell diameter of 28.2 ± 2.4 µm in control rats and 28.6 ± 3.3 µm in diabetic rats, respectively. As shown in Fig. 1A, the inward current activated by the application of capsaicin (1 µM, 2 s) was significantly larger in neurons from diabetic rats compared with controls. The mean peak current density of capsaicin-induced currents was 372.6 ± 32.1 pA/pF (n = 15) in diabetic neurons and 256.5 ± 22.8 pA/pF (n = 18) in controls, respectively. This peak current amplitude was increased 45% in diabetic rats compared with the control group, and the difference between these two values was significant (p < 0.05) (Fig. 1B). Compared with controls, the whole-cell inward current activated by capsaicin also displayed a longer duration in DRG neurons from diabetic rats. The average duration of whole-cell current activated by capsaicin was 28.9 ± 5.6 s (n = 14) in control rats and 83.7 ± 17.6 s (n = 15) in diabetic rats, respectively. The duration of inward current was increased ~2-fold in diabetic rats compared with the control group (p < 0.05) (Fig. 1B). Moreover, capsazepine, a competitive capsaicin antagonist, blocked capsaicin-induced inward currents almost completely in DRG neurons from both control and diabetic rats. Fig. 1C shows the neurons from control and diabetic rats that did not respond to capsaicin (1 µM, 2 s) in the presence of 10 µM capsazepine (n = 6). This result indicates that the capsaicin-binding gate of VR1 receptors is not altered in DRG neurons of diabetic rats.

Increased Low pH-evoked Currents in DRG Neurons from Diabetic Rats—As the VR1 receptor can be activated by low pH as well as capsaicin (31), we recorded whole-cell currents activated by delivering the bath solution at a low pH (5.0). Fig. 2A shows representative currents evoked by pH 5.0 in the absence or presence of capsazepine (10 µM) in DRG neurons from control and diabetic rats. Low pH bath solution (2-s application) evoked large inward currents in DRG neurons from both control and diabetic rats. The peak amplitude of low pH-evoked whole-cell current was 255.2 ± 29.2 pA/pF (n = 10) in control rats and 365.3 ± 32.6 pA/pF (n = 11) in diabetic rats, respectively. The level of inward current was increased 43% in diabetic rats compared with the control group, and the difference between them was significant (p < 0.05) (Fig. 2B). The low pH-evoked inward currents were blocked by capsazepine (Fig. 2A). In the presence of capsazepine (10 µM), the low pH-evoked inward current was substantially reduced in DRG neurons from both control and diabetic rats. The peak current density of pH-evoked current was 10.3 ± 1.3 pA/pF (n = 12) in control rats and 12.1 ± 2.2 pA/pF (n = 12) in diabetic rats, respectively (Fig. 2B). Compared with the inward current in the absence of capsazepine, 96% of the pH-activated current was blocked in DRG neurons from both control and diabetic rats by capsazepine. The difference in the reduced pH-activated currents in the presence of capsazepine was not significantly different between control and diabetic rats (p > 0.1), suggesting the pore-gating property of VR1 is not changed in DRG neurons in diabetic rats.

Increased Oligomeric Structure and Phosphorylation of VR1 Receptor in DRGs from Diabetic Rats—To test whether the increased function of the VR1 receptor was because of increased protein expression, we extracted crude DRG homogenates and plasma membranes, and we analyzed VR1 expression level by Western blot. As shown in Fig. 3A, the expression level of VR1 was substantially decreased in diabetic rats (55 ± 10% of the control; n = 8, p < 0.05), when total protein from DRGs (including both cytoplasm and membrane protein) was analyzed using crude homogenates. When plasma membrane...
extracts were subjected to analysis, the level of VR1 expression was significantly increased in DRGs of diabetic rats (151 ± 6% of the control levels; n = 5, p < 0.05), indicating that more VR1 receptor was aggregated in cell membranes of diabetic DRG neurons as compared with the control. These results support a redistribution of VR1 protein from cytoplasm to cell surface membranes in diabetic rats, whereas total VR1 expression is significantly increased in DRGs (170 ± 22% of the control; n = 5, p < 0.05) as shown in Fig. 3B. These results suggest that more VR1 receptor-channel complexes are formed at the cell membranes in diabetic DRG neurons.

