Transient Receptor Potential Vanilloid Type 1 Activation Down-regulates Voltage-gated Calcium Channels through Calcium-dependent Calcineurin in Sensory Neurons*

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Calcium influx through voltage-activated Ca\(^{2+}\) channels (VACCs) plays a critical role in neurotransmission. Capsaicin application inhibits VACCs and desensitizes nociceptors. In this study, we determined the signaling mechanisms of the inhibitory effect of capsaicin on VACCs in primary sensory neurons. Whole-cell voltage clamp recordings were performed in acutely isolated rat dorsal root ganglion neurons. Capsaicin caused a profound decrease in the Ca\(^{2+}\) current (I\(_{Ca}\)) density in capsaicin-sensitive, but not -insensitive, dorsal root ganglion neurons. At 1 \(\mu\)M, capsaicin suppressed about 60% of N-, P/Q-, L-, and R-type I\(_{Ca}\) density. Pretreatment with iodo-resiniferatoxin, a specific transient receptor potential vanilloid type 1 (TRPV1) antagonist, or intracellular application of 1,2-bis(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid blocked the inhibitory effect of capsaicin on I\(_{Ca}\). However, neither W-7, a calmodulin blocker, nor KN-93, a CaMKII inhibitor, attenuated the inhibitory effect of capsaicin on I\(_{Ca}\). Furthermore, intracellular dialysis of deltamethrin or cyclosporin A, the specific calcineurin (protein phosphatase 2B) inhibitors, but not okadaic acid (a selective protein phosphatase 1/protein phosphatase 2B) inhibitors, abolished the effect of capsaicin on I\(_{Ca}\). Interestingly, 1,2-bis(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid, deltamethrin, cyclosporin A, and okadaic acid each alone significantly increased the I\(_{Ca}\) density and caused a depolarizing shift in the voltage dependence of activation. Immunofluorescence labeling revealed that capsaicin induced a rapid internalization of Ca\(^{2+}\) channels on the membrane. Thus, this study provides novel information that VACCs are tonically modulated by the intracellular Ca\(^{2+}\) level and endogenous phosphatases in sensory neurons. Stimulation of TRPV1 by capsaicin down-regulates VACCs by dephosphorylation through Ca\(^{2+}\)-dependent activation of calcineurin.

TRPV1\(^1\) is a nonselective cation channel with high Ca\(^{2+}\) permeability and is the molecular target of capsaicin, the main pungent ingredient in chili peppers. The TRPV1 channel is expressed in subsets of primary sensory neurons and nerve terminals and plays an essential role in detecting noxious heat and several other nociceptive stimuli (1, 2). Capsaicin can excite nociceptive sensory neurons and produce transient pain in animals and humans (3, 4). Paradoxically, exposure to capsaicin desensitizes nociceptive sensory neurons and results in long lasting pain relief. For example, topical or local application of capsaicin is effective in treating many acute and chronic pain syndromes in patients (5–7). Capsaicin also causes an unexplained synaptic transmission block in the spinal cord dorsal horn (8).

The voltage-activated Ca\(^{2+}\) channels (VACCs) play a critical role in signal transmission, synaptic neurotransmitter release, and nociceptive transmission (9–11). VACCs also are an important molecular target of many analgesic drugs such as opioids (12, 13). Interestingly, capsaicin causes a profound inhibition of VACC currents in dorsal root ganglion (DRG) neurons (14). However, the cellular and signaling mechanisms of the capsaicin effect on VACCs remain poorly understood.

Protein kinases and phosphatases are key enzymes in signal transduction pathways for a wide range of cellular processes. The enzymatic addition or removal of phosphate esters on serine and threonine hydroxyls alters the activity of many proteins that are essential to the characteristic structure and function of neurons. An important mechanism regulating VACC function is through phosphorylation by protein kinases and phosphatases (15–18). We now show that a Ca\(^{2+}\)-dependent serine/threonine phosphatase, calcineurin (protein phosphatase 2B), is critically involved in down-regulation of high voltage-activated Ca\(^{2+}\) channels (HVACCs) by capsaicin in native DRG neurons. Furthermore, the basal intracellular Ca\(^{2+}\) level and endogenous protein phosphatases tonically modulate the HVACC current in DRG neurons. These findings are not only important to our understanding of the functional interaction between TRPV1 and HVACCs in primary nociceptors but are also significant to our understanding of the Ca\(^{2+}\)-dependent feedback regulation of neuronal Ca\(^{2+}\) channels in general.

MATERIALS AND METHODS

Isolation of DRG Neurons—All procedures conformed to the guidelines of the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the Animal Care and Use Committee of the Pennsylvania State University College of Medicine. Male Sprague-Dawley rats (5–6 weeks old; Harlan, Indianapolis, IN) were anesthetized with halothane and then rapidly decapitated. The thoracic and lumbar segments of the vertebral column were dissected. The DRGs were quickly removed and transferred immediately into anoxic/ethane-N,N,N',N'-tetracetic acid; W-7, N-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide; TEA, tetraethylammonium; pF, picofarads.

