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# Role of Transient Receptor Potential Channels Trpv1 and Trpm8 in Diabetic Peripheral Neuropathy

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## Abstract

**Objective:** Transient Receptor Potential (Vanilloid 1) TRPV1 and (Melastatin 8) TRPM8 are heat and cold sensing non-selective cation channels, respectively. We sought to correlate the modulation of TRPV1- and TRPM8-mediated membrane currents and altered thermal sensitivity in Diabetic Peripheral Neuropathy (DPN).

**Method:** Streptozotocin (STZ)-induced diabetic mice were used and thermal (heat and cold) pain sensitivities were determined using hot plate and acetone drop test, respectively. Membrane currents were recorded using patch-clamp techniques.

**Results:** First, we tested thermal pain sensitivities to implicate possible role of TRPV1 and TRPM8 in DPN. Paw withdrawal latency on a hot plate test was decreased and acetone-induced cold sensitivity was enhanced in diabetic mice as compared to non-diabetic mice. Dorsal Root Ganglion (DRG) neurons dissociated from diabetic hyperalgesic mice exhibited an increase in TRPV1-mediated current and a decrease in TRPM8-mediated currents as compared to non-diabetic mice. Then, we determined the modulation of TRPV1- and TRPM8-mediated currents using HEK cells heterologously expressing TRPV1 by promoting of PKC- and PKA-mediated phosphorylation. Both Phorbol 12,13-Dibutyrate (PDBu), a PKC activator and forskolin, a PKA activator upregulated TRPV1-mediated currents, but downregulated TRPM8-mediated currents. In diabetic mice, intraplantar injection of capsaicin, a TRPV1 agonist induced nocifensive behavior but the severity of this behavior was significantly lower when co-administered with menthol, a TRPM8 agonist.

**Conclusions:** These findings suggest that diabetic thermal hyperalgesia mediated by up regulation of TRPV1 function may be further aggravated by the down regulation of TRPM8 function. Targeting TRPV1 may be a useful approach to alleviate pain associated with DPN.

**Keywords:** Capsaicin; Diabetic Peripheral Neuropathy; Menthol; TRPV1; TRPM8

## Introduction

One of the major complications of diabetes is Diabetic Peripheral Neuropathy (DPN) [1]. The symptoms of DPN range from spontaneous pain, allodynia, hyperalgesia, numbness, dysesthesia, paresthesia, and ulceration in the extremities [2-5]. Recent studies [6,7], including our own [8] suggest that specific types of neurons carry particular sensation and this distinction occurs at the level of peripheral sensory neurons. A subset of small and medium diameter sensory neurons (C and Aγ) respond solely to noxious stimuli. It is becoming apparent that C-fibers that express TRPV1 carry inflammatory thermal hypersensitivity, whereas a population of non-TRPV1 expressing neurons that express the G-protein coupled receptor MrgprD carry mechanical sensitivity [9]. On the other hand, TRPM8 which was previously called menthol and cold receptor 1 (CMR1) was shown to play a role in cold sensation and is activated by temperatures lower than ~25-28°C, cooling agents such as menthol and icilin [10-14]. Activation of either TPRV1 or TRPM8 allows flux of $Na^+$ and $Ca^{2+}$, resulting in the membrane depolarization and tachyphylaxis.

Previous studies using STZ-induced diabetic models indicate that there are two pain phenotypes in rodents with hyperalgesia followed by hypoalgesia [15-17]. Studies suggest that hyperalgesia phenotype is characterized by the greater release of capsaicin-evoked CGRP from the hind paw [18], increased TRPV1 activity in primary sensory neurons of the spinal cord and dorsal root ganglions [15,18]. However, the role of TRPM8 in diabetic neuropathy is not known. Both TRPV1 and M8 are modulated by cellular pH, $PIP_2$, PKC and PKA altering threshold of activation and in turn affecting the sensitization of neuron. Menthol-induced TRPM8 sensitization in the presence of acidic pH, $PIP_2$ and PKC was reduced whereas capsaicin-induced TRPV1 sensitization was increased [19-21]. These studies suggest that inflammatory mediators while up regulating TRPV1 activity might down regulate TRPM8 activity aggravating the pain condition resulting in hyperalgesia phenotype [21]. Localized and topical treatments are becoming recognized treatment options for painful conditions. Recently, high dose of capsaicin (8%) transdermal patch, which is a localized treatment that provides effective durable pain relief from single application in patients with peripheral neuropathic pain [22-25]. In a direct comparison, the capsaicin 8% patch has been shown to be noninferior to pregabalin in the control of neuropathic pain but has a faster onset of analgesia with fewer systemic side effects [25,27].

The primary aim of this study is to investigate the molecular mechanisms underlying deficiencies in temperature sensation in diabetic mice. We have used STZ-induced diabetic mouse models to identify altered heat and cold sensitivities. We have measured TRPV1 and TRPM8 channel currents and their modulation by PKC and PKA in the presence and absence of extracellular calcium from dorsal root ganglion neurons. Our results suggest that in the first week of diabetes mice exhibited increased thermal sensitivities to both heat and cold. Diabetic thermal hyperalgesia is associated with upregulation of TRPV1 but down regulation of TRPM8 activity. Furthermore, PKC- and PKA-mediated modulation of TRPV1 and TRPM8 alter thermal sensitivities was confirmed using heterologous expression system.

## Materials and Methods

### Induction of Diabetes by STZ

The experiments were performed according to the National Institutes of Health guidelines and approved by the animal welfare committee of Southern Illinois University School of Medicine. Age-matched mice were used for induction of diabetes with STZ. STZ (Sigma, St Louis) was prepared fresh in saline adjusted to pH 4.5 with 0.1N citrate buffer [28]. A single intraperitoneal injection of STZ (200mg/kg) in mice induced diabetes in 38 of 51 mice. 0.1 N citrate buffer injected mice served as control. Glucose levels were quantitated with a One Touch Ultra blood glucose monitoring system (Life Scan, California) using whole blood obtained from the tail. Diabetes was defined as blood glucose concentrations greater than 299 mg/dl (16.7 mM) [22,28].