It has been reported that phosphorylation of VR1 modulates VR1 current to mediate pain sensation (20–22, 33, 34). To investigate the phosphorylation level of VR1 receptors, crude DRG homogenates were immunoprecipitated with anti-VR1 antibodies or anti-phosphoserine antibodies, and the blots containing the immunoprecipitated complexes were probed with the reciprocal antibodies. The results are shown in Fig. 3C. In DRG neurons from diabetic rats, the phosphorylation level of VR1 receptor increased substantially (256 ± 23% of the control; n = 4, p < 0.05) when the data were normalized to the VR1 protein loaded on gels. This result supports the hypothesis that increased phosphorylation of VR1 may contribute to the increased current of VR1 receptor in DRG neurons from diabetic rats.

**Impaired Capsaicin-induced Desensitization of VR1 Receptor in DRG Neurons—Extracellular Ca\(^{2+}\)-dependent desensitization of VR1 has been observed in patch clamp experiments when using both heterologous expression systems and native DRG neurons (35). The inactivation of nociceptive neurons by capsaicin has generated extensive research on the possible therapeutic effectiveness of capsaicin as a clinical analgesic tool (12, 13). To test the desensitization of VR1 receptors in diabetic rats, we examined whole-cell currents induced by capsaicin 2 min after the initial brief application of capsaicin in the presence of extracellular Ca\(^{2+}\). Application of a high concentration of capsaicin (10 \(\mu\)M) completely desensitized VR1 in DRG neurons in both control and diabetic rats (data not shown). In order to obtain the whole-cell current in response to a secondary capsaicin application, a low concentration of capsaicin (0.5 \(\mu\)M) and a short time application (1 s duration) were used. Fig. 4A shows the original traces of inward currents induced by consecutive application of capsaicin (0.5 \(\mu\)M, 1 s) in DRG neurons from control and diabetic rats. Consistent with the increased currents induced by 1 \(\mu\)M capsaicin in diabetic rats, the current induced by 0.5 \(\mu\)M capsaicin was also significantly larger in diabetic rats compared with the controls (n = 10, p < 0.001). The peak current density was 76.3 ± 6.8 pA/pF in control rats and 138.1 ± 23.3 pA/pF in diabetic rats, respectively. The inward currents evoked by secondary application of capsaicin decreased significantly in both control and diabetic rats as compared with the initial currents, indicating that desensitization of VR1 receptors occurs in both groups. When the ratio of the second current to the initial current was calculated, it was 29 ± 4% in diabetic rats and 12 ± 2% in control rats, respectively (Fig. 4B). As compared with the control, this value increased 141% in diabetic rats (n = 10, p < 0.01).

It has been reported recently that VR1 current and desensitization can be modulated by phosphorylation via PKC-dep

![Image](http://www.jbc.org/)

**Fig. 2. Low pH-activated inward currents in small diameter DRG neurons from control and diabetic rats.** A, typical current traces of neurons responding to external solution buffered at pH 5.0 in the absence (left) or presence (right) of 10 \(\mu\)M capsaicin (CZP) preincubated for 2 min. Capsazepine almost completely blocked low pH-activated currents in neurons from both control and diabetic rats. B, mean peak current densities of low pH (5.0) activated inward currents in the presence or absence of capsaicin in DRG neurons from control and diabetic rats. The current density of low pH-activated inward current in diabetic rats was significantly larger than that of the control (p < 0.05). There was no difference in the low pH-activated current in the presence of capsaicine between control and diabetic rats (p > 0.05). Values are mean ± S.E. * indicates significant difference (p < 0.05).
calculated, its value ($I_{2nd}/I_{1st}$) increased significantly from 12 ± 2 to 60 ± 9% in control DRG neurons ($n = 9, p < 0.05$), whereas this value increased from 29 ± 4% to 94 ± 10% in diabetic rats ($n = 11, p < 0.05$). This potentiation was probably mediated by PKC because no increase of currents was observed by the inactive analogue 4α-phorbol ($n = 4$, data not shown) and the increase by PMA was inhibited by the use of the specific inhibitor bisindolylmaleimide I (BIM), which inhibits PKC at a concentration of 200–500 nM and inhibits cAMP-dependent protein kinase only at concentrations exceeding 2 μM (21, 36, 38). Exposure to BIM (500 nM, 30 s) inhibited the potentiation of capsaicin-induced current by PMA in both control and diabetic DRG neurons (Fig. 4A). The values of the ratio ($I_{2nd}/I_{1st}$) were reversed back to the levels of the normal conditions in the absence of PMA treatment (Fig. 4B). These data suggest that VR1 receptors in DRG neurons from diabetic rats are more resistant to the consecutive application of capsaicin, possibly due to the enhanced phosphorylation of VR1 receptors in a PKC-dependent manner.