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\(^1\) The abbreviations used are: TRPV1, transient receptor potential vanilloid type 1; DRG, dorsal root ganglion; Ib, Grifonia simplicifolia isoelectric Ib; VACCs, voltage-activated calcium channels; HVACCs, high voltage-activated calcium channels; BAPTA, 1,2-bis(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid; W-7, N-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide; TEA, tetraethylammonium; pF, picofarads.
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Dulbecco’s modified Eagle’s medium (Invitrogen). After removal of attached nerves and surrounding connective tissues, the DRGs were minced with fine spring scissors, and the ganglion fragments were placed in a flask containing 5 ml of Dulbecco’s modified Eagle medium in which trypsin (type I, 0.5 mg/ml; Sigma), collagenase (type Ix, 1 mg/ml; Sigma), and DNase (type I, 0.1 mg/ml; Sigma) had been dissolved. After incubation at 34 °C in a shaking water bath for 30 min, soybean trypsin inhibitor (type II-s, 1.25 mg/ml; Sigma) was then added to stop trypsin digestion. The cell suspension was centrifuged (500 rpm, 5 min) to remove the supernatant and replated with Dulbecco’s modified Eagle’s medium. The cultured DRG cells were replated with B-27 medium containing neurobasal medium (Invitro- gen) with B-27 supplement (2%; Invitrogen) and penicillin/streptomycin/gluta- mine supplement (1%; Invitrogen). Cells were then plated onto a 35-mm culture dish containing poly-L-lysine (50 μg/ml) precoated coverslips and kept for at least 30 min before electrophysiological recordings. Recordings were performed 1 to 2 h after subcultivation, at room temperature (22, 23), or 4 °C (21). Tyrode solution for 10 min and then rinsed for at least 3 min. IB4-conotoxin GVIA (M iodoresiniferatoxin, a specific TRPV1 antagonist (19), or 1 μM capsaicin or vehicle in phosphate-buffered saline. The cells were incubated with 1–10 μM capsaicin or vehicle in phosphate-buffered saline for 20 s or 2 min. Additional cells were pretreated with either 10 μM capsaicin, a specific TRPV1 antagonist (19), or 1 μM deltamethrin, a membrane-permeable inhibitor of calcineurin (20), for 5 min before capsaicin incubation. After rinsing, the cells were immediately fixed with 4% formaldehyde for 10 min. The cells were quenched in 1% H\(_2\)O\(_2\) in Tris-buffered saline for 10 min and permeabilized in 0.1% Triton X-100 in Tris-buffered saline for another 10 min at room temperature. Then the cells were blocked in 4% normal goat serum for 30 min at room temperature. For Ca\(_{\text{v}2.2}\) and IB\(_{\text{A}}\) double labeling, the cells were incubated overnight at 4 °C with the primary antibody (rabbit anti-Ca\(_{\text{v}2.2}\), 1:200 dilution; Chemicon (Temecula, CA) or Alomone (Jerusalem, Israel)) diluted in 2% normal goat serum. Subsequently, the cells were washed and incubated with peroxidase-conjugated AffiniPure goat anti-rabbit IgG (1:100 dilution; Jackson Immunoresearch, West Grove, PA) for 1 h at room temperature. The cells were then rinsed and incubated with fluorescein isothio- cyanate-conjugated tyramide (1:100 dilution; PerkinElmer Life Sciences) for 5 min at room temperature and washed. Finally, the cells were incubated with Alexa 594 conjugated to IB4 (1 μg/ml; Molecular Probes, Inc., Eugene, OR) for 1 h at room temperature.

For Ca\(_{\text{v}2.2}\) and TRPV1 double immunolabeling, the cells were incubated overnight at 4 °C with a mixture of two primary antibodies (rabbit anti-Ca\(_{\text{v}2.2}\) and guinea pig anti-TRPV1, 1:1000 dilution; Jackson Immunoresearch) for 1 h at room temperature. After rinsing, cells were incubated with Alexa 594 conjugated to streptavidin for 1 h at room temperature. Subsequently, cells were washed and incubated with the ProLong mounting medium. Negative controls were performed by omitting the primary antibodies. The labeled cells were examined on a laser-scanning confocal microscope (Leica, Germany), and areas of interest were photodocumented. Confocal laser scanning microscopy was used for accurate localization of fluorescent markers, because the thin (0.3-μm) optical sectioning generated by the confocal microscope eliminated out-of-focus labeling fixed at 1 focal plane.