### Hot Plate Test for Thermal Sensitivity in Mice

All the mice used in this study were housed in the barrier facility and were tested on the days the cages were not cleaned. Mice were placed individually on a Hot Plate Analgesia Meter (Harvard Apparatus, Boston, MA) maintained at a constant temperature of 52±0.3°C after observing them for 5 five minutes in the cage. The Paw Withdrawal Latency (PWL) was recorded as described previously [15]. These studies were blinded and after completing the test, the ear tags were read to place them in the appropriate groups. Since PWL of male and female mice did not have significant differences, the data were combined.

### Acetone Test for Cold Sensation in Mice

Acetone drop (50-70µL) was applied on the left plantar surface of the mice placed on a metal wire mesh enclosed in a chamber having a perforated roof. Acetone bubble was formed at the tip of the 1cc syringe and gently applied while the animal stood on the mesh. Foot lift frequency (flicking, licking or biting the limb) was counted in a period of 5 minutes after administering acetone on the plantar surface of the paw. This procedure was repeated for three times with a gap 10minutes. The average of the responses obtained from 3 acetone applications was taken as foot lift frequency and plotted against days of testing [29,30].

### Measurement of Nocifensive Behavior

Nocifensive behavior was examined in mice after intraplantar injection of TRPV1 and TRPM8 agonists, capsaicin (2 mM), and menthol (5 mM), respectively. 20µL of the drug was injected into the hind limb paw by using Hamilton syringe (Hamilton Company, Reno, Nevada, USA). Nocifensive behavior was characterized by the latency, duration, and number of licking and/or shaking of the injected hindlimb.

### Adult DRG Neuronal Cultures

DRG were dissected from diabetic and control mice euthanized by deep anesthesia with isoflurane followed by decapitation. Isolated DRG were collected in HBSS (calcium- and magnesium-free) on the ice and then enzymatically digested for 45 minutes at 37°C in 0.1% collagenase D, Worthington type 2 (Roche Diagnostics, Indianapolis, IN) and 0.1% trypsin, type 1 (Sigma-Aldrich, St. Louis, MO). After trituration using fire-polished Pasteur pipettes, DRG were plated on 12 mm poly D-lysine coated glass coverslips placed in 24 well plates and grown in neurobasal medium (Gibco BRL, Grand Island, NY) supplemented with L-glutamine (2 mM, Invitrogen, Grand Island, NY) and 10 µl/ml B27-supplement (Gibco, Invitrogen corporation, GrandIsland, N.Y). Neurons were maintained at 37°C in a humidified atmosphere of 5% $CO_2$ and were used within 8-10 hours.

### HEK 293 Cells Culture

HEK293 cells, stably expressing rat TRPM8 cDNA, were obtained from Dr D. Julius (University of California, San Francisco, San Francisco, CA). They were and cultured in growth medium containing 90% DMEM, 10% fetal bovine serum, 50 U/ml penicillin, 25 µg/ml streptomycin (all from Invitrogen, Grand Island, NY). Cells were grown at 37°C, in the presence of 5% $CO_2$ and 95% ambient air. Cells were cultured in the presence of G418 sulfate, neomycin-resistant cells were expanded, and frozen stocks were kept in liquid nitrogen.

### Whole Cell Patch Clamp Recording

Whole-cell patch-clamp recordings were performed in DRGs that were isolated from age matched diabetic and control mice. The bath and pipette were filled with sodium based solution and were prepared as described previously [15,21]. $Ca^{2+}$-free solutions were used to avoid desensitization and tachyphylaxis of capsaicin-induced currents. Currents were recorded using a WPC 100 patch-clamp amplifier (E.S.F. Electronic, Goettingen, Germany) using borosilicate pipettes as described previously [15,21]. Capacitance compensation and series resistance compensation (>70%) were done before recording currents. Membrane capacitance as obtained from the compensations and also calculated by determining the $\tau$ to an applied square pulse ($\tau$=RC). Data were filtered at 2.5kHz, digitized at 5 kHz (VR-10B; Instrutech, Great Neck, NY) and stored on the computer using a Labview interface (National Instruments, Austin, TX). Current amplitudes were measured using Channel 2 (software kindly provided by Michael Smith, Australian National University, Canberra, Australia). The traces and graphs were made using Origin (Microcal Software, Northampton, MA).

### Data Analysis

For behavioral experiments, a mixed model analysis was performed using SAS software, which includes both fixed events (age and time after diabetes onset) and random events (number of subjects) with repeated measures of ANOVA. The comparisons were made between the control group and the diabetic groups. Data are shown as mean±S.E.M. (Standard Error of Mean). The current amplitudes were normalized and expressed as fold change as compared to control responses. Significance was tested using unpaired Student's t-test, and the data are considered significant at $p<0.05$.

All the chemicals used in this study were obtained from Sigma (St. Louis, MO).

## Results

### Altered Heat and Cold Pain Sensitivity Following STZ-Induced Diabetes

Blood glucose levels were significantly elevated by 1 week after STZ treatment and remained elevated during the course of the study (pre- STZ injection, 187±2.7 mg/dl; day 12, 532.5±23.9 mg/dl; n=10 mice, $p<0.001$) as compared to vehicle injected mice (pre-vehicle injection, 178.8±2.2 mg/dl; day 12, 194.6±12.5 mg/dl, n=10 mice) (Figure 1A). As disease progressed, body weights of STZ-treated diabetic mice decreased, whereas the body weights of control animals increased steadily (diabetic mice; pre- STZ injection, 22.1±0.3 gms; day 12, 20.8±0.7 gms; n=10 mice) (pre-vehicle injection, 22.7±0.6 gms; day 12, 23.7±0.4 gms, n=10 mice, $p<0.001$) (Figure 1B).

Diabetic mice tested on hotplate were hyperalgesic within a week indicated by a decrease in PWL (prediabetic 9.6±0.3s; day 12, 7.1±0.4s, n=33, $p<0.001$ compared with control group). Vehicle injected mice did not show altered thermal pain sensitivity (pretreatment 9.4±0.3 s; day 12, 9±0.3s) (Figure 1C). Following acetone administration, the number of flickerings and lickings was significantly higher in diabetic mice (day 0, 11.7±0.6; day 12, 17.8±1.4, n=10 mice, $p<0.01$) as compared to non-diabetic mice (day 0, 10.7±0.4; day 12, 11.7±1.3, n=10 mice) (Figure 1D).