Enhanced Re-sensitization of VR1 Receptors by PKC-dependent Pathway in DRG Neurons in Diabetic Rats—Fig. 5A shows the representative current traces of the DRG neurons desensitized by consecutive application of capsaicin and re-sensitized by PMA. After delivery of PMA (100 nM) to the cell for 20 s, VR1 receptor was re-activated by capsaicin (2 μM) in both control and diabetic rats, with a large inward current in diabetic rats compared with the controls (right panel). The data were normalized to the VR1 protein loaded on gels. CT, control; DM, diabetic. Values of the right panels (mean ± S.E.) are pixel densities corresponding to the protein bands at the left panel. * indicates significant difference ($p < 0.05$).
in the presence of PMA in both control and diabetic rats (Fig. 5). These data suggest that a PKC-dependent pathway modulates the properties of VR1 receptor through phosphorylation, and this pathway is altered in DRG neurons in diabetic rats.

Differential Changes of VR1 Expression in Subpopulations of DRG Neurons in Diabetic Rats—In parallel studies, we examined the VR1 expression level in DRG neurons by immunofluorescence staining using frozen-cut tissues. As shown in Fig. 6A, the neurons that were immunoreactive (IR) for VR1 were mostly small sized (diameter less than 30 μm). The total number of VR1 IR-positive neurons in DRGs in diabetic rats was reduced compared with that of control rats. The percentage of VR1-positive neurons was 52.1 ± 5.0% in control rats and 42.7 ± 1.9% in diabetic rats, respectively, among the 6300 DRG neurons counted in each group (Table I). This reduction in VR1 IR-positive neurons in DRGs in diabetic rats was significant (p < 0.01). We did not observe clear staining of VR1 at the cell surface by using frozen DRG sections as some studies demonstrated using VR1-transfected cell lines (39, 40), despite testing different titers of the anti-VR1 antibody. To explore the subcellular localization of VR1 receptor in DRG neurons, we acutely dissociated DRG neurons and plated them on cover glasses for culture for 24–48 h. When these cultured DRG neurons were immunostained using anti-VR1 antibody, a ring-like pattern of staining was clearly visible in some neurons (Fig. 6B). To analyze the cellular distribution of VR1, the ring-like stained neurons were viewed using a confocal fluorescence microscope. As shown in Fig. 6C, VR1 protein localized largely at the plasma membrane and was also present in intracellular structures, probably the endoplasmic reticulum or cytoplasmic vesicles (41, 42). Most interestingly, more neurons had a ring-like pattern of VR1 expression in diabetic rats than in control rats, as indicated by the increased percentage of VR1-positive neurons in diabetic rats compared with control rats (42.7 ± 1.9% vs. 52.1 ± 5.0%, p < 0.01).

