

Propofol Restores Transient Receptor Potential Vanilloid Receptor Subtype-1 Sensitivity *via* Activation of Transient Receptor Potential Ankyrin Receptor Subtype-1 in Sensory Neurons

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ABSTRACT

Background: Cross talk between peripheral nociceptors belonging to the transient receptor potential vanilloid receptor subtype-1 (TRPV1) and ankyrin subtype-1 (TRPA1) family has been demonstrated recently. Moreover, the intravenous anesthetic propofol has directly activates TRPA1 receptors and indirectly restores sensitivity of TRPV1 receptors in dorsal root ganglion (DRG) sensory neurons. Our objective was to determine the extent to which TRPA1 activation is involved in mediating the propofol-induced restoration of TRPV1 sensitivity.

Methods: Mouse DRG neurons were isolated by enzymatic dissociation and grown for 24 h. F-11 cells were transfected with complementary DNA for both TRPV1 and TRPA1 or TRPV1 only. The intracellular Ca^{2+} concentration was measured in individual cells *via* fluorescence microscopy. After TRPV1 de-

What We Already Know about This Topic

- Members of the transient receptor potential family are coexpressed in peripheral sensory neurons and function as transducers of noxious stimuli, such as capsaicin.

What This Article Tells Us That Is New

- Propofol dose dependently restores transient receptor potential vanilloid receptor subtype-1 sensitivity to agonist stimulation *via* a transient receptor potential ankyrin receptor subtype-1-dependent signaling pathway.
- Interactions between transient receptor potential channels are important in modulating nociceptor sensitivity and provide a potential target for novel analgesic treatments.

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Received from the Department of Biological Sciences, Kent State University, Kent, Ohio. Submitted for publication May 27, 2010. Accepted for publication December 6, 2010. Supported by grant HL-65701 from the National Heart, Lung, and Blood Institute, Bethesda, Maryland (to Dr. Damron). F-11 cells were a gift from Probal Banerjee, Ph.D., Professor, Department of Chemistry, College of Staten Island, New York, New York. Anti-TRPA1 antibody was purchased from Novus Biologicals, Littleton, Colorado. Fura-2 acetoxy methyl ester was obtained from Texas Fluorescence Laboratories, Austin, Texas. Anti-TRPV1 antibody, capsaicin, and AITC were purchased from σ Chemical Co, St. Louis, Missouri. HC-030031 was purchased from Enzo Life Sciences, Plymouth Meeting, Pennsylvania. Propofol (Diprivan) and Intralipid were obtained from Cleveland Clinic Pharmacy, Cleveland, Ohio. Collagenase was obtained from Worthington Biochemical Corp, Lakewood, New Jersey. TRPV1 and TRPA1 cDNA were provided by David Julius, Ph.D., Professor, Department of Physiology, University of California at San Francisco, San Francisco, California. Drs. Zhang and Wickley contributed equally to this work.

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sensitization with capsaicin (100 nM), cells were treated with propofol (1, 5, and 10 μM) alone or with propofol in the presence of the TRPA1 antagonist, HC-030031 (0.5 μM), or the TRPA1 agonist, allyl isothiocyanate (AITC; 100 μM); capsaicin was then reapplied.

Results: In DRG neurons that contain both TRPV1 and TRPA1, propofol and AITC restored TRPV1 sensitivity. However, in DRG neurons containing only TRPV1 receptors, exposure to propofol or AITC after desensitization did not restore capsaicin-induced TRPV1 sensitivity. Similarly, in F-11 cells transfected with both TRPV1 and TRPA1, propofol and AITC restored TRPV1 sensitivity. However, in F-11 cells transfected with TRPV1 only, neither propofol nor AITC was capable of restoring TRPV1 sensitivity.

Conclusions: These data demonstrate that propofol restores TRPV1 sensitivity in primary DRG neurons and in cultured F-11 cells transfected with both the TRPV1 and TRPA1 receptors *via* a TRPA1-dependent process. Propofol's effects on sensory neurons may be clinically important and may contribute to peripheral sensitization to nociceptive stimuli in traumatized tissue.

TWO prominent members of the transient receptor potential (TRP) family, the vanilloid subtype-1 (TRPV1) receptor and the ankyrin subtype-1 (TRPA1) receptor, are extensively coexpressed in peripheral sensory neurons and function as sensory transducers of noxious stimuli.^{1–4} The relative sensitivity of these receptors to their respective agonists plays an important role in nociceptive signal transduction. The cellular mechanisms

mediating sensitization, desensitization, and resensitization of TRPV1 receptors are complex and involve calcium-dependent and calcium-independent mechanisms.^{5–9} Recent evidence^{10,11} has suggested that TRPV1 and TRPA1 receptors exhibit reciprocal regulation, indicating cross talk between the two receptors. Specifically, these receptors have cross sensitized or cross desensitized each other, whereby activation of one enhances or inhibits the sensitivity of the other to agonist stimulation.^{10,12–16}

Propofol is one of the most commonly used intravenous anesthetics for the induction and maintenance of general anesthesia and sedation. Apart from its anesthetic properties, propofol has several nonanesthetic effects, one of which is the modulation of TRP receptor function. A recent study¹⁷ indicated that propofol directly activates TRPA1 receptors in transfected human embryonic kidney 293 cells. In addition, our laboratory recently showed that propofol restores the sensitivity of TRPV1 receptors after agonist-induced desensitization and attenuates agonist-induced desensitization *via* a protein kinase C ε -dependent signaling pathway in mouse dorsal root ganglion (DRG) sensory neurons.¹⁸ However, to our knowledge, no study has investigated the role of TRPA1 receptor activation on restoration of TRPV1 sensitivity or the role that TRPA1 may play in the propofol-induced restoration of TRPV1 sensitivity.

In the current study, we tested the hypothesis that propofol restores TRPV1 receptor sensitivity to agonist stimulation *via* a TRPA1-dependent pathway. Moreover, we also tested the hypothesis that TRPA1 activation restores TRPV1 receptor sensitivity to agonist stimulation. The major finding is that in DRG neurons containing both TRPV1 and TRPA1, pretreatment with propofol or allyl isothiocyanate (AITC) restores TRPV1 sensitivity to agonist stimulation in the previously desensitized neurons. In contrast, restoration of TRPV1 sensitivity to agonist stimulation by propofol or AITC was not observed in DRG neurons containing only TRPV1 receptors (*i.e.*, those lacking TRPA1). Moreover, the TRPA1 antagonist, HC-030031, prevents the propofol-induced restoration of TRPV1 sensitivity. In F-11 cells (DRG neuronal cell line) transfected with TRPV1 complementary DNA (cDNA) only (*i.e.*, those lacking TRPA1), neither propofol nor AITC restores TRPV1 sensitivity. However, in F-11 cells transfected with both TRPV1 and TRPA1, propofol and AITC restore TRPV1 sensitivity. Our current findings indicate that propofol can resensitize TRPV1 receptors, thereby restoring TRPV1 function *via* a TRPA1-dependent signaling pathway. In addition, our findings indicate that TRPA1 activation can restore TRPV1 receptor sensitivity to agonist stimulation.