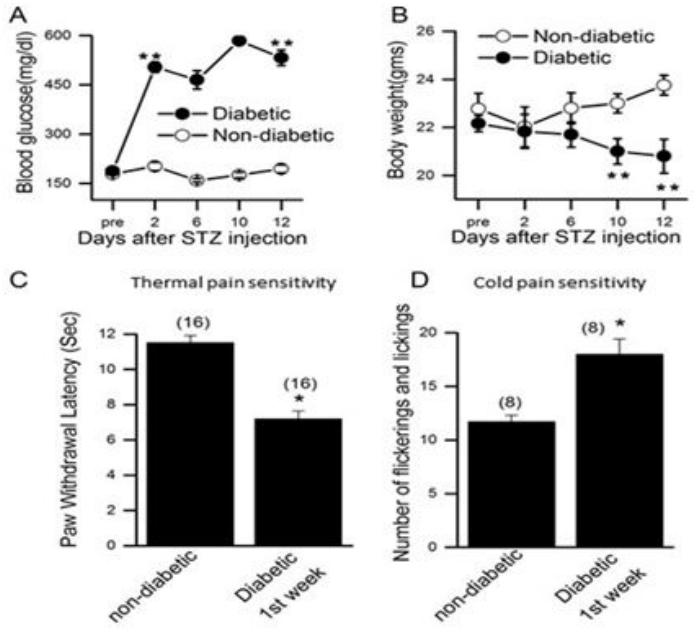


**Figure 1(A-D):** Blood Glucose Levels, Body Weight Changes, Heat and Cold Pain Sensitivities in STZ-Induced Diabetic Mice.A. Blood glucose levels of STZ-injected diabetic mice were significantly increased (filled circles, n = 10 mice, $p<0.01$) in a week and remained constant throughout the course of the experiment as compared to non-diabetic mice (open circles, n = 10 mice) B. Body weights of diabetic mice decreased (filled circles, n = 10 mice) as compared to non-diabetic mice (open circles, n = 10 mice). C, D. Bar graph of the PWL (C) and number of flickerings and lickings (D) in the first week of diabetes compared to non-diabetic mice. *$p < 0.05$, **$p < 0.001$ compared with control group.

### Altered TRPV1 Currents in Diabetic Hyperalgesia

The peak currents in response to application of capsaicin (1 µM) were significantly larger (3.74±0.27 fold, n=5 cells from 2 mice, $p<0.03$) in hyperalgesic mice as compared to control mice

(1±0.12 fold n=12 cells from 4 mice) (Figure 2A, B). In order to take the cell size into consideration, we normalized the current to the cell capacitance. The current density is found to be significantly higher in hyperalgesic (2.81±0.19 fold, n=5, p<0.05) as compared to normal non-diabetic mice (1±0.18 fold n=12) (Figure 2C).

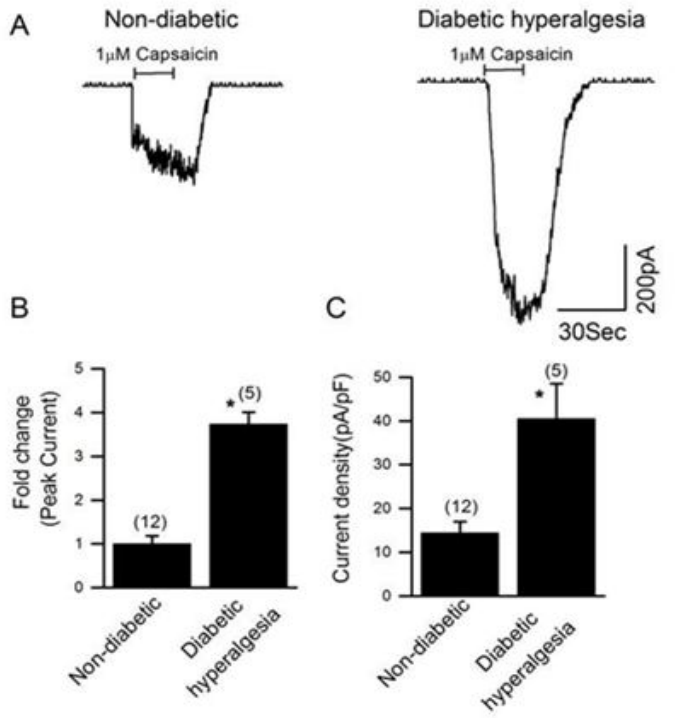


**Figure 2(A-D):** Diabetic Hyperalgesic Mice Exhibit Increased TRPV1 Currents. **A**. Representative current traces of increased 1μM capsaicin-evoked TRPV1 currents in diabetic hyperalgesic mice as compared to control mice. **B, C**. Summary graph showing fold change in increased TRPV1 peak currents and current densities (non-diabetic: n=12 cells; diabetic: n=5 cells) in diabetic hyperalgesic mice as compared to non-diabetic control mice. The number in the parenthesis represents the number of DRG neurons studied and the asterisk (*) represents p<0.05 obtained from students unpaired t-test.

## Altered TRPM8 Currents in Diabetic Hyperalgesia

To determine whether TRPM8 channel function is altered in diabetes-induced hyperalgesia, we recorded 1mM menthol-induced TRPM8 whole cell currents from acutely dissociated DRG from diabetic hyperalgesic and non-diabetic mice. In contrast to our hypothesis, menthol-induced TRPM8-mediated peak currents were significantly smaller (0.54±0.13 fold, n=7 cells from 2 mice, p<0.03) (Figure 3A and B) in diabetic hyperalgesic mice as compared to non-diabetic mice (1±0.09 fold, n=6 cells from 4 mice). When the currents were normalized to the capacitance of the cells, we noticed a similar decrease (0.33±0.15 fold, n=7, p<0.05) as compared to normal non-diabetic mice (1±0.09 fold n=6) (Figure 2C).