![Fig. 4. Desensitization of VR1 receptor in small diameter DRG neurons from control and diabetic rats.](http://www.jbc.org/)

![Fig. 5. Re-sensitization of VR1 receptors by PMA after consecutive capsaicin-induced desensitization.](http://www.jbc.org/)
**Fig. 6.** Differential changes of VR1 expression in DRG neurons from diabetic rats. A, comparison of the representative immunofluorescence staining of DRG neurons between control (left) and diabetic rats (right) using anti-VR1 antibody. Scale bar, 50 μm. B, cultured DRG neurons from diabetic rats showed a clear ring-like staining pattern of VR1 receptors. Scale bar, 35 μm. C, confocal image of cultured DRG neurons that have clear ring-like staining pattern of VR1. Scale bar, 20 μm. D, statistical analysis of the percentage of DRG neurons that had a clear ring-like staining pattern of VR1 receptor. The percentage of the neurons that had a ring-like staining pattern significantly increased in diabetic rats compared with the control (n = 4, p < 0.01). E, double immunofluorescence labeling for VR1 and VRL-1 in DRG neurons. Compared with VR1, VRL-1 IR-positive neurons were mostly large diameter neurons. Scale bar, 50 μm. ***, p < 0.01.

**TABLE I**

Statistical analysis of the percentage of DRG neurons from control and diabetic rats after double immunofluorescence labeling using anti-VR1, anti-VRL-1, anti-peripherin (marker for C-fiber neurons), and anti-NF200 (marker for A-β fiber neurons) antibodies

The percentage counted for double labeling was based on the following criteria: in VR1 IR-positive neurons, neurons were also IR-positive for peripherin (peripherin-p) or NF200 (NF200-p). Compared with the control, the change in increase is marked as ↑, and the change in decrease is marked as ↓ in neurons from diabetic rats. Values are means ± S.E.

|                  | VR1 IR-positive | VRL-1 IR-positive | VR1 IR-positive |
|------------------|----------------|-------------------|----------------|
|                  | %              | %                 | Peripherin-p    |
| Control          | 52.1 ± 5.0     | 28.3 ± 2.5        | 70.7 ± 2.9      |
| Diabetic         | 42.7 ± 1.9*    | 29.1 ± 1.8        | 56.4 ± 2.6*     |
| Change           | ↓              | ↓                 | 23.8 ± 1.5*     |

* Value indicates significant difference between control and diabetic rats (p < 0.05).
staining in diabetic rats compared with the control. The number of neurons that had a ring-like staining pattern was 15 ± 3% in control rats and 40 ± 7% in diabetic rats (Fig. 6D), respectively, and the difference between them was significant (n = 4, p < 0.01). These results together with increased tetramer form of VR1 receptor support the increased distribution of VR1 receptor on cell surface membranes in diabetic peripheral sensory neuropathy.

Inflamed tissue demonstrates increased expression of VR1 analogue VRL-1 (43), which is sensitive to higher temperature (>52 °C) but insensitive to capsaicin and protons (44). To test the possible up-regulation of VRL-1 in diabetic rats, we localized VR1 and VRL-1 in DRG neurons by immunofluorescence staining. Fig. 6E shows the colocalization of VR1 and VRL-1 in DRG neurons from control and diabetic rats. The VRL-1 IR-positive were mostly large diameter cells in both control and diabetic rats, which is consistent with previous studies (44–46). The percentage of VRL-1 IR-positive neurons did not change in DRGs in diabetic rats compared with the controls (Table I). These results indicate that the expression pattern of VRL-1 is not altered in DRGs in painful diabetic neuropathy.

**Diabetic Neuropathy Is Associated with Increased Expression of VR1 in A-fiber Neurons**—Generally, peripheral sensory DRG neurons are classified as large myelinated A-fiber neurons that transmit signals about non-noxious stimuli and small unmyelinated C-fiber or thinly myelinated A-δ fiber neurons that transmit information about thermal and noxious stimuli (47, 48). However, the respective roles of A-fibers and C-fibers in chronic pain sensation remain unresolved (43, 49). To examine the expression of VR1 in large versus small DRG neurons, we colocalized VR1 by immunofluorescence staining with peripherin, a marker of small unmyelinated C-fiber and thinly myelinated A-δ fiber neurons (14, 16, 50), and NF200, a marker of large myelinated A-fiber neurons (43, 51). As shown in Fig. 7A, the neurons double IR-positive for VR1 and peripherin decreased in diabetic rats as compared with the control. In VR1 IR-positive neurons, the neurons also IR-positive for peripherin was 70.7 ± 2.9% in control rats, and this percentage was significantly lower in diabetic rats (56.4 ± 2.6%) as shown in Table I (n = 5, p < 0.05). Fig. 7B shows the double labeling for VR1 and NF200 in DRGs from control and diabetic rats. An up-regulation of VR1 expression in large sized DRG neurons was observed in diabetic rats compared with the control. In VR1 IR-positive neurons, the neurons also IR-positive for NF200 was 23.8 ± 1.5% in diabetic rats and 11.7 ± 2.3% in control rats, respectively, and the difference between these two values was significant (n = 5, p < 0.05) (Table I). The up-regulation of VR1
expression in A-fiber neurons suggests that large DRG neurons may play a role in the painful diabetic neuropathy.