Data Analysis—Data were analyzed using the PulseFit software program (HEKA). Whole-cell current-voltage (I-V) relationships for individual neurons were constructed by calculating the mean peak inward current at each test potential and normalized for cell capacitance. Conductance-voltage (G-V) curves were calculated by dividing current (and normalized for cell capacitance) by the voltage to construct the whole-cell conductance. The percent decrease in total conductance, \(G_{\text{max}}\), was calculated as the ratio of capsaicin-inhibited \(I_{\text{Ca}}\) to the peak total \(I_{\text{Ca}}\) or subtypes of VACC currents during control, respectively. Statistical data are presented as means ± S.E. All comparisons between means were tested for significance using Student’s unpaired t test or one-way analysis of variance unless otherwise indicated. p < 0.05 was considered to be statistically significant.

RESULTS

Capsaicin Inhibits VACC Currents through TRPV1—The whole-cell VACC currents were elicited by a series of depolarizing pulses (from ~70 to 50 mV for 150 ms in 10-mV increments) from a holding potential of ~90 mV. The steady-state inactivation of VACCs was obtained by depolarizing cells to a series of prepulse potentials from ~100 to 20 mV for 500 ms followed by a command potential to 0 for 150 ms. Most drugs were dissolved in distilled water at 1,000 times the final concentration and kept frozen in aliquots. Nimodipine, thapsigargin, and KN-93 were initially dissolved in Me\(_2\)SO to make the stock solution. The capsaicin, cyclopamine A, and deltamethrin stock solutions were further diluted in the extracellular or intracellular solution just before use and held in a series of independent syringes connected to corresponding pipettes in the pipette compartments. The end of the parallel columns was connected to a common column. The distance from the column mouth to the cell examined was about 100 μm. Cells in the recording chamber were continuously bathed in extracellular solution. Each drug solution was delivered to the recording chamber by gravity, and rapid solution exchange was achieved by controlling the corresponding valve switch (World Precision Instruments). Drugs and chemicals were purchased from Sigma, except o-conotoxin GVIA, α-conotoxin IVA, and α-conotoxin MVIIIC (Alomone Labs, Jerusalem, Israel) and W-7 and deltamethrin (Calbiochem).

Double Fluorescence Labeling of Ca\(_{\text{v}2.2}\) and TRPV1/IB\(_{\text{A}}\) in DRG Cells—The N-type current (Ca\(_{\text{v}2.2}\) channel) is the most predominant subtype of VACC currents in DRG neurons (22). We determined the effect of capsaicin on the spatial distribution of Ca\(_{\text{v}2.2}\) using immunofluorescence and confocal microscopy. DRG neurons cultured on glass coverslips were rinsed three times with phosphate-buffered saline. The cells were incubated with 1–10 μM capsaicin or vehicle in phosphate-buffered saline for 20 s or 2 min. Additional cells were pretreated with either 10 μM capsaicin, a specific TRPV1 antagonist (19), or 1 μM deltamethrin, a membrane-permeable inhibitor of calcineurin (20), for 5 min before capsaicin incubation. After rinsing, the cells were immediately fixed with 4% formaldehyde for 10 min. The cells were quenched in 1% H\(_2\)O\(_2\) in Tris-buffered saline for 10 min and permeabilized in 0.1% Triton X-100 in Tris-buffered saline for another 10 min at room temperature. Then the cells were blocked in 4% normal goat serum for 30 min at room temperature. For Ca\(_{\text{v}2.2}\) and IB\(_{\text{A}}\) double labeling, the cells were incubated overnight at 4 °C with the primary antibody (rabbit anti-Ca\(_{\text{v}2.2}\), 1:200 dilution; Chemicon (Temecula, CA) or Alomone (Jerusalem, Israel)) diluted in 2% normal goat serum. Subsequently, the cells were washed and incubated with peroxidase-conjugated AffiniPure goat anti-rabbit IgG (1:100 dilution; Jackson Immunoresearch) for 1 h at room temperature. After rinsing, cells were incubated with Alexa 594 conjugated to IB4 (1 μg/ml; Molecular Probes, Inc., Eugene, OR) for 1 h at room temperature.

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RESULTS

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FIG. 1. Capsaicin inhibits HVACC currents in IB4-positive DRG neurons. A, representative traces showing $I_{\text{Ca}}$ before and after application of 1 μM capsaicin in a capsaicin-sensitive DRG neuron. B, mean $I_{\text{Ca}}$ density before and after application of capsaicin at different test potentials ($n = 15$). C, original recordings of $I_{\text{Ca}}$ before and after capsaicin application in a capsaicin-insensitive neuron. D, treatment with 500 ng/ml pertussis toxin had no effect on capsaicin-induced inhibition of $I_{\text{Ca}}$ ($n = 9$). The peak current was divided by cell capacitance to yield the current density. All of the neurons were voltage-clamped at −90 mV and depolarized from −70 to 50 mV for 150 ms with 10-mV increments. Inset, the double-pulse voltage protocol with a depolarizing prepulse to 100 mV for 50 ms preceding the second test pulse. *, $p < 0.05$ compared with corresponding values before capsaicin application.