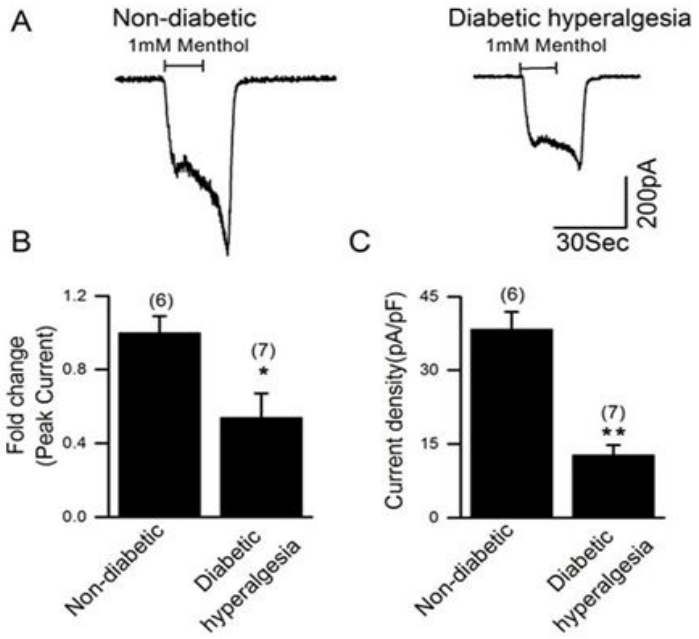


**Figure 3(A-C):** Diabetic Hyperalgesic Mice Exhibit Reduced TRPM8 Currents. **A**. Representative traces showing reduced 1mM Menthol-evoked TRPM8 currents in DRG of diabetic hyperalgesic mice as compared to control mice. **B, C**. Summary graph showing fold change in reduced TRPM8 peak currents and current densities (diabetic n=7 cells) in diabetic hyperalgesic mice as compared to non-diabetic mice (n = 6 cells). The number in the parenthesis represents the number of DRG neurons studied, and the asterisks (*, **) denote p<0.05 and p<0.004 obtained from students unpaired t-test.

## Modulation of TRPV1 by PKC

To determine the modulation by PKC, we used HEK cells heterologously expressing TRPV1. First, whole cell currents were recorded in the presence (2mM calcium) and absence of calcium (1.5mM EGTA) as it has been shown that TRPV1 currents undergo desensitization/tachyphylaxis ($-Ca^{2+}$, pre 1±0.13, n = 4 cells; post, 0.96±0.04 fold, n = 4 cells; $+Ca^{2+}$, pre 1±0.1, n = 4 cells; post, 0.37±0.04 fold, n = 4 cells) (Figure 4). We recorded currents before and after activation of PKC by incubating cultured DRG neurons for 5 minutes with PKC activator PDBu (1μM). Both in the absence and presence of calcium, PKC activation potentiated capsaicin-induced (100nm) TRPV1-mediated currents ($-Ca^{2+}$ pre PDBu 1±0.1, n = 8 cells; post PDBu 2.33±0.26 fold, n = 8 cells, p<0.05; $+Ca^{2+}$ pre PDBu 1±0.1, n = 8 cells; post PDBu 2.2±0.3 fold, n = 8 cells, p<0.05) (Figure 5 A, B, C).

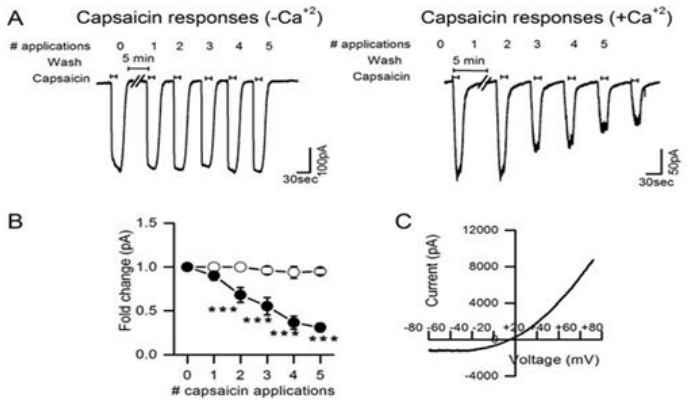


**Figure 4(A-C):** TRPV1-Mediated Whole Cell Currents in the Absence and Presence of Calcium. A. Representative capsaicin (1μM)-evoked TRPV1 channel currents in the absence (left trace) and presence (right trace) of calcium (2 mM) (a gap of 5 min is denoted by a break in the trace). B. Repeated capsaicin applications did not show any change in the current amplitude in the absence of calcium (open circles, n=8 cells), but in the presence of calcium, there is gradual decrease in the current amplitude (tachyphylaxis) (closed circles, n=8 cells. C. A ramp (-80-+80 mv) current voltage relationship of capsaicin induced currents showing outward rectification. Data are represented as mean±SEM. 0 represents capsaicin application at time 0 and 1 to 6 represents number of capsaicin applications.

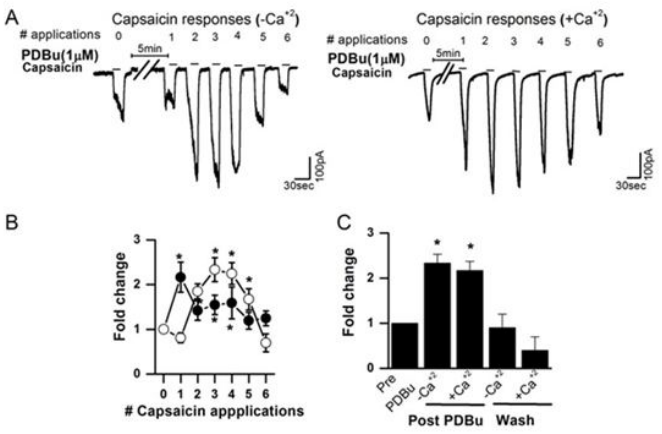


**Figure 5(A-C):** Activation of PKC Potentiates TRPV1-Mediated Whole Cell Currents both in the Absence and Presence of Calcium. **A**. Representative capsaicin (1μM)-evoked TRPV1 channel currents before and after phorbol 12,13-dibutyrate (PDBu) incubation (1μM for 5 min denoted by a break in the trace) in the absence (left trace) and presence (right trace) of calcium. **B**. Changes in repeated capsaicin-induced currents after PDBu incubation in the presence (filled circles, n = 8 cells) and absence of calcium (open circles, n = 8 cells). **C**. Summary graph showing capsaicin-induced currents are potentiated significantly after PDBu incubation ($p < 0.05$). Data are represented as mean±SEM. 0, represents capsaicin application before PDBu and 1 to 6 represents the number of capsaicin applications after PDBu incubation. The number in the parenthesis represents the number of cells and the asterisk (*) denotes $p < 0.05$. PDBu denotes Phorbol 12,13-dibutyrate.