Materials and Methods

All experimental procedures and protocols were approved by the Kent State University Institutional Animal Care and Use Committee, Kent, Ohio; and conform to the international guidelines for the care and use of animals.

Animals

Male C57BL/6 mice, aged 12 weeks, were used. All animals were housed at an animal care facility at Kent State University that is accredited by the American Association for Accreditation of Laboratory Animal Care.

DRG Cell Isolation and Culture

DRG neurons from adult mice (weight, 30–40 g) were used in this study. The ganglia were dissected from the lumbar (L1–L6) segments of the spinal cord; incubated with type 4 collagenase, 0.15%, at 37°C for 50 min; and dissociated by gentle trituration. Neurons were cultured on coverslips precoated with poly-D-lysine and laminin at 37°C in F-12/Dulbecco's modified eagle's medium (50/50) medium supplemented with fetal bovine serum, 10%; 100 ng/ml nerve growth factor; and antibiotics in a humidified atmosphere of 5% CO₂ and 95% air. Proliferation of fibroblasts and Schwann cells was prevented by including cytosine arabinoside (5–10 μ M) in the medium. Cells began to develop neurites within approximately 24 h, and studies were performed within 24 h from the time of isolation.

F-11 Cell Transfection with TRPV1 or TRPA1 cDNA

Cultured F-11 cells (DRG neuronal cell line) were transfected with TRPV1 or TRPA1 cDNA *via* electroporation using a commercially available system (Neon Transfection System; Invitrogen, Carlsbad, CA), per manufacturer's instructions for F-11 cells. Briefly, cultured F-11 cells at 80% confluence were harvested and washed with phosphate-buffered saline without Ca²⁺ or Mg²⁺. The cells were resuspended in 100- μ l electrolytic Buffer R in a sterile Eppendorf tube and 5 μ g either TRPV1 or TRPA1 cDNA was then added. Electroporation was performed using a pulse voltage of 1500 V, a pulse width of 35 ms, and a pulse number of 2. After electroporation, the cells were placed in F-12/DMEM (50/50) supplemented with fetal bovine serum, 10%, at 37°C. A proportion of the cells was seeded into a six-well culture plate and incubated for 48 h at 37°C in a humidified atmosphere of 5% CO₂ and 95% air. These cells were used for intracellular Ca²⁺ measurements. The remaining cells were used for immunoblot analysis to confirm the presence of TRPV1 and TRPA1.

Intracellular Ca²⁺ Measurements

DRG neurons or F-11 cells were incubated at room temperature (23°C) for 15 min with fura-2 acetoxy methyl ester (2 μ M) in HEPES-buffered saline containing the following: 118 mM NaCl, 4.8 mM KCl, 1.2 mM MgCl₂, 1.25 mM CaCl₂, 11.0 mM dextrose, 5 mM pyruvate, and 25 mM HEPES (pH 7.35). Coverslips containing the fura-2 acetoxy methyl ester-loaded DRG neurons were placed in a temperature-regulated (30°C) chamber (Warner Instruments, Hamden, CT) mounted on the stage of an inverted fluorescence microscope (Olympus IX-81; Olympus America, Lake Success, NY). The cells were superfused continuously with HEPES-buffered saline at a flow rate of

2 ml/min. Drugs were delivered by switching from control buffer to drug-containing buffer for 20 s unless noted otherwise. Intracellular free Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) measurements were obtained simultaneously for multiple individual DRG neurons using a fluorescence imaging system (Easy Ratio Pro; Photon Technology International, Lawrenceville, NJ) equipped with a multiwavelength spectrofluorometer (DeltaRAM X) and an electron-multiplying charged-coupled device camera (QuantEM 512SC; Photometrics, Tuscon, AZ). Images and photometric data were acquired by alternating excitation wavelengths between 340 and 380 nm (20 Hz) and monitoring an emission wavelength of 510 nm. Because calibration procedures rely on several assumptions, the ratio of the light intensities at the two wavelengths was used to measure qualitative changes in $[\text{Ca}^{2+}]_i$. Just before data acquisition, background fluorescence was measured and automatically subtracted from the subsequent experimental measurement using computer software (Easy Ratio Pro; Photon Technology International).

Immunoblot Analysis of TRPA1 and TRPV1

Immunoblot analysis was performed on whole F-11 cell lysates, as described previously in DRG neurons.¹⁸ Protein concentration was assessed using the Bradford method.¹⁹ All samples were adjusted to a protein concentration of 1–2 mg/ml in sample buffer, boiled for 5 min, and then kept at -20°C until use. Equal amounts of protein (50 μg) from each fraction were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis on polyacrylamide gels, 12%, and transferred to nitrocellulose membranes. Nonspecific binding was blocked with Tris-buffered saline solution (vol/vol, 0.1%), Tween 20 in 20 mM Tris base, and 137 mM NaCl adjusted to pH 7.6 with HCl, containing bovine serum albumin, 3% (wt/vol), for 1 h at room temperature. Antibodies against total TRPV1 and TRPA1 were diluted 1:1,000 in Tris-buffered saline containing 1% bovine serum albumin for immune-blotting (2 h). After washing in Tris-buffered saline three times (10 min each), membranes were incubated for 1 h at room temperature with horseradish peroxidase-linked secondary antibody (goat antimouse and goat antirabbit; 1:5,000 dilution in Tris-buffered saline containing bovine serum albumin, 1%). Membranes were washed again, and bound antibody was detected by enhanced chemiluminescence. Immunoreactivity was quantified by scanning densitometry and analyzed using software (ImageJ; National Institutes of Health, Washington, DC).

Statistical Analysis

All Ca^{2+} imaging experimental protocols were repeated in a minimum of five DRG neurons obtained from at least five different mice or in four separate coverslips of F-11 cells. Results obtained from each animal (for DRG experiments) or coverslips of cells (for F-11 cell experiments) were averaged so that all animals or coverslips of cells were weighted equally. Within-group comparisons were made using repeated-measures one-way analysis of variance and the Bonferroni *post hoc* test. Comparisons between groups were made using

two-way analysis of variance and the Bonferroni *post hoc* test. Differences were considered statistically significant at $P < 0.05$. All results are expressed as mean \pm SD. The error bars in all of the figures refer to the variability in the peak Ca^{2+} response compared with the control response or a specific intervention, as noted in the legends. Statistical analysis was conducted using software (NCSS, Kaysville, UT).