Capsaicin Inhibits All Subtypes of HVACCs—We next determined which subtypes of HVACC currents are inhibited by capsaicin. The subtype-selective Ca$^{2+}$ channel blockers nifedipine (5 μM, L-type), $\omega$-conotoxin GVIA (2 μM, N-type), $\omega$-agatoxin IVA (100 nM, P/Q-type), and $\omega$-conotoxin MVIIIC (500 nM, N- and P/Q-type) were appropriately combined to pharmacologically isolate L-, N-, P/Q-, and R-type Ca$^{2+}$ currents, as described previously (22, 28, 29). Because 100 nM $\omega$-agatoxin IVA alone is not sufficient to block the R-type Ca$^{2+}$ channel (29), $\omega$-conotoxin MVIIIC was co-applied with $\omega$-agatoxin IVA to define L- and R-type VACC currents. In this protocol, the cells were depolarized from −90 to 0 mV for 150 ms. A desired subtype of $I_{\text{Ca}}$ was isolated before application of 1 μM capsaicin for 30 s. After capsaicin current was washed out, the $I_{\text{Ca}}$ was reexamed in capsaicin-sensitive neurons.

To determine the effect of 1 μM capsaicin on R-type $I_{\text{Ca}}$, $\omega$-conotoxin MVIIIC and $\omega$-agatoxin IVA were co-applied with

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Fig. 2. Iodoresiniferatoxin (I-RTX) blocked the inhibitory effect of capsaicin on \( I_{\text{ca}} \). A, original current traces showing that 10 \( \mu \text{M} \) I-RTX abolished the effect of 1 \( \mu \text{M} \) capsaicin on \( I_{\text{ca}} \) in a DRG cell. B, summary data showing lack of effect of 1 \( \mu \text{M} \) capsaicin on \( I_{\text{ca}} \) at different test potentials in the presence of 10 \( \mu \text{M} \) I-RTX in 13 IB\(_4\)-positive DRG neurons. C, mean \( I_{\text{ca}} \) density before and after application of 1 \( \mu \text{M} \) capsaicin at different test potentials in nine IB\(_4\)-negative but capsaicin-sensitive DRG neurons. The peak current was divided by cell capacitance to yield the current density. Data are presented in mean \( \pm \) S.E. \textit{Caps}, capsaicin. *, \( p < 0.05 \) compared with corresponding values before capsaicin application.

Fig. 3. Effect of capsaicin on four subtypes of HVACC currents. A, representative current traces showing the original recording and time course of the effect of 1 \( \mu \text{M} \) capsaicin on pharmacologically isolated R-, L-, P/Q-, and N-type \( \text{Ca}^{2+} \) currents in separate capsaicin-sensitive DRG neurons. \textit{Caps}, capsaicin; \textit{con}, control; \textit{Cd}, cadmium; \textit{Nim}, nimodipine; \textit{IVA}, \( \alpha\)-agatoxin IVA; \textit{GVIA}, \( \alpha\)-conotoxin GVIA; \textit{MVIIC}, \( \alpha\)-conotoxin MVIIC. B, summary data showing the inhibitory effect of 1 \( \mu \text{M} \) capsaicin on the total and different subtypes of \( I_{\text{ca}} \) in capsaicin-sensitive neurons. Percentage inhibition was calculated as the ratio of capsaicin-inhibited currents to either the total or the isolated subtype \( I_{\text{ca}} \) before capsaicin application. The number of cells tested in each group is indicated in columns. Data are presented as mean \( \pm \) S.E.
duced inhibition of capsaicin currents (14), we determined the role of Ca_{\text{Ca}}. The N-type I_{\text{Ca}} was isolated by application of nimodipine and \omega-agatoxin IVA in a separate group of DRG neurons. Capsaicin inhibited 61.9 \pm 11.6\% N-type I_{\text{Ca}} in nine DRG neurons tested (Fig. 3). Furthermore, the L-type Ca^{2+} current, isolated with \omega-agatoxin GVIA, \omega-agatoxin MVIIC, and \omega-agatoxin IVA, was inhibited 48.5 \pm 8.9\% (n = 5; Fig. 3) by 1 \mu M capsaicin. Additionally, 1 \mu M capsaicin inhibited 57.1 \pm 14.9\% P/Q-type Ca^{2+} current, isolated using \omega-agatoxin GVIA and nimodipine (n = 5; Fig. 3).