## Modulation of TRPV1 by PKA

In our studies we questioned whether TRPV1 sensitization by PKA activation plays a role in diabetic hyperalgesia. To address this question, TRPV1-mediated whole cell currents were recorded in cultured DRG neurons incubated in the presence of an adenylyl cyclase activator (FSK-forskolin, 20μM) and phosphodiesterase inhibitor (IBMX, 50μM) for 5 minutes. Forskolin activates adenylyl cyclase increasing the levels of cAMP, which in turn increases PKA activity and IBMX prevents the breakdown of cAMP by inhibiting the phosphodiesterase activity. Our results suggest that activation of PKA sensitizes TRPV1 activity both in the presence and absence of calcium ($-Ca^{2+}$ , pre FSK+IBMX 1±0.13, n = 5 cells; $-Ca^{2+}$ , Post FSK+IBMX, 1.89±0.43, n = 5 cells, $p<0.05$) ($+Ca^{2+}$, pre FSK+IBMX, 1±0.13, n = 7 cells; $+Ca^{2+}$ , Post FSK+IBMX, 2.45±0.5, n = 7 cells, $p<0.05$) (Figure 6 A, B, C).

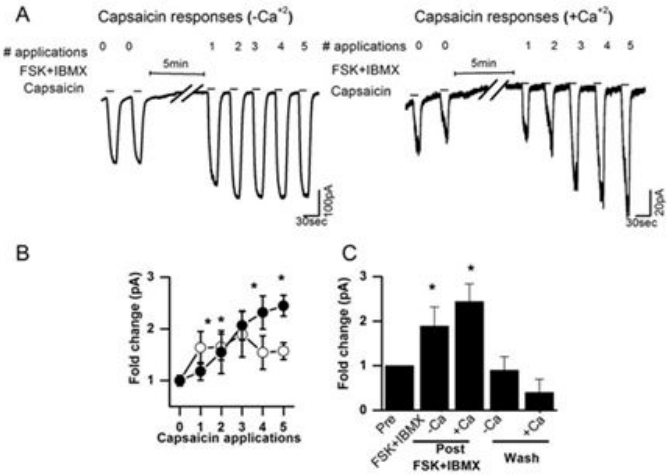


**Figure 6(A-C):** Activation of PKA Potentiates TRPV1-Mediated Whole-Cell Current both in the Absence and Presence of Calcium. **A**. Representative capsaicin (1μM )-evoked TRPV1 channel currents before and after 20μM FSK+ 50μM IBMX incubation (5 min denoted by a break in the trace) in the (1μM; 5 min denoted by a break in the trace) in the absence (left trace) and presence (right trace) of calcium **B**. repeated capsaicin applications induces currents after 20μM FSK+ 50μM IBMX incubation in the presence (filled circles, n = 7 cells) and absence of calcium (open circles, n = 5 cells) C. Summary graph showing capsaicin-induced currents are potentiated ($p < 0.05$) after 20μM FSK+ 50μM IBMX incubation and no change after wash application both in the presence and absence of calcium. 0, represents capsaicin application before FSK+IBMX and 1 to 5 represents the number of capsaicin applications after FSK+IBMXC. The number in the parenthesis represents the number of cells studied and the asterisk (*) denotes $p < 0.05$. FSK: Forskolin; IBMX: 3-isobutyl-1-methylxanthine.

## Modulation of TRPM8 by PKC

In the presence of extracellular Ca2+, menthol induced currents in HEK cells heterologously expressing TRPM8 showed tachyphylaxis ($-Ca^{2+}$, pre, 1±0.11, n = 3 cells; post, 0.91±0.04 fold, n = 3 cells; $+Ca^{2+}$, pre 1±0.1, n = 4 cells; post, 0.67±0.09 fold, n =

4 cells) (Figure 7). To study the modulation of TRPM8-mediated currents by PKC, menthol-induced TRPM8 whole cell currents were recorded from HEK293 cells expressing TRPM8 following incubation with a PKC activator PDBu (1μM) for 5 minutes. We recorded currents both in the presence (2mM $Ca^{2+}$) and absence of calcium (1.5mM EGTA), as Ca2+ induces desensitization/tachyphylaxis of TRPM8 induced currents (Figure 7). PKC activation decreases TRPM8 channel activity (-$Ca^{2+}$ pre PDBu 1, n = 3; post PDBu, 0.44±0.13, n = 3, p<0.05; +Ca2+ pre PDBu 1, n = 4; post PDBu, 0.53±0.13, n = 4, p<0.05) (Figure 8A, B and C).

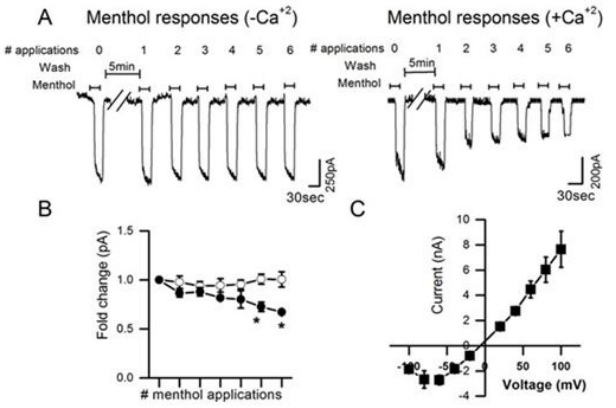


**Figure 7:** TRPM8-Mediated Whole-Cell Current in the Absence and Presence of Calcium. **A**. Representative Menthol (1 mM)-evoked TRPM8 channel currents (a 5-min gap is denoted by a break in the trace) in the absence (left trace) and presence (right trace) of calcium (2 mM). **B**. Repeated menthol applications did not show any change in the current amplitude in the absence of calcium (open circles, n=3 cells), but in the presence of calcium, there is a gradual decrease in the current amplitude (tachyphylaxis) (closed circles, n=4 cells. **C**. Current-voltage relationship of menthol-induced currents showing outward rectification. Data are represented as mean±SEM. 0, represents menthol application at time 0 and 1 to 6 represents the number of menthol applications.