Experimental Protocols

DRG neurons were chosen for study by distinguishing small ($<30\text{-}\mu\text{m}$) nociceptive neurons (capsaicin sensitive) from large ($>40\text{-}\mu\text{m}$) mechanosensitive neurons (capsaicin insensitive) using a precalibrated eyepiece reticle to measure the diameter of the neuronal cell body. The lipid emulsion form of propofol (Diprivan; Astra Zeneca, London, United Kingdom) (56 mM [10 mg/ml] propofol; soybean oil, 10%; glycerol, 2.25%; and purified phospholipid, 1.2%) was used in all protocols and will be referred to as propofol throughout this article. Intralipid vehicle was used as a control. The effect of Intralipid was examined at concentrations equivalent to those used for the propofol–Intralipid mixture.

Selection Criteria for Determining Subpopulations of DRG or F-11 Neurons. Before each protocol was initiated, DRG neurons (protocols 1 and 2) or F-11 cells (protocols 3 and 4) containing TRPV1 receptors only or both TRPV1 and TRPA1 receptors were identified first by exposing the cells to single applications of capsaicin, AITC, and propofol. Cells responsive to only capsaicin were deemed to contain only functional TRPV1 receptors, whereas cells that were responsive to capsaicin, AITC, and propofol were deemed to contain both TRPV1 and TRPA1 receptors. Cells that did not respond to capsaicin, AITC, or propofol were excluded from the study and, therefore, were not used in the statistical analysis.

Protocol 1: Effect of Propofol on Restoration of TRPV1 Sensitivity to Agonist Stimulation in Mouse DRG Neurons Containing Both Functional TRPV1 and TRPA1 Receptors.

To determine the extent to which TRPA1 is involved in the propofol-induced restoration of TRPV1 receptor sensitivity, $[\text{Ca}^{2+}]_i$ was monitored in DRG neurons that were predetermined to contain both functional TRPV1 and TRPA1 receptors. The DRG neurons were exposed repeatedly to capsaicin (100 nM) every 30 s to induce desensitization of the channel. After desensitization, neurons were exposed to propofol (10 μM , 10 min) and capsaicin was reapplied. Parallel experiments demonstrating the effect of the specific TRPA1 activator, AITC (100 μM),³ on restoration of TRPV1 receptor sensitivity in the absence of propofol were also performed. Summarized results are expressed as a percentage of the response to the final application of capsaicin in untreated cells (control).

Protocol 2: Effect of Propofol on Restoration of TRPV1 Sensitivity to Agonist Stimulation in Mouse DRG Neurons Containing Only Functional TRPV1 Receptors. To determine further the extent to which TRPA1 is involved in the propofol-induced restoration of TRPV1 receptor sensitivity,

protocol 1 was repeated in DRG neurons that were predetermined to contain only functional TRPV1 receptors (*i.e.*, those lacking TRPA1). In addition, the effect of the specific TRPA1 inhibitor (HC-030031, 0.5 μ M, 5 min)²⁰ on restoration of TRPV1 receptor sensitivity in the presence of propofol was also performed. Summarized results are expressed as a percentage of the response to the final application of capsaicin in untreated cells (control).

Protocol 3: Effect of Propofol on Restoration of TRPV1 Sensitivity to Agonist Stimulation in Cultured F-11 Cells Expressing Both TRPA1 and TRPV1. To determine further the extent to which TRPA1 is involved in the propofol-induced restoration of TRPV1 receptor sensitivity, F-11 cells that were transfected with both TRPV1 and TRPA1 cDNA were exposed repeatedly to capsaicin, as described in protocol 1. After desensitization, neurons were exposed to propofol (1, 5, and 10 μ M) before restimulation of TRPV1 receptors with capsaicin. Parallel experiments demonstrating the effect of the specific TRPA1 activator, AITC (100 μ M), on restoration of TRPV1 receptor sensitivity in the absence of propofol were also performed. Before the start of the protocol, F-11 cells that contained both functional TRPV1 and TRPA1 receptors were identified first by treating the cells with single applications of capsaicin (100 nM), AITC (100 μ M), and propofol (10 μ M). Only cells that were responsive to capsaicin, AITC, and propofol were chosen for the protocol. Summarized results are expressed as a percentage of the response to the final application of capsaicin during the initial desensitization of TRPV1.

Protocol 4: Effect of Propofol on Restoration of TRPV1 Sensitivity to Agonist Stimulation in Cultured F-11 Cells Expressing TRPV1 Only. To determine further the extent to which TRPA1 is involved in the propofol-induced restoration of TRPV1 receptor activity, protocol 2 was repeated in F-11 cells that were transfected with only TRPV1 cDNA. Before the start of the protocol, F-11 cells that contained only functional TRPV1 receptors were identified first by treating the cells with a single application of capsaicin (100 nM), AITC (100 μ M), and propofol (10 μ M). Only cells that were responsive to capsaicin were chosen for the protocol. Summarized results are expressed as a percentage of the response to the final application of capsaicin during the initial desensitization of TRPV1.

Results

Effect of Propofol on Restoration of TRPV1 Sensitivity to Agonist Stimulation in Mouse DRG Neurons Containing Both Functional TRPV1 and TRPA1 Receptors

DRG neurons containing both functional TRPV1 and TRPA1 receptors were identified first by treating the cells with single applications of capsaicin (100 nM), AITC (100 μ M), and propofol (10 μ M). DRG neurons that responded with a transient increase in $[Ca^{2+}]_i$ subsequent to capsaicin (100 nM), AITC, and propofol treatment were selected for the protocol (fig. 1A). Verification of TRPA1 and TRPV1

protein expression in cultured DRG neurons was determined *via* immunoblot analysis of DRG cell lysates (fig. 1A, inset). Repetitive stimulation of the preselected DRG neurons with capsaicin resulted in a progressive decrease (desensitization) in peak $[Ca^{2+}]_i$ that was maintained after a 10-min pause in capsaicin stimulation, as indicated by the lack of any response to capsaicin after reapplication of capsaicin to the bath (fig. 1B). In contrast, when propofol (10 μ M) was added to the bath during the 10-min pause in stimulation, subsequent reapplication of capsaicin resulted in a robust transient increase in $[Ca^{2+}]_i$ (resensitization) (fig. 1C). Pretreatment with HC-030031 (0.5 μ M) inhibited the propofol-induced restoration of TRPV1 sensitivity to capsaicin compared with DRGs treated only with propofol (fig. 1D). In addition, pretreatment with AITC (100 μ M) restored TRPV1 sensitivity (fig. 1E). Intralipid vehicle alone (amount of Intralipid equivalent to that added with 10 μ M propofol) was unable to restore TRPV1 sensitivity to capsaicin ($99.1 \pm 6\%$ [mean \pm SD] of control). Summarized data depicting the effect of propofol alone (10 μ M), propofol in the presence of HC-030031 (0.5 μ M), or AITC (100 μ M) on restoration of TRPV1 sensitivity to capsaicin are depicted in figure 1F.