Capsaicin Increases Steady-state Inactivation of I_{\text{Ca}}—To further characterize the inhibitory effect of capsaicin on I_{\text{Ca}}, we examined the effect of capsaicin on steady-state activation and inactivation kinetics of I_{\text{Ca}} illustrated in Fig. 4. Although 1 \mu M capsaicin caused a pronounced inhibition of I_{\text{Ca}}, it did not significantly alter the voltage dependence of activation (Fig. 4A, n = 15). The decay phase of I_{\text{Ca}} was fitted with one exponential function, and the decay time constant of I_{\text{Ca}} was significantly reduced by 1 \mu M capsaicin (from 51.6 \pm 3.0 to 39.9 \pm 3.3 ms, p < 0.05; Fig. 4B; n = 15). In 13 additional capsaicin-sensitive neurons, the steady-state inactivation of I_{\text{Ca}} was examined using a series of prepulse potentials (−100 to 20 mV for 500 ms) followed by depolarizing the cell to 0 mV for 150 ms. Following application of 1 \mu M capsaicin, the voltage-dependent steady-state inactivation of HVACC currents was shifted significantly to the left (more negative potentials) (Fig. 4B).

Both Extracellular and Intracellular Ca^{2+} Are Required for the Effect of Capsaicin on I_{\text{Ca}}—The TRPV1 channel is highly permeable to Ca^{2+} (1). Because the degree of capsaicin-produced inhibition of I_{\text{Ca}} was proportional to the amplitude of capsaicin currents (14), we determined the role of Ca^{2+} influx in the inhibitory effect of capsaicin on I_{\text{Ca}}. BAPTA, a rapid Ca^{2+} chelator (30), was included in the pipette solution. Following the membrane rupture to achieve the whole-cell configuration, the intracellular solution exchange was allowed for 5 min before examining the effect of capsaicin on I_{\text{Ca}}. In 11 capsaicin-sensitive neurons tested, 1 \mu M capsaicin had no significant effect on I_{\text{Ca}} when 10 mM BAPTA was included in the pipette solution (Fig. 5A). Capsaicin also failed to alter the inactivation kinetics of I_{\text{Ca}} (Fig. 5B). Notably, I_{\text{Ca}} was activated at about −30 mV and reached its peak at 10 mV in the presence of 10 mM BAPTA. Compared with the peak I_{\text{Ca}} density and I-V curve recorded in the absence of BAPTA (95.4 \pm 10.7 pA/pF at 0 mV, n = 15), intracellular dialysis of BAPTA significantly shifted the I-V curve to the right (more positive potentials) and increased the density of I_{\text{Ca}} (164.3 \pm 22.2 pA/pF at 10 mV, n = 11; Fig. 5A).

TRPV1 is present on both the cell membrane and endoplasmic reticulum in the DRG (31, 32). A rapid rise in intracellular Ca^{2+} levels may be due to either Ca^{2+} influx or release of Ca^{2+}...
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from an intracellular store. In 12 capsaicin-sensitive DRG neurons, we replaced Ca\textsuperscript{2+} with Ba\textsuperscript{2+} in the extracellular solution to determine the role of extracellular Ca\textsuperscript{2+} in the effect of capsaicin on HVACCs. \(I_{\text{Ba}}\) was elicited at \(-40\) mV and reached its peak at \(-10\) mV (Fig. 6A). In the Ca\textsuperscript{2+}-free external solution, 1 \(\mu\)M capsaicin still significantly inhibited \(I_{\text{Ba}}\), although its inhibitory effect on \(I_{\text{Ba}}\) was significantly attenuated (37.5 \pm 5.0\% at \(-10\) mV, \(n = 6\); Fig. 6A). However, the capsaicin effect can be completely washed out in 3 min. Capsaicin did not significantly alter the steady-state inactivation kinetics of \(I_{\text{Ba}}\) (Fig. 6B).

We subsequently determined whether the remaining inhibitory effect of capsaicin on \(I_{\text{Ba}}\) was mediated by Ca\textsuperscript{2+} released from the intracellular stores, such as the endoplasmic reticulum. The intracellular Ca\textsuperscript{2+} store was depleted with thapsigargin, a specific Ca\textsuperscript{2+}-ATPase inhibitor (33). In a total of 15 capsaicin-sensitive neurons, we pretreated the DRG cells with 5 \(\mu\)M thapsigargin for 10 min and washed it out for at least another 3 min using Ba\textsuperscript{2+} external solution before testing the effect of 1 \(\mu\)M capsaicin on \(I_{\text{Ba}}\). The inhibitory effect of capsaicin on \(I_{\text{Ba}}\) was completely blocked in all 15 neurons treated with thapsigargin (Fig. 6C).

Furthermore, to determine whether Ca\textsuperscript{2+} release from the endoplasmic reticulum independent of TRPV1 activation can inhibit HVACCs, we examined the effect of caffeine, a selective activator for endoplasmic reticulum ryanodine receptors (34, 35), on \(I_{\text{Ba}}\) in 12 additional DRG cells. Bath perfusion of 30 mM caffeine for 1–2 min caused a significant and reversible reduction of \(I_{\text{Ba}}\) (23.7 \pm 3.2\% inhibition at \(-10\) mV) (Fig. 6D).