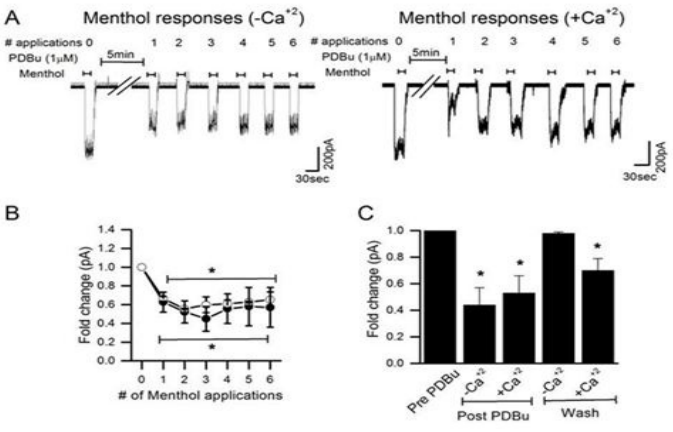


**Figure 8(A-C):** Activation of PKC Downregulates TRPM8-Mediated Whole-Cell Current both in the Absence and Presence of Calcium. **A**. Representative Menthol (1mM)-evoked TRPM8 channel currents before and after phorbol 12,13-dibutyrate (PDBu) incubation (1μM; 5 min denoted by a break in the trace) in the absence (left trace) and presence (right trace) of calcium. **B**. Repeated applications of menthol-induced currents after PDBu incubation in the presence (filled circles, n = 3 cells) and absence of calcium (open circles, n =4 cells) **C**. Summary graph showing menthol-induced currents are inhibited significantly after PDBu incubation ($p < 0.05$) and no change after wash application both in the presence and absence of calcium. 0, represents capsaicin application before PDBu and 1 to 6 represents the number of capsaicin applications after PDBu incubation. The number in the parenthesis represents the number of cells and the asterisk (*) denotes $p < 0.05$. PDBu denotes Phorbol 12,13-dibutyrate.

## Modulation of TRPM8 by PKA

TRPM8 whole cell currents mediated by TRPM8 were recorded from HEK293 cells incubated (5 min) with an adenylyl cyclase activator (FSK-forskolin, 20μM) and phosphodiesterase inhibitor (IBMX, 50μM) (increases the levels of cAMP by preventing it from degradation by phosphodiesterases). Both in the presence and absence of calcium we noticed a decrease in TRPM8 activity (-$Ca^{2+}$, pre FSK+IBMX, 1±0.04, n = 6 cells; post FSK+IBMX, 0.43±0.05, n = 6 cells, p<0.05) (+$Ca^{2+}$, pre FSK+IBMX, 1±0.03, n = 3 cells; post FSK+IBMX, 0.53±0.17, n = 3 cells, p<0.05) (Figure 9 A, B and C). The current amplitude remained constant in experiments where the kinases were not activated either in the presence or absence of calcium (TRPM8; –$Ca^{2+}$, pre wash 1±0.11, n = 3 cells; post wash, 0.91±0.04 fold, n = 3 cells; +Ca2+, pre wash 1±0.1, n = 4 cells; post wash, 0.67±0.09 fold, n = 4 cells).

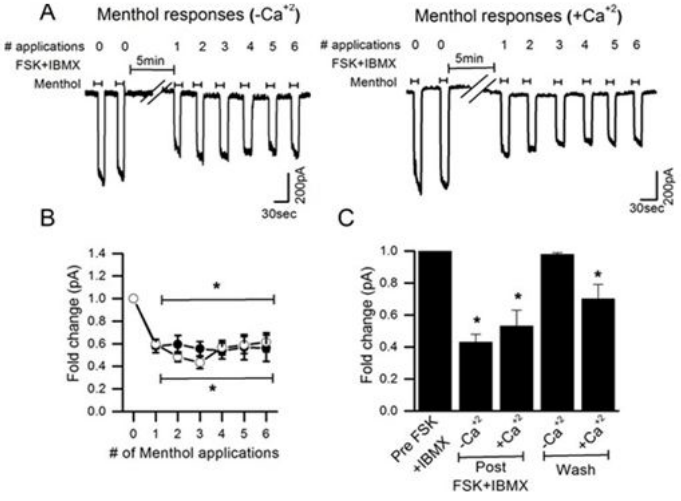


**Figure 9(A-C):** Activation of PKA Downregulates TRPM8-Mediated Whole-Cell Current both in the Absence and Presence of Calcium. **A**. Representative Menthol (1mM)-evoked TRPM8 channel currents before and after application of FSK + IBMX (20μM and 50μM; 5 min denoted by a break in the trace) in the absence (left trace) and presence (right trace) of calcium **B**. repeated applications of menthol-induced currents before and after 20μM FSK+ 50μM IBMX incubation in the presence (filled circles, n = 6 cells) and absence of calcium (open circles, n = 3 cells) and C. Summary graph showing menthol-induced currents are inhibited significantly after 20μM FSK+ 50μM IBMX incubation and no change after wash ap-

plication both in the presence and absence of calcium. The number in the parenthesis represents the number of cells responses and the asterisk (*) represents $p < 0.05$. 0 represents pre FSK+IBMX applications, and 1 to 6 represents the number of menthol applications after FSK+IBMX.

## Capsaicin Alone or Combined with Menthol-Induced Nocifensive Behavior is Altered in Diabetic Mice

To determine the physiological relevance of the interaction between TRPM8 and TRPV1, nocifensive behavior was examined after intraplantar injection of capsaicin or capsaicin with menthol. The latency of onset of pain, the number, and the duration of flickerings and lickings were recorded after intraplantar injection of 20 µl of capsaicin (2 mM) or capsaicin plus menthol (2mM and 5 mM, respectively) in non-diabetic and diabetic mice.

In non-diabetic mice onset of pain symptoms such as licking and flickering was significantly increased from 3.07±0.29 s (n = 8 mice) after capsaicin alone to 10.7±0.8 s (n =8 mice) after capsaicin plus menthol. Similar results were obtained in diabetic mice also, although there is a reduction in the onset of pain with both capsaicin (1.3±0.2 s, n = 8 mice) and capsaicin plus menthol (2.3±0.4 s, n = 8 mice) compared to non-diabetic mice (Figure 10A). The total duration of nocifensive behavior was also significantly decreased from 32.5±1.9 with capsaicin to 19.1±2.1 s with capsaicin plus menthol in non-diabetic mice. A similar decrease was noticed in diabetic mice (capsaicin, 62±7.5 s, n = 8 mice; capsaicin plus menthol, 39.5±7.8 seconds) although there was an increase in the duration of lickings and flickerings in diabetic mice (Figure 10B). The number of hind paw licks and flickerings was significantly decreased from 34±2.3 to 19.1±2.1 in non-diabetic mice and similar findings were noticed in diabetic mice (capsaicin, 60±7.3 s, n = 8 mice; capsaicin plus menthol, 35±4.1 sec n = 8 mice) although there were more number of lickings and flickerings in diabetic mice (Figure 10C).