Effect of Propofol on Restoration of TRPV1 Sensitivity to Agonist Stimulation in Mouse DRG Neurons Containing Only Functional TRPV1 Receptors

DRG neurons containing only functional TRPV1 receptors were identified first by treating the cells with single applications of capsaicin (100 nM), AITC (100 μ M), and propofol (10 μ M). DRG neurons that responded with a transient increase in $[Ca^{2+}]_i$ subsequent to capsaicin treatment only were selected for the protocol (fig. 2A). Repetitive stimulation of the preselected DRG neurons with capsaicin resulted in a progressive decrease (desensitization) in peak $[Ca^{2+}]_i$ that was maintained after a 10-min pause in capsaicin stimulation, as indicated by the lack of any response to capsaicin after reapplication to the bath (fig. 2B). When propofol (10 μ M) was added to the bath during the 10-min pause in stimulation, subsequent reapplication of capsaicin to the bath did not result in a robust transient increase in $[Ca^{2+}]_i$ (fig. 2C). In addition, pretreatment with AITC (100 μ M) did not restore TRPV1 sensitivity (fig. 2D). Summarized data depicting the effect of propofol alone (10 μ M) or AITC (100 μ M) on restoration of TRPV1 sensitivity to capsaicin are depicted in figure 2E.

Effect of Propofol on Restoration of TRPV1 Sensitivity to Agonist Stimulation in Cultured F-11 Cells Expressing Both TRPA1 and TRPV1

First, verification of TRPV1 and TRPA1 protein expression was determined *via* immunoblot analysis of F-11 cell lysates obtained from the same group of transfected cells that was used for measurement of $[Ca^{2+}]_i$ (fig. 3A). TRPV1 and TRPA1 were both expressed in F-11 cells transfected with both TRPV1 and TRPA1 cDNA. Second, AITC (100 μ M), capsaicin (100 nM),

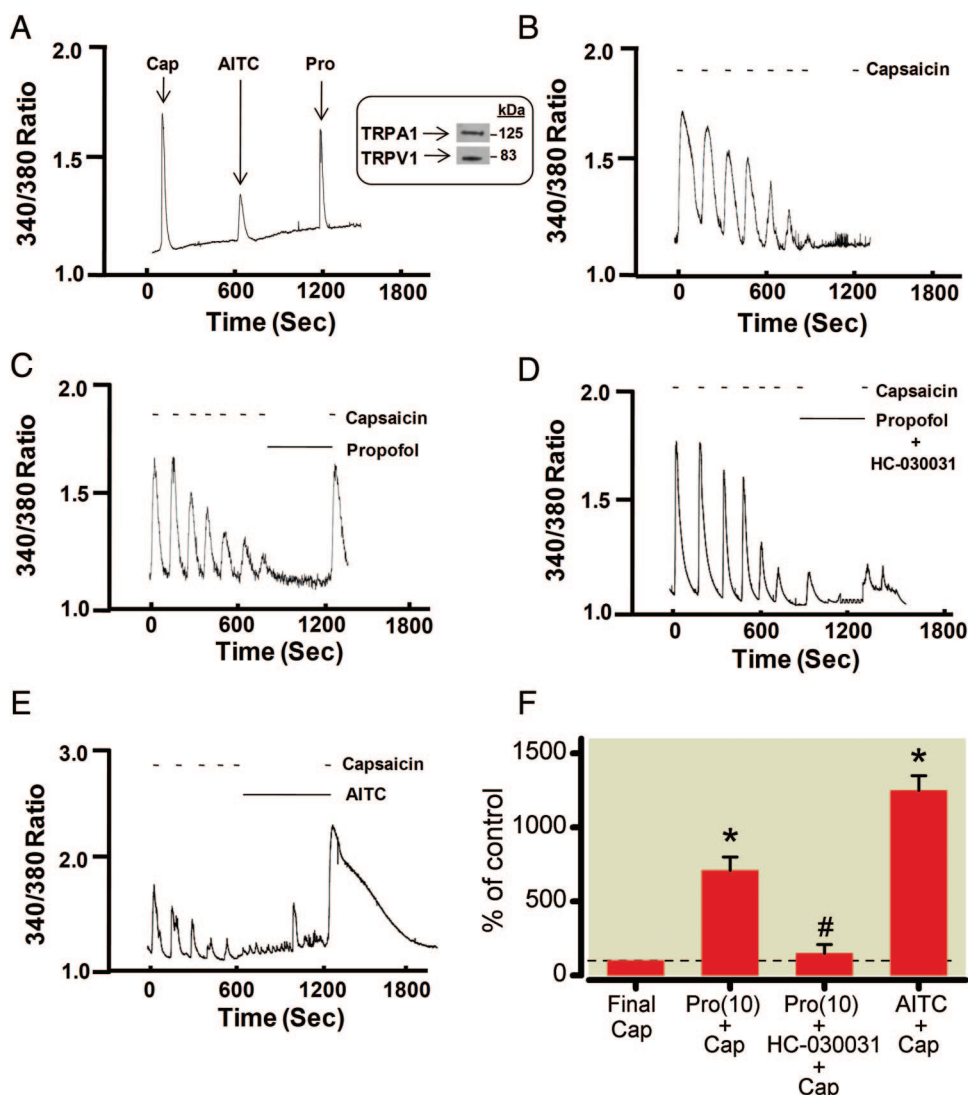


Fig. 1. (A) Representative trace depicting the effect of a single application of capsaicin (Cap; 100 nM), the specific transient receptor potential ankyrin receptor subtype-1 (TRPA1) activator allyl isothiocyanate (AITC; 100 μ M), or propofol (Pro; 10 μ M) on intracellular free calcium concentration in mouse dorsal root ganglion (DRG) neurons. Inset: Representative immunoblot depicting TRPA1 and transient receptor potential vanilloid receptor subtype-1 (TRPV1) protein expression in cultured DRG neurons. (B through E) Representative traces depicting the effect of time, Pro (10 μ M), Pro in the presence of the specific TRPA1 inhibitor HC-030031 (0.5 μ M), and AITC (100 μ M), respectively, after capsaicin-induced (100 nM) desensitization on restoration of TRPV1 sensitivity in mouse DRG neurons that contain functional TRPV1 and TRPA1 receptors. (F) Summarized data for B through E. Data are expressed as a percentage of the response to the final application of capsaicin (Final Cap) in the untreated control and reported as the mean \pm SD. * $P < 0.05$ versus Final Cap in the untreated control. # $P < 0.05$ versus Pro plus Cap. (The DRG neurons were obtained from five different mice.)

and propofol (10 μ M) stimulation of F-11 cells transfected with both TRPV1 and TRPA1 cDNA all resulted in a robust transient increase in $[Ca^{2+}]_i$, thus identifying F-11 cells containing fully functional TRPV1 and TRPA1 receptors (fig. 3B). In the F-11 cells containing both TRPV1 and TRPA1, propofol (1, 5, and 10 μ M) restored TRPV1 sensitivity to capsaicin (fig. 3C). Summarized data depicting the effect of propofol (1, 5, and 10 μ M) on restoration of TRPV1 sensitivity in F-11 cells containing both TRPV1 and TRPA1 are depicted in figure 3D. In addition, AITC (100 μ M) restored TRPV1 sensitivity to capsaicin in the F-11 cells containing both TRPV1 and TRPA1 (fig. 4A). Summarized data depicting the effect of AITC (100

μ M) on restoration of TRPV1 sensitivity in F-11 cells containing both TRPV1 and TRPA1 are depicted in figure 4B.