Calmodulin and Ca\textsuperscript{2+}/Calmodulin-dependent Protein Kinase II Are Not Involved in the Effect of Capsaicin on \(I_{\text{Ca}}\)—Because the increase in intracellular Ca\textsuperscript{2+} appeared essential for the inhibitory effect of capsaicin on VACCs, we next determined if calmodulin and Ca\textsuperscript{2+}/calmodulin-dependent protein kinase II are involved in this effect. The specific calmodulin antagonist W-7 (36) or the selective Ca\textsuperscript{2+}/calmodulin-dependent protein kinase II inhibitor KN-93 (37) was included in the pipette solution. In the presence of W-7 (200 \(\mu\)M, \(n = 7\)) or KN-93 (50 \(\mu\)M, \(n = 7\)), 1 \(\mu\)M capsaicin still produced a large inhibition of \(I_{\text{Ca}}\), which was not significantly different from the effect of capsaicin on \(I_{\text{Ba}}\) recorded using regular pipette solution without W-7 and KN-93 (Fig. 7A). W-7 and KN-93 alone did not result in inhibition of capsaicin current. Furthermore, intracellular application of a higher concentration of W-7 (500 \(\mu\)M, \(n = 4\)) or KN-93 (100 \(\mu\)M, \(n = 4\)) also failed to attenuate the inhibitory effect of 1 \(\mu\)M capsaicin on \(I_{\text{Ca}}\) in additional DRG neurons tested (data not shown).

Endogenous Protein Phosphatases Play a Critical Role in Regulation of HVACCs and Capsaicin-induced Inhibition of \(I_{\text{Ca}}\)—We tested the effect of okadaic acid, a specific inhibitor for protein phosphatase 1 and protein phosphatase 2A (38), on capsaicin-induced inhibition of \(I_{\text{Ca}}\). Compared with the I-V relationship of \(I_{\text{Ca}}\) using regular pipette solution, intracellular dialysis of 0.5 \(\mu\)M okadaic acid significantly increased the den-
the inhibitory effect of 1 μM capsaicin on $I_{Ca}$. Percentage inhibition was calculated as the ratio of capsaicin-inhibited currents to the total peak current during the control. B, representative current traces and summary data showing that intracellular dialysis with 0.5 μM okadaic acid failed to block the inhibitory effect of 1 μM capsaicin on $I_{Ca}$ at different test potentials (from −70 to 50 mV for 150 ms, n = 6). Note the 1-V current shift and increased $I_{Ca}$ density by okadaic acid. Data were presented in mean ± S.E. *p < 0.05 compared with corresponding values before capsaicin application.

Calcineurin (protein phosphatase 2B) is a Ca$^{2+}$-sensitive protein phosphatase and can be activated by a rise in intracellular Ca$^{2+}$. To determine if capsaicin inhibits $I_{Ca}$ through dephosphorylation mediated by calcineurin, 1 μM deltamethrin, a specific inhibitor of calcineurin (20), was included in the pipette solution. Deltamethrin completely blocked the inhibitory effect of 1 μM capsaicin on $I_{Ca}$ in all six capsaicin-sensitive neurons tested (Fig. 7B).

Capsaicin Induces Internalization of CaV2.2—Because capsaicin caused a profound and long lasting decrease in $I_{Ca}$ density, the pronounced inhibition of VACCs by capsaicin exposure cannot be fully explained with increased inactivation of HVACCs. Thus, we determined whether redistribution (e.g., internalization) of HVACCs plays a role in the effect of capsaicin on $I_{Ca}$ in DRG cells. Because the N-type Ca$^{2+}$ current (mediated by CaV2.2 channels) is the most prominent subtype of HVACC currents, we chose to examine the effect of 1–10 μM capsaicin on the spatial distribution of CaV2.2 in DRG cells. The CaV2.2 distribution in TRPV1- and IB4-positive DRG neurons was studied using double immunofluorescence labeling and confocal microscopy. All negative controls (omitting primary antibodies) displayed no detectable staining. In vehicle-treated DRG neurons, the cross-sectional confocal images of CaV2.2 immunoreactivity showed a bright staining pattern with a clear and well defined CaV2.2 immunoreactivity on the plasma membrane (Fig. 9). By contrast, the cells treated with 1–10 μM capsaicin for 20 s and 2 min displayed a more diffuse and dummer immunoreactivity of CaV2.2 in the cytoplasm near the cell membrane surface (Fig. 9). The inward spread of CaV2.2 immunoreactivity was more evident when compared with the labeling of TRPV1 and IB4 present in the same DRG cell (Fig. 9). Furthermore, in DRG cells pretreated with either 10 μM iodesiniferatoxin or 1 μM deltamethrin, the spatial distribution of CaV2.2 immunoreactivity on the cell membranes was not altered by capsaicin (data not shown). In DRG cells exposed to 1–10 μM capsaicin for 3–5 min, CaV2.2 immunoreactivity completely disappeared from the plasma membrane. The cell membrane also became disintegrated following prolonged exposure to capsaicin, suggesting permanent damage to the cells (data not shown).