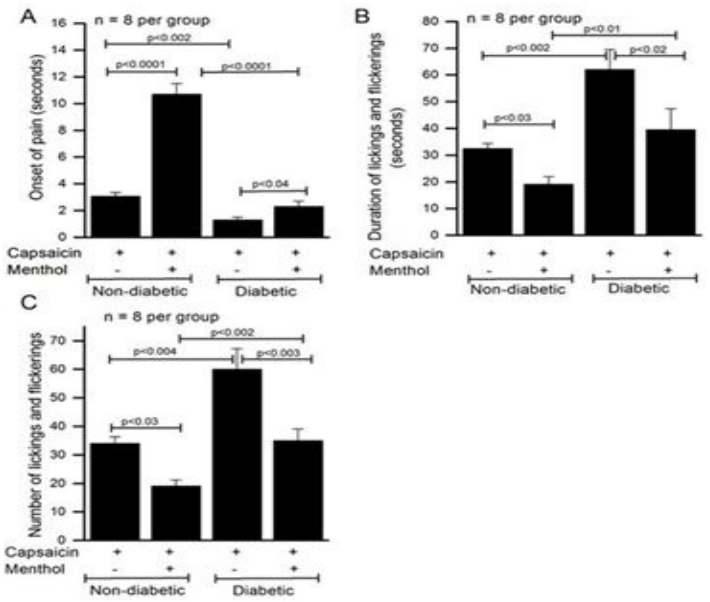


**Figure 10(A-C):** Capsaicin-Induced Nocifensive Pain Behavior is Reduced by Co-administration with Menthol in Diabetic and Non-Diabetic Mice. Onset, duration, and the number of lickings and/or flickerings of the injected hind paw were determined after intraplantar injection of 20µl of capsaicin (2 mM) or capsaicin plus menthol (5 mM) in diabetic and non-diabetic mice. **A**. In non-diabetic mice onset of pain symptoms such as licking and flickering was significantly increased from 3.07±0.29 s (n = 8 mice) after capsaicin alone to 10.7±0.8 s (n =8 mice) after capsaicin plus menthol. Similar results were obtained in diabetic mice also although the onset of pain with both capsaicin (1.3±0.2 s, n = 8 mice) and capsaicin plus menthol (2.3±0.4 s, n = 8 mice) is much quicker compared to non-diabetic mice. **B**. The total duration of nocifensive behavior was also significantly decreased from 32.5±1.9 with capsaicin to 19.1±2.1 s with capsaicin plus menthol in non-diabetic mice. A similar decrease was noticed in diabetic mice (capsaicin, 62±7.5 s, n = 8 mice; capsaicin plus menthol, 39.5±7.8 sec) although there was an increase in the duration of

lickings and flickerings in diabetic mice. C. We also compared the total number of hind paw lickings and flickerings for the duration of nocifensive behavior in diabetic and non-diabetic mice. The number of hind paw licks and flickerings was significantly decreased from 34±2.3 to 19.1±2.1 in non-diabetic mice and similar findings were noticed in diabetic mice (capsaicin, 60±7.3 s, n = 8 mice; capsaicin plus menthol, 35±4.1 sec n = 8 mice) although there were more number of lickings and flickerings in diabetic mice. Data are represented as mean±SEM. N = 8 mice per group. *p<0.05 obtained from students unpaired t-test.

## Discussion

The present study identified the role of TRPV1 and TRPM8 and their reciprocal regulation by PKC and PKA in DPN in STZ-induced diabetic mice. Our results suggest that STZ-induced mice exhibit heightened sensitivity to both heat and cold. There was an increase in TRPV1-mediated currents but a decrease in TRPM8-mediated currents in DRG isolated from diabetic hyperalgesic mice. While both PKA and PKC increased TRPV1 activity, they decreased TRPM8 channel activity. Finally, intraplantar injection of capsaicin exaggerated diabetic pain but was ameliorated when combined with menthol.

### Diabetic Peripheral Neuropathy and the Role of TRPV1 and TRPM8

Diabetic peripheral neuropathy, a disorder characterized by altered thermal, mechanical, and chemical sensation, is the most common chronic complication of diabetes mellitus. The symptoms of DPN can be categorized as either painful or painless [31-35]. In animal models of diabetes, hypersensitivity occurs at an earlier stage following the onset of diabetes as compared to hyposensitivity, which occurs at a later stage [15-17]. Although measurement of heat-induced pain sensitivity using hot plate analgesia meter is well established, methods to study cold-induced pain are not clear. We used acetone test to measure cold pain sensitivity in which a drop of acetone is placed on the hind paw and responses (number of lickings and flickerings) are measured [29,30,35]. Consistent with previous studies, we observed a marked increase in the sensitivity to acetone application indicating a cold hyperalgesia following diabetes [36-38]. However, several caveats surround the interpretation of behavioral responses evoked by acetone, namely the influence of mechanical stimulation during application, possible direct chemical effects of acetone on the skin, the effect of ambient temperature on the rate of evaporation, and the prospect of olfactory cues accompanying application involvement of other cold sensitive channels [39]. In our studies influence of mechanical sensation was ruled out as saline application did not induce any nocifensive behavior.

The ability to sense hot and cold temperatures distinctly suggests the presence of ion channels in specialized neurons within the peripheral nervous system. TRPV1 is involved in detecting noxious heat stimuli and is found in DRG neurons [40]. Much less is known about channels that sense cold. However, cloning of a menthol- and cold-activated channel, TRPM8 (CMR1), its activation at 25°C, and by menthol products suggests a discovery of cold sensing ion channel [13,14]. By using knock out strategy it has been shown that both TRPM8 and TRPA1 plays a role in inflammation or chronic constrictive injury (CCI) or acetone-induced hypersensitivity and activation of which relieves painful condition [29,30,35,37]. Although diabetic animals exhibited cold hyperalgesia, we noticed a reduction in menthol-induced TRPM8 currents and increased capsaicin-mediated TRPV1 currents as compared with non-diabetic mice. These results demonstrate that a decrease in TRPM8 and increase in TRPV1 function might aggravate the hyperalgesia observed in early stages of diabetes. Extracellular calcium has been shown to modulate TRP channels. We have demonstrated in this study that extracellular calcium reduces both TRPV1 and TRPM8 currents with repeated application a phenomenon known as tachyphylaxis. Therefore, we have compared the differences in the TRPV1 and TRPM8 currents in the presence or absence of calcium while studying the role of PKC or PKA-mediated phosphorylation using HEK cells heterologously expressing TRPV1(Figure 4-9).