Effect of Propofol on Restoration of TRPV1 Sensitivity to Agonist Stimulation in Cultured F-11 Cells Expressing Only TRPV1

First, verification of TRPV1 protein expression was determined *via* immunoblot analysis of F-11 cell lysates obtained from the same group of TRPV1-transfected cells that was used for measurement of $[Ca^{2+}]_i$ (fig. 5A). TRPV1 was expressed in F-11 cells transfected with TRPV1 cDNA while TRPA1 was not present. Second,

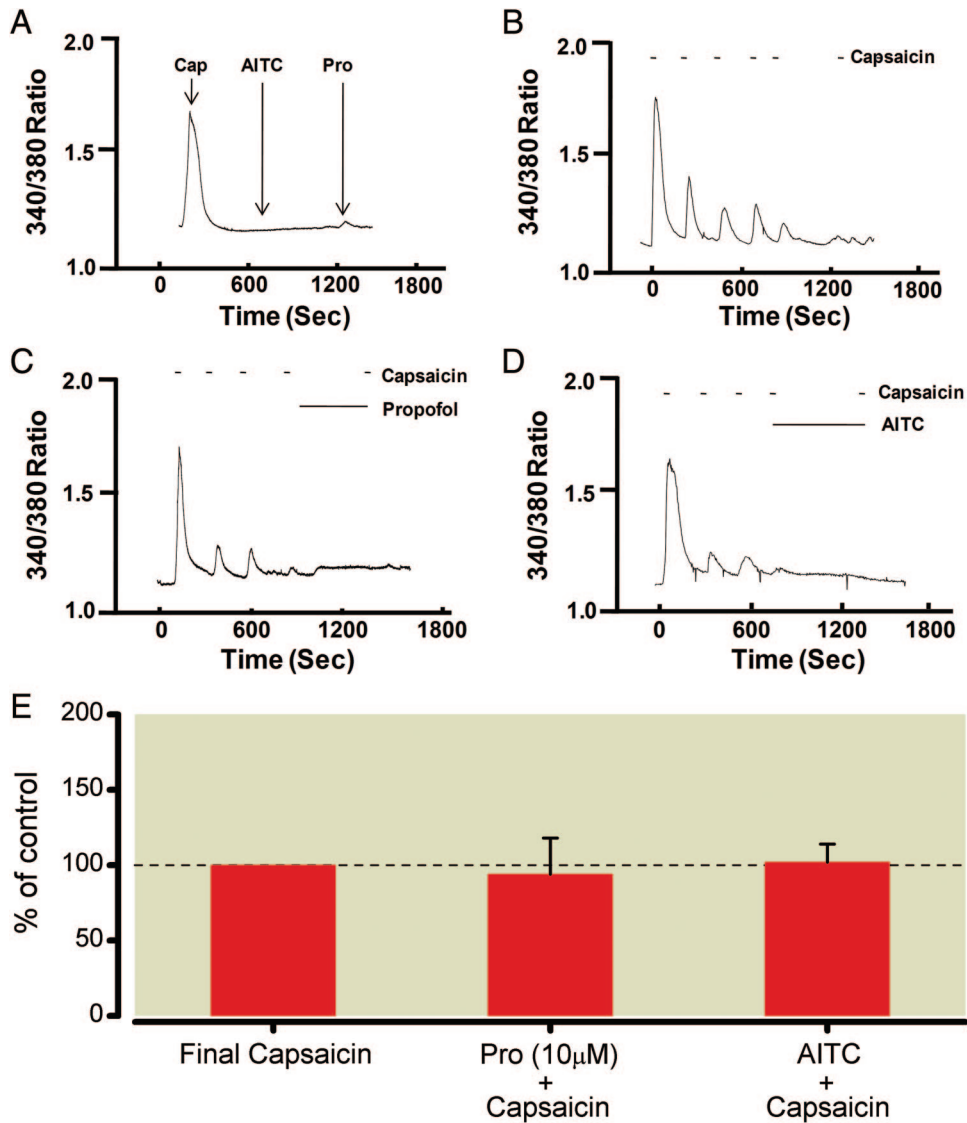


Fig. 2. (A) Representative trace depicting the effect of a single application of capsaicin (Cap; 100 nM), the specific transient receptor potential ankyrin subtype-1 (TRPA1) activator allyl isothiocyanate (AITC; 100 μ M), or propofol (Pro; 10 μ M) on intracellular free calcium concentration in mouse dorsal root ganglion (DRG) neurons. (B through D) Representative traces depicting the effect of time, Pro (10 μ M), and AITC (100 μ M), respectively, after capsaicin-induced (100 nM) desensitization on restoration of transient receptor potential vanilloid receptor subtype-1 (TRPV1) sensitivity in mouse DRG neurons that contain only functional TRPV1 receptors. (E) Summarized data for B through D. Data are expressed as a percentage of the response to the final application of capsaicin (Final Capsaicin) in the untreated control and reported as the mean \pm SD. (The DRG neurons were obtained from five different mice.)

AITC (100 μ M) or propofol (10 μ M) stimulation of F-11 cells transfected with only TRPV1 cDNA did not result in a transient increase in $[Ca^{2+}]_i$; stimulation with capsaicin (100 nM) resulted in a robust transient increase in $[Ca^{2+}]_i$, thus identifying F-11 cells containing only functional TRPV1 receptors that were used in the protocol (fig. 5B). In nontransfected F-11 cells, neither AITC nor capsaicin elicited a Ca^{2+} response (data not shown). In the F-11 cells containing TRPV1, but lacking TRPA1, propofol failed to restore TRPV1 sensitivity to capsaicin (fig. 5C). Summarized data depicting the effect of propofol (1, 5, and 10 μ M) on restoration of TRPV1 sensitivity in F-11 cells containing TRPV1, but lacking TRPA1, are

depicted in figure 5D. In addition, AITC (100 μ M) failed to restore TRPV1 sensitivity to capsaicin in F-11 cells containing TRPV1 but lacking TRPA1 (fig. 6A). Summarized data depicting the effect of AITC (100 μ M) on restoration of TRPV1 sensitivity in F-11 cells containing TRPV1, but lacking TRPA1, are depicted in figure 6B.