DISCUSSION

We have investigated the signaling mechanism underlying down-regulation of HVACCs by TRPV1 stimulation in native DRG neurons. Small sized DRG neurons are richly endorsed with TRPV1 (41, 42). Application of capsaicin produced a profound and sustained suppression of HVACC currents in DRG cells. We found that this inhibition was only limited to capsaicin-sensitive DRG neurons, and iodesiniferatoxin, a highly specific TRPV1 antagonist, eliminated the effect of capsaicin. Hence, capsaicin inhibits HVACC currents specifically through TRPV1 in DRG neurons. Furthermore, we observed that capsaicin produced a similar degree of inhibition in all the subtypes (N-, P/Q-, L-, and R-type) of HVACCs. This suggests that a common mechanism is probably responsible for the effect of capsaicin on different subtypes of HVACCs. Although capsaicin had no effect on the voltage dependence activation of HVACCs, it increased the steady-state inactivation of HVACC currents.

Thus, the inhibitory effect of capsaicin on HVACC currents appears to be due in part to a decrease in the open state probability of HVACCs in DRG neurons.

What then are the downstream signaling mechanisms responsible for the down-regulation of HVACCs by capsaicin in DRG neurons? Stimulation of G protein-coupled receptors inhibits HVACCs through G protein βγ-subunits (25, 26). However, we obtained no evidence that G proteins are involved in the effect of capsaicin on HVACCs. This is because depolarizing pulses did not lead to a prepulse-induced facilitation (relief of
inhibition) of Ca\(^{2+}\) currents. Furthermore, pretreatment of DRG neurons with pertussis toxin to inactivate inhibitory G\(_{i/o}\) proteins had no effect on capsaicin-induced inhibition on HVACC currents. Thus, the G proteins are not involved in the rapid inhibition of HVACCs by capsaicin.

Because TRPV1 is highly permeable to Ca\(^{2+}\) (1, 2), capsaicin can induce substantial Ca\(^{2+}\) influx into the DRG cell. In our study, intracellular dialysis with BAPTA, a rapid Ca\(^{2+}\) chelator (30), abolished the inhibitory effect of capsaicin on Ca\(^{2+}\) currents, suggesting that the rise in intracellular Ca\(^{2+}\) is the key event in capsaicin-induced inhibition of HVACC currents. Although these data clearly indicate the importance of intracellular Ca\(^{2+}\) in the effect of capsaicin on HVACCs, they do not discriminate the sources of Ca\(^{2+}\). Capsaicin can activate TRPV1 located on both the plasma membrane and endoplasmic reticulum (31, 32). Consequently, we examined more closely the relative contribution of intracellular and extracellular Ca\(^{2+}\) to the effect of capsaicin on HVACC currents. When the extracellular Ca\(^{2+}\) was replaced with barium, the inhibitory effect of capsaicin was significantly attenuated, and its effect was completely washed out. Furthermore, depletion of intracellular Ca\(^{2+}\) with thapsigargin, a highly specific inhibitor of Ca\(^{2+}\)-ATPase (39), abolished the capsaicin-induced inhibition on HVACC currents. Thus, the inhibitory effect of capsaicin on HVACCs is triggered by both Ca\(^{2+}\) influx and release of Ca\(^{2+}\) from intracellular stores. We found that stimulation of endoplasmic reticulum ryanodine receptors with caffeine (34, 35), significantly inhibited \(I_{\text{Ca}}\) in DRG neurons. These data strongly suggest that Ca\(^{2+}\) release from the endoplasmic reticulum contributes to the inhibitory effect of capsaicin on HVACCs. In addition, capsaicin increases the steady-state inactivation of HVACC currents through an increase in intracellular Ca\(^{2+}\), which highlights a direct Ca\(^{2+}\)-dependent inactivation of HVACCs in primary sensory neurons.

The most salient finding of this study is that calcineurin plays a pivotal role in down-regulation of HVACCs caused by TRPV1 stimulation in DRG neurons. Calcineurin is a Ca\(^{2+}\)-dependent protein phosphatase and is enriched in the DRG neurons (43). We found that inhibition of calcineurin with intracellular dialysis of either deltamethrin or cyclosporin A, two structurally dissimilar calcineurin inhibitors, completely blocked the effect of capsaicin on HVACC currents. On the other hand, intracellular application of okadaic acid, a specific inhibitor for protein phosphatases protein phosphatase 1 and protein phosphatase 2A, failed to attenuate the effect of capsaicin. Deltamethrin and structurally related Type II pyrethroids are potent inhibitors of calcineurin (20, 44, 45). Cyclosporin A specifically binds to the intracellular receptor protein cyclophilin A, a member of the immunophilin protein family (39), and the resulting complex is an effective inhibitor of calcineurin. Interestingly, rapamycin, an inhibitor of FK506-binding protein but ineffective in inhibiting calcineurin activity (40), did not alter the effect of capsaicin on Ca\(^{2+}\) currents. Calcineurin may be activated by Ca\(^{2+}\), calmodulin, and inhibitory G proteins (46, 47). However, it appears that Ca\(^{2+}\) is a direct activator of calcineurin following stimulation of TRPV1 in DRG neurons, since treatment with W-7, KN-93, or pertussis toxin failed to attenuate the effect of capsaicin. These data strongly suggest that an increase in intracellular Ca\(^{2+}\) by TRPV1 leads to down-regulation of HVACCs by protein dephosphorylation mediated by Ca\(^{2+}\)-dependent calcineurin.