**DISCUSSION**

To our knowledge, this is the first report demonstrating differential changes in the protein levels and function of VR1 receptor in DRG neurons in painful diabetic neuropathy. The function of VR1 receptor-channel complex was significantly enhanced in small sized DRG neurons as both capsaicin and protons (low pH) activated significantly larger inward currents in neurons from diabetic rats compared with the controls. Of interest, the total protein level of VR1 was decreased in DRGs from diabetic rats, but a significant increase in the VR1 level was observed on the cell surface membranes, possibly representing the formation of more channels. Consistent with this interpretation, the tetrameric form of VR1 (most likely the native structure of the functional VR1 receptors) was increased in DRGs from diabetic rats, supporting increased numbers of functional VR1 receptors on the cell surface membranes. Moreover, impaired desensitization and enhanced re-sensitization of the VR1 receptor via a PKC-dependent mechanism also likely contributed to the increased function of this receptor in diabetic rats. Therefore, both the number and function of VR1 channels were increased. Previous studies in our lab and in others demonstrated the development of thermal hyperalgesia and mechanical allodynia in the early stages of diabetic mellitus (5, 6). These data suggest that modulation of VR1 function and/or expression is involved in the sensation of neuropathic pain.

Like other ion channels the activity of VR1 is modulated by phosphorylation and/or dephosphorylation. It has been reported recently that the phosphorylation of VR1 is required for its ligand binding (35). Increased phosphorylation of VR1 in DRGs from diabetic rats suggests that the number of channels available for ligand binding is enhanced in diabetic rats because only modest phosphorylation of VR1 was observed in DRGs from control rats. The increased phosphorylation of VR1 increases the open probability of the channel gating (34) or promotes and/or stabilizes channel cell surface expression, resulting in increased cation influx into the cell, which may contribute to the cellular basis for hyperalgesia or allodynia observed in painful diabetic neuropathy. Furthermore, desensitization of VR1 was impaired in diabetic rats (Fig. 4). After the VR1 channel has been opened by capsaicin, an increase in intracellular Ca$^{2+}$ could activate the phosphatase calcineurin, which leads to dephosphorylation of VR1 and therefore desensitization (35). With repeated stimuli, the impairment of VR1 desensitization would keep channels open and allow continued cation influx into the cell, contributing to persistent hyperactivity. In addition, capsaicin activates larger inward current in diabetic DRG neurons than the controls when VR1 receptor is re-sensitized in a PKC-dependent manner after desensitization. This suggests that phosphorylation/dephosphorylation process that regulates the function of VR1 is altered in painful diabetic neuropathy. Consistent with this, we observed increased phosphorylation of VR1 at serine residues in diabetic rats. This likely occurs at Ser$^{502}$ and Ser$^{800}$ because application of PMA triggers the phosphorylation of VR1 at these two sites and potentiates the capsaicin-evoked current in transfected HEK293 cells. The double Ser$^{502}$/Ser$^{800}$ mutant demonstrated no potentiation of VR1 currents evoked by capsaicin and PMA application (37).