Discussion

To our knowledge, this is the first study to assess the role of TRPA1 receptor activation on TRPV1-dependent Ca^{2+} signaling and mediation of propofol-induced restoration of TRPV1 sensitivity to agonist stimulation. Recent studies^{10–16} have shown that TRPA1 and TRPV1 exhibit cross

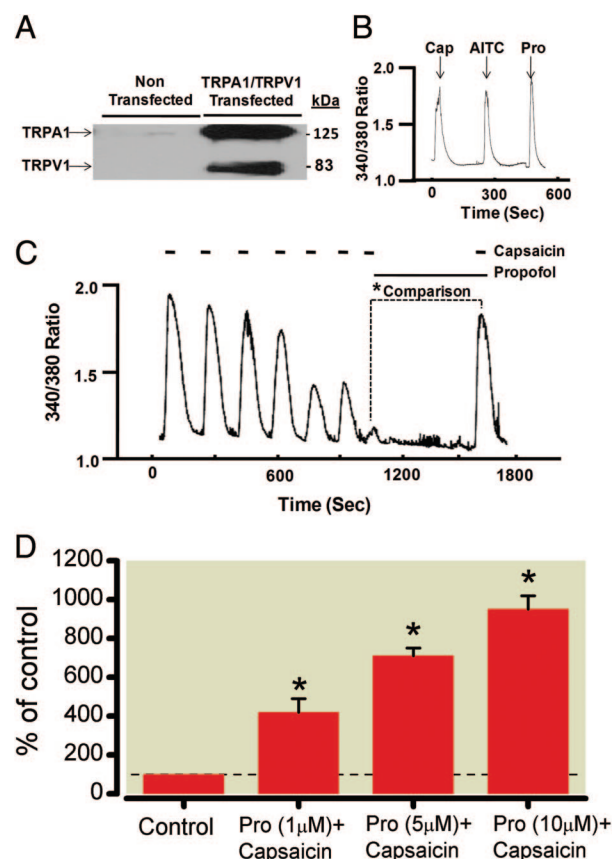


Fig. 3. (A) Representative immunoblot depicting transient receptor potential ankyrin subtype-1 (TRPA1) and transient receptor potential vanilloid receptor subtype-1 (TRPV1) protein expression in F-11 cells transfected with both TRPA1 and TRPV1 complementary DNA (cDNA). (B) Representative trace depicting the effect of a single application of capsaicin (Cap; 100 nM), the specific TRPA1 activator allyl isothiocyanate (AITC; 100 μM), or propofol (Pro; 10 μM) on intracellular free calcium concentration in F-11 cells transfected with both TRPA1 and TRPV1 cDNA. (C) Representative trace depicting the effect of Pro (5 μM) after Cap-induced (100 nM) desensitization on restoration of TRPV1 sensitivity in F-11 cells transfected with both TRPA1 and TRPV1 cDNA. The dashed line indicates the statistical comparison that was made. (D) Summarized data depicting the effect of Pro (1, 5, and 10 μM) after Cap-induced desensitization on restoration of TRPV1 sensitivity in F-11 cells transfected with both TRPA1 and TRPV1 cDNA. Data are expressed as a percentage of the response to the final application of capsaicin before propofol treatment (Control) and reported as the mean \pm SD. * $P < 0.05$ versus Control. (Nine separate coverslips of F-11 cells were used.)

talk between each other, resulting in modulation of receptor sensitivity to agonist stimulation. Recent studies^{15,16} have indicated that short-term TRPA1 activation can desensitize TRPV1 receptors and *vice versa*. However, to our knowledge, the role of TRPA1 activation on restoration of TRPV1 sensitivity in previously desensitized TRPV1 receptors has not been investigated. In addition, more recent studies have shown that propofol activates TRPA1 receptors in DRG neurons¹⁷ and our laboratory recently demonstrated that propofol can modulate TRPV1 sensi-

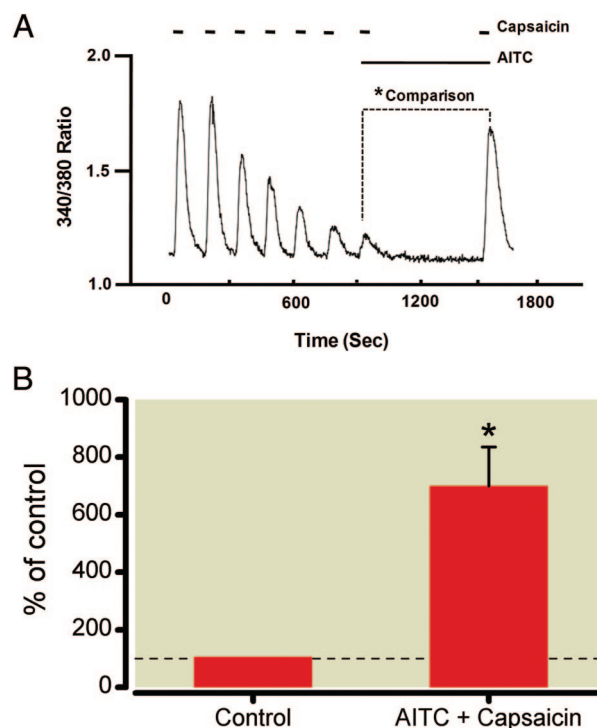


Fig. 4. (A) Representative trace depicting the effect of the specific transient receptor potential ankyrin receptor subtype-1 (TRPA1) activator allyl isothiocyanate (AITC; 100 μM) after capsaicin-induced (100 nM) desensitization on restoration of transient receptor potential vanilloid receptor subtype-1 (TRPV1) sensitivity in F-11 cells transfected with both TRPA1 and TRPV1 cDNA. Data are expressed as a percentage of the response to the final application of capsaicin before AITC treatment (Control) and reported as the mean \pm SD. * $P < 0.05$ versus Control. (Four separate coverslips of F-11 cells were used).

tivity.¹⁸ Therefore, we tested the hypothesis that propofol restores TRPV1 receptor sensitivity to agonist stimulation *via* a TRPA1-dependent pathway. Moreover, we tested the hypothesis that TRPA1 activation restores TRPV1 receptor sensitivity to agonist stimulation. Our novel findings are that propofol dose dependently restores TRPV1 sensitivity to agonist stimulation *via* a TRPA1-dependent signaling pathway and that TRPA1 activation restores TRPV1 sensitivity to agonist stimulation in sensory neurons. Our findings also imply that TRPA1 and TRPV1 could directly interact and form a heteromer, as described previously.^{10,21}

Effect of Propofol on Restoration of TRPV1 Sensitivity to Agonist Stimulation in Mouse DRG Neurons Containing Both Functional TRPV1 and TRPA1 Receptors

To determine the extent to which TRPA1 is involved in the propofol-induced restoration of TRPV1 receptor sensitivity, DRG neurons that were predetermined to contain both