We also demonstrate for the first time that stimulation of TRPV1 causes a rapid internalization of HVACCs in DRG neurons. Using double immunofluorescence labeling and confocal microscopy, we found that capsaicin induced a rapid internalization of Ca\(_{\text{v}}\),2.2 immunoreactivity in IB\(_{\text{b}}\) and TRPV1-positive DRG neurons. Since capsaicin caused a similar degree of down-regulation in all four subtypes of HVACCs, it is probable that internalization occurs in the other three subtypes of HVACCs. It is not clear if the substrate of calcineurin is the HVACCs or a regulatory phosphoprotein that interacts with the channel in the DRG. We found that capsaicin shifted the
reversal potential of HVACCs, which is probably due to dephosphorylation of HVACCs, resulting in a structural change of channel proteins, through activation of Ca\(^{2+}\)-dependent calcineurin. In addition to direct phosphorylation and dephosphorylation of HVACCs (16, 48), protein kinases and calcineurin can phosphorylate and dephosphorylate, respectively, the cytoskeletal proteins, which in turn affect the HVACC activity (49, 50). Therefore, internalization of HVACCs appears to be an important mechanism responsible for capsaicin-induced profound loss of the HVACC density in DRG neurons. Further studies are warranted to identify the exact substrates dephosphorylated by calcineurin in the DRG neurons.

Another intriguing and important finding is that the basal intracellular Ca\(^{2+}\) and endogenous calcineurin tonically modulate the HVACC currents in DRG neurons. In this regard, intracellular dialysis with BAPTA, deltamethrin, or cyclopiazonic acid resulted in increased density of HVACC and induced a depolarizing shift in the voltage dependence of activation. The observation that BAPTA and calcineurin inhibitors resulted in increased density of Ca\(^{2+}\) currents points to a basal activity of calcineurin in DRG cells in the absence of any prior evoked increases in intracellular Ca\(^{2+}\). Notably, a similar effect was observed when okadaic acid was applied intracellularly, suggesting that the phosphatases protein phosphatase 1 and protein phosphatase 2A may also be involved in the basal regulation of HVACCs. Therefore, a substantial proportion of HVACCs or closely associated proteins in the DRGs are present in the dephosphorylated form in the DRG. Consistent with our findings, overexpression of calcineurin in NG108-15 cell lines causes a decreased current density of HVACCs. By contrast, the current density of HVACCs is increased in those cells transfected with the calcineurin antisense (16). HVACCs in the dynamic equilibrium of calcineurin-protein kinase activity allow DRG cells to both increase and reduce Ca\(^{2+}\) influx and, by this mechanism, contribute to regulation of neurotransmitter release and nociceptive inputs to spinal dorsal horn neurons.

In summary, this study provides substantial new evidence that calcineurin, constitutively expressed in the cytoplasm of DRG neurons, is a key feedback regulator of intracellular Ca\(^{2+}\) and plays an important role in down-regulation of HVACCs by TRPV1 stimulation. Increased calcineurin activity produced by TRPV1 activation could limit Ca\(^{2+}\) influx through HVACCs in the plasma membrane by down-regulation of HVACCs through dephosphorylating the HVACC or a closely associated cytoskeletal protein. This new information is important for our understanding of the molecular mechanism of the analgesic action and diminished spinal synaptic transmission produced by capsaicin and its related analogs. This study highlights the pivotal role of intracellular Ca\(^{2+}\) level and calcineurin in negative modulation of HVACCs in primary sensory neurons.

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FIG. 9. Representative high magnification confocal images showing the spatial distribution of CaV2.2 immunoreactivity in IB4-positive (A) and TRPV1-positive (B) DRG neurons treated with the vehicle or 1 μM capsaicin for 20 s and 2 min. Digitally merged images from CaV2.2 (green) and IB4/TRPV1 (red) labeling are shown on the right. Scale bar, 10 μm. All images are single confocal optical sections.

A

B

Vehicle

Capsaicin (20 s)

Capsaicin (2 min)

Cav2.2

IB4

Overlay

Capsaicin (20 s)

Capsaicin (2 min)

Cav2.2

TRPV1

Overlay

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Transient Receptor Potential Vanilloid Type 1 Activation Down-regulates Voltage-gated Calcium Channels through Calcium-dependent Calcineurin in Sensory Neurons

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