The binding domain in VR1 for capsaicin and protons are different (52, 53). The capsaicin binding domain appears to be intracellular (40), whereas protons are thought to act by binding to the extracellular surface of the protein (15, 16, 54). In diabetic rats, both capsaicin- and proton-activated inward currents were significantly up-regulated and completely blocked by the capsaicin antagonist capsazepine, indicating that capsazepine is binding to a site different from the capsaicin-binding site. Our findings differ with earlier functional studies on sensory neurons (55) but are consistent with recent studies (16, 56). It is likely that capsazepine interacts with a distinct site that conformationally stabilizes the channel in a closed state that prevents the channel from opening after application of agonists that act either intra- or extracellularly (53). Single-channel analysis of VR1 current reveals that protons enhance the binding affinity of capsaicin, promote the channel opening, and stabilize the open conformations of the channel (57). It is not known whether diabetes alters the binding affinity of protons and/or capsaicin sites in DRG neurons. In addition, the mechanisms underlying the endogenous activation of the VR1 receptor under normal physiological conditions are also not known. Currently, three classes of putative endogenous ligands for VR1 have been identified, i.e. N-arachidonoyldopamine, lipoxigenase products of arachidonic acid, and anadamide (58, 59). We propose that in diabetic rats the levels of the endogenous ligands of VR1 are increased in DRGs and thus VR1 receptors maintain high activity that leads to hyperalgesia. Further studies are required to investigate the nature of the activation pathways of the channel evoked by different stimuli as well as the mechanisms underlying the cross-sensitization in painful diabetic neuropathy.

Several studies have reported that the functional VR1 receptor is most likely composed of more than one subunit, forming homomultimeric or heteromultimeric ion channels (27, 60). Consistent with this observation, we found that VR1 did oligomerize to tetrameric form in DRG neurons at plasma membrane in both control and diabetic rats. Moreover, the tetramer form of VR1 increased in DRG neurons from diabetic rats, suggesting that more functional channels were formed, although the total protein was down-regulated. The subcellular localization of VR1 channels remains controversial. By using acutely dissociated DRG neurons, we observed localization of VR1 on cell surface plasma membrane of DRG neurons that significantly increased in diabetic rats, supporting the requirement of VR1 localization at cell surface plasma membrane in patch clamp single-channel analysis studies (14). It is likely that the localization of VR1 is cell type-specific because VR1 is predominantly endogenous in transfected COS-7 cells (61) but localizes specifically on plasma membrane in DRG-derived F-11 cells (39). We propose that the localization of VR1 is also likely modulated by functional requirements under certain conditions such as hyperalgesia.

In the peripheral nervous system VR1 is expressed mainly on unmyelinated C-fibers and thinly myelinated A-δ fibers (14). C-fiber hyperactivity has been reported in diabetic neuropathic pain (49). The expression of VR1 was decreased in small C-fibers and A-δ fibers (peripherin IR-positive) neurons in DRGs from diabetic rats, which is consistent with the down-regulation of total VR1 protein. This suggests the possibility of a compensatory mechanism to reduce the increased activity of VR1 channels in painful diabetic neuropathy, given that C-fibers are the primary conveyors of pain in diabetes mellitus. In contrast, the expression of VR1 increased in large myelinated A-β fiber (NF200 IR-positive) neurons in diabetic rats. This subpopulation of primary afferent neurons demonstrates a low level of expression of VR1 in controls. It is possible that VR1 receptors expressed in large myelinated A-β fiber neurons may act as modulators to transmit pain signals or maintain the peripheral and central sensitization in neuropathy states because desensitization of nociceptive C-fibers by resiniferatoxin could not prevent the development of hyperalgesia in diabetic rats (4). However, it is not likely that A-β fiber neurons assume...
the role of C-fiber neurons in the painful diabetic neuropathy because a large population of small C-fiber neurons still expresses VR1 receptor in diabetic rats. Further detailed analysis of the expression and function of VR1 on C-fiber and Aβ fiber neurons are necessary to clarify the roles of subpopulations of DRG neurons in painful diabetic neuropathy.

In conclusion, we demonstrate that early painful diabetic neuropathy is associated with enhanced function of VR1 in DRG neurons. The enhanced function of VR1 receptor involves increased phosphorylation, oligomerization, and reallocation channels to cell surface plasma membrane. Impaired desensitization of VR1 may also contribute to regulation of the excitability of sensory neurons that mediates neuropathic pain. These data provide a rationale for targeted therapeutic intervention to modulate VR1 expression and/or function.

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