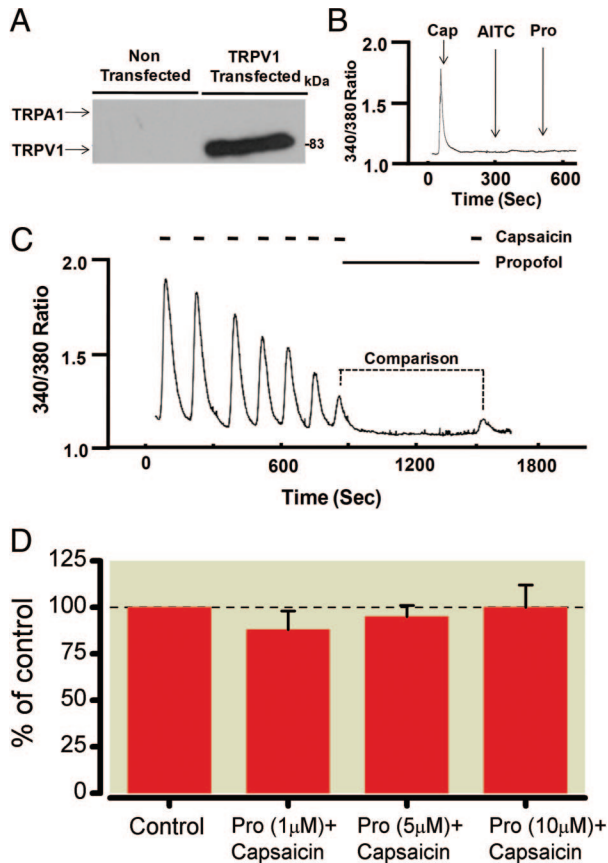


Fig. 5. (A) Representative immunoblot depicting transient receptor potential ankyrin subtype-1 (TRPA1) and transient receptor potential vanilloid receptor subtype-1 (TRPV1) protein expression in F-11 cells transfected with TRPV1 complementary DNA (cDNA) only. (B) Representative trace depicting the effect of a single application of capsaicin (Cap; 100 nM), allyl isothiocyanate (AITC; 100 μM), or propofol (Pro; 10 μM) on intracellular free calcium concentration in F-11 cells transfected with TRPV1 cDNA only. (C) Representative trace depicting the effect of Pro (5 μM) after Cap-induced (100 nM) desensitization on restoration of TRPV1 sensitivity in F-11 cells transfected with TRPV1 cDNA only. The dashed line indicates the statistical comparison that was made. (D) Summarized data depicting the effect of Pro (1, 5, and 10 μM) after Cap-induced desensitization on restoration of TRPV1 sensitivity in F-11 cells transfected with TRPV1 cDNA. Data are expressed as a percentage of the response to the final application of Cap before Pro treatment (Control) and reported as the mean ± SD. (Nine separate coverslips of F-11 cells were used.)

functional TRPV1 and TRPA1 receptors were treated with propofol after desensitization with capsaicin; in addition, the effect of the TRPA1 agonist, AITC, on restoration of TRPV1 sensitivity was also assessed. Our findings indicate that in DRG neurons that contain both functional TRPV1 and TRPA1 receptors, both propofol and AITC were able to restore the sensitivity of previously desensitized TRPV1 receptors. In addition, the propofol-induced restoration of TRPV1 sensitivity to agonist stimulation is markedly attenuated in these DRG neurons treated with HC-030031. One

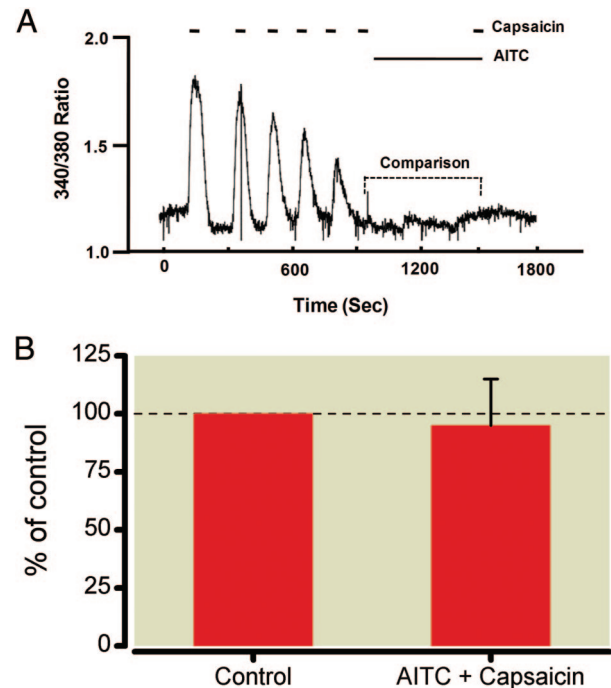


Fig. 6. (A) Representative trace depicting the effect of the specific transient receptor potential ankyrin subtype-1 (TRPA1) activator allyl isothiocyanate (AITC; 100 μM) after capsaicin-induced (100 nM) desensitization on restoration of transient receptor potential vanilloid receptor subtype-1 (TRPV1) sensitivity in F-11 cells transfected with TRPV1 complementary DNA (cDNA) only. The dashed line indicates the statistical comparison that was made. (B) Summarized data depicting the effect of AITC after capsaicin-induced desensitization on restoration of TRPV1 sensitivity in F-11 cells transfected with TRPV1 cDNA only. Data are expressed as a percentage of the response to the final application of capsaicin before AITC treatment (Control) and reported as the mean ± SD. (Four separate coverslips of F-11 cells were used.)

unexpected finding was that the addition of propofol or AITC after TRPV1 desensitization did not elicit any transient increase in $[Ca^{2+}]_i$ (fig. 2, C and E), which was indicative of an activation of TRPA1 receptors, as noted in a previous study.¹¹ This is somewhat concerning considering that this lack of response to AITC and propofol could be because of the lack of TRPA1 receptors in the population of DRG neurons being studied. However, the population of DRG neurons used in this protocol responded to single applications of AITC and propofol, indicating the presence of functional TRPA1 receptors. The finding that the addition of propofol or AITC after TRPV1 desensitization did not elicit any transient increase in $[Ca^{2+}]_i$ may be because of TRPV1 desensitization-induced cross talk with the TRPA1 receptor that may prevent a propofol-induced TRPA1-dependent Ca^{2+} response but still may allow for a Ca^{2+} -independent signaling mechanism that can modulate TRPV1 activity. In fact, a recent study²¹ demonstrated that TRPA1 is regulated by TRPV1 independent of intracellular Ca^{2+} . In

addition, it was demonstrated recently that propofol restores TRPV1 sensitivity to agonist stimulation *via* activation of protein kinase C ϵ , which is a Ca^{2+} -independent protein kinase C isoform.²² Therefore, propofol or AITC may be activating protein kinase C ϵ *via* a TRPA1 Ca^{2+} -independent process. These questions remain unanswered but are being addressed in our laboratory. However, these data obtained from DRG neurons indicate that TRPA1 receptors are required for the propofol-induced restoration of TRPV1 receptor sensitivity to agonist stimulation. Our findings are consistent with previous studies^{10–16} indicating cross talk between TRPV1 and TRPA1 receptors that regulates and modulates nociceptive ion channel function.