### Modulation of TRPM8 and TRPV1 Function by PKC and PKA

Our findings support that both PKC and PKA upregulate TRPV1 function suggesting that hyperalgesia noticed in early stages of diabetic neuropathy is due to enhanced activity of TRPV1 function. These results are in line with previous studies that have suggested that an elevated activity of PKC and cAMP-PKA pathway may underlie the mechanism involved in hyperalgesia noticed in early stages of diabetes [41,43]. Also, diabetic hyperalgesia is attenuated by PKC inhibitors *in vivo* [44]. Specific blockade of PKCε diminishes PKC-mediated enhancement of heat-induced currents in sensory neurons and epinephrine-induced hypersensitivity *in vivo* [45]. PKCε knockout mice exhibit reduced hyperalgesia after intracutaneous injection of epinephrine and nerve growth factor [46]. Furthermore, increased enzyme activity from hyperglycemia, specifically PKCβ, is implicated in several diabetic complications including thermal hyperalgesia [46,47]. On the other hand, potentiation of TRPV1 activity by PKC has been associated with unmasking of silent channels [48,49], increased channel gating [50,51] and the recruitment of vesicular receptors to the plasma membrane by regulated exocytosis [52].

Studies demonstrate that proinflammatory prostaglandin $E_2$ ($PGE_2$) induces sensitization of sensory neuron responses to heat (heat hyperalgesia) by activating $G_s$-coupled prostaglandin E (EP) receptor subtypes (EP3C and EP4) and subsequently the cAMP/PKA cascade [53]. Accordingly, sensitization to heat also occurs in the presence of membrane-permeant cAMP analogs activating PKA [54]. Moreover, mice carrying a null mutation for type I β PKA regulatory subunit (PKA-RI β) no longer exhibit increased heat-induced pain behavior after $PGE_2$ administration, suggesting a crucial role of the cAMP/PKA second-messenger system in $G_s$-

mediated hyperalgesia [55]. PKA induced TRPV1 potentiation was shown to be due to increase in translocation of catalytic PKA subunit to the plasma membrane tethered to AKAP (A-kinase anchoring protein) [56]. It was also shown that activation of PKA increases the gating of the TRPV1 channel through direct phosphorylation [57, 58]. In conclusion, sensitization of TRPV1 by PKC and PKA enhances its activity, which may result in increased sensory neuron excitability leading to the exaggerated pain sensation.

Our findings indicate a downregulation of TRPM8 by PKC- and PKA-mediated phosphorylation, which in turn reveals that hyperalgesia noticed in early stages of diabetic neuropathy may be due to enhanced activity of TRPV1 as a result of impaired activity of TRPM8. PKC mediated phosphatase (PP1) activity was shown to involve in dephosphorylation of TRPM8 [21]. Furthermore, we suggest that inflammatory mediators while up-regulating TRPV1 might downregulate TRPM8 aggravating the pain condition resulting in hyperalgesia phenotype [21]. However, mechanisms that downregulate TRPM8 activity are not clear. The events such as decreased membrane fraction, phosphorylation, and the vesicular release of TRPM8 channels might be associated with decreased PKC-induced activity of TRPM8. PKA induced desensitization of TRPM8 might be due to either reduced gating or decreased translocation of TRPM8 and requires further investigation.

### Reciprocal Interaction of TRPV1 and TRPM8 Contributes to Diabetic Hyperalgesia

Our studies suggest that upon activation of PKA or PKC in vitro, TRPV1-mediated currents were up-regulated and TRPM8-mediated currents were downregulated. It has been shown that intraplantar injection of capsaicin induces severe pain and this pain is less severe when co-administered with menthol, suggesting a reciprocal interaction of TRPV1 and TRPM8 [21]. We noticed a similar decrease in nocifensive behavioral reflexes in diabetic mice treated with capsaicin plus menthol when compared with mice treated with capsaicin alone. In heterologous expression systems, TRPM8 is activated, whereas TRPV1 is tonically inhibited by $PIP_2$ [20]. Acidification (pH<6.5) of neurons leads to potentiation of TRPV1 and desensitization of TRPM8. Potentiation of TRPV1 results in lowered thermal threshold activation, whereas inhibition of TRPM8 leads to an increase in the thermal threshold of activation [19-21,48-61]. In normal conditions, TRPM8, which is almost exclusively expressed in a subset of neurons, may mediate a cool/soothing sensation and could function to counter the painful behavior via activation of TRPV1. In chronic pain conditions, elevated NGF could induce co-expression of TRPM8 and TRPV1 in the same neuron that may jeopardize the counter-balance mechanism we are proposing. Overall our studies support the hypothesis that diabetic hyperalgesia is associated with elevated TRPV1 activity and this hyperalgesia may be exacerbated by the decrease in TRPM8 function as the latter contributes to the much needed soothing sensation. TRPV1 expressed at peripheral terminals can be targeted to treat painful peripheral neuropathies [23-27].

## Conclusions

The present study identified the role of TRPV1 and TRPM8 and their reciprocal regulation by PKC and PKA in DPN in STZ-induced diabetic mice. Our results suggest that STZ-induced diabetic mice exhibit heightened sensitivity to both heat and cold. There was an increase in TRPV1-mediated currents but a decrease in TRPM8-mediated currents in DRG neurons isolated from diabetic hyperalgesic mice. While both PKA- and PKC-mediated phosphorylation increased TRPV1 channel activity, but they decreased TRPM8 channel activity. Finally, intraplantar injection of capsaicin exaggerated diabetic pain but was ameliorated when combined with menthol. Therefore, targeting TRPV1 or TRPM8 could be a useful approach to treat pain associated with DPN.

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