Effect of Propofol on Restoration of TRPV1 Sensitivity to Agonist Stimulation in Mouse DRG Neurons Containing Only Functional TRPV1 Receptors

To determine the extent to which TRPA1 is involved in the propofol-induced restoration of TRPV1 receptor sensitivity, DRG neurons that were predetermined to contain only functional TRPV1 receptors (*i.e.*, those lacking TRPA1) were treated with propofol or AITC after desensitization with capsaicin; $[\text{Ca}^{2+}]_i$ was measured in response to another challenge with capsaicin. Our findings indicate that in DRG neurons that contain only functional TRPV1 receptors (*i.e.*, those lacking TRPA1), neither propofol nor AITC was able to restore the sensitivity of previously desensitized TRPV1 receptors, unlike the propofol- and AITC-induced restoration of TRPV1 sensitivity in DRG neurons containing both TRPV1 and TRPA1. These findings indicate that the presence of TRPA1 receptors may be required for propofol to restore TRPV1 sensitivity and are in agreement with a recent finding indicating that propofol directly activates TRPA1 receptors.¹⁷

Effect of Propofol on Restoration of TRPV1 Sensitivity to Agonist Stimulation in Cultured F-11 Cells Expressing Both TRPA1 and TRPV1

To substantiate further our findings in DRG neurons that the presence of TRPA1 is required for the propofol-induced restoration of TRPV1 sensitivity, we incorporated the use of a heterologous cell line in which we could experimentally manipulate the expression of TRPV1 and TRPA1 receptors to characterize further the role of TRPA1 in the propofol-induced restoration of TRPV1 sensitivity. Subsequently, we transfected F-11 cells with both TRPV1 and TRPA1 cDNA and assessed whether the presence of TRPA1 is required for propofol or AITC to restore TRPV1 sensitivity. Our findings indicate that clinically relevant concentrations of propofol dose dependently restored TRPV1 sensitivity to agonist stimulation in F-11 cells containing both functional TRPV1 and TRPA1 receptors. In addition, AITC restored TRPV1 sensitivity to agonist stimulation in F-11 cells containing both TRPA1 and TRPV1 receptors. These findings are consistent with our finding of a propofol- and AITC-in-

duced restoration of TRPV1 sensitivity in DRG neurons that were predetermined to contain both TRPV1 and TRPA1 receptors.

Effect of Propofol on Restoration of TRPV1 Sensitivity to Agonist Stimulation in Cultured F-11 Cells Expressing Only TRPV1

To determine whether the presence of TRPA1 receptors is required for propofol to restore TRPV1 sensitivity, we incorporated the use of F-11 cells transfected with only TRPV1 cDNA. In F-11 cells containing TRPV1 receptors but lacking TRPA1 receptors, both propofol and AITC failed to restore TRPV1 sensitivity, indicating that propofol is incapable of restoring TRPV1 receptor sensitivity to agonist stimulation if the TRPA1 receptor is absent. These findings substantiate further the TRPA1 receptor as a key component of the signaling mechanism by which propofol restores TRPV1 sensitivity to agonist stimulation. These results, in combination with the results obtained from DRG neurons, provide definitive evidence that propofol restores TRPV1 sensitivity to agonist stimulation *via* a TRPA1-dependent process.

However, our finding that TRPA1 activation can restore the sensitivity of previously desensitized TRPV1 receptors is somewhat surprising considering that recent studies^{15,16} have indicated that TRPA1 activation desensitizes TRPV1 receptors. An important distinction that needs to be addressed is that these studies investigated the effect of short-term TRPA1 activation on the sensitivity of TRPV1 receptors that were not previously desensitized, which is different from our current study. As such, the state of responsiveness of the TRPV1 receptor (*i.e.*, either desensitized or not desensitized) to agonists could also affect how other receptors, most notably TRPA1, respond to particular regulatory pathways, such as TRPV1 activation. The process of TRPV1 desensitization could initiate an intracellular signaling pathway that alters the functional characteristics of the TRPA1 receptor. Whether desensitization of TRPV1 alters TRPA1 sensitivity to agonists is being investigated by our laboratory. In addition, in DRG neurons, TRPV1 receptors appear to desensitize more rapidly when TRPA1 receptors are not present, as indicated by fewer capsaicin applications needed to fully desensitize the receptor. This observation substantiates our finding that TRPA1 activation can restore the sensitivity of desensitized TRPV1 receptors. Moreover, propofol and AITC also failed to elicit any transient increase in $[\text{Ca}^{2+}]_i$ in the F-11 cells transfected with both TRPV1 and TRPA1 after desensitization, a finding identical to that observed in DRG neurons that contained both TRPV1 and TRPA1 receptors. Given that propofol has directly activated TRPA1 receptors¹⁷ but did not cause an increase in $[\text{Ca}^{2+}]_i$ after capsaicin-induced desensitization of TRPV1 in both DRG neurons and F-11 cells, propofol appears to initiate a Ca^{2+} -independent signaling cascade *via* TRPA1 that restores the

sensitivity of TRPV1 in sensory neurons. As stated previously, these questions are being assessed. In addition, the overall importance of our findings regarding propofol's modulation of TRPV1 function would be strengthened by assessing the effect of endogenous activators of TRPV1, such as protons. In the current study, we only investigated one activator of the TRPV1 signaling pathway that is not an endogenous mediator of the receptor. Therefore, the translational impact of this investigation may be limited. Further studies are under way using other endogenous activators of TRPV1, including acid load and heat.

In total, our novel finding that propofol dose dependently restores TRPV1 sensitivity to agonist stimulation *via* a TRPA1-dependent signaling pathway and that TRPA1 activation restores TRPV1 sensitivity to agonist stimulation in sensory neurons could have a few important clinical implications. First, the mechanisms of TRPV1 and TRPA1 interactions are of great importance because knowledge of their interactions may prove vital to uncover novel treatment modalities for various clinically important pathological conditions, such as diabetic neuropathy and inflammatory pain.^{23–25} Second, the current findings imply that propofol may contribute to peripheral sensitization to nociceptive endogenous inflammatory mediators at the site of tissue injury. The broad translational impact of the current findings may be extended into other organ systems, including the cardiovascular system.^{26–28} Recent evidence has indicated that the TRPV1 and TRPA1 receptors are important in blood pressure regulation,^{27,28} and defective cross talk between these receptors may occur during the metabolic syndrome^{29,30} and explain some of the pathophysiological characteristics associated with autonomic system reflexes and potentially vasovagal/neurocardiogenic syncope disorders.²⁷ Moreover, a recent review article³¹ highlighting the importance of the TRP cation channels in disease indicates a plethora of organs systems that may be affected, including the immune, respiratory, reproductive, urinary, and endocrine systems.

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