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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—Crosstalk between peripheral nociceptors belonging to the transient receptor potential vanilloid receptor subtype-1 (TRPV1) and ankyrin subtype-1 (TRPA1) family has recently been demonstrated. Moreover, the intravenous anesthetic propofol has been shown to directly activate TRPA1 receptors, and indirectly restore 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. Intracellular Ca²⁺ concentration was measured in individual cells *via* fluorescence microscopy. Following TRPV1 de-sensitization with capsaicin (100 nM), cells were treated with propofol (1, 5 and 10 μM) alone, propofol in the presence of the TRPA1 antagonist, HC-030031 (0.5 μM) or the TRPA1 agonist, Allyl isothiocyanate (AITC, 100 μM) and 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 following de-sensitization 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 were 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 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 mechanism(s) mediating sensitization, de-sensitization and resensitization of TRPV1 receptors is complex and involve calcium-dependent and independent mechanisms.^{5–9} Recent evidence has suggested that TRPV1 and TRPA1 receptors exhibit reciprocal regulation, indicating cross-talk between the two receptors.^{10,11} Specifically, these receptors have been shown to cross-sensitize or cross-desensitize each other in which 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 study indicated that propofol directly activates TRPA1 receptors in transfected human embryonic kidney 293 cells.¹⁷ In addition, our laboratory has recently shown that propofol restores sensitivity of TRPV1 receptors following agonist-induced de-sensitization and attenuates agonist-induced de-sensitization *via* a protein kinase C ϵ -dependent signaling pathway in mouse dorsal root ganglion (DRG) sensory neurons.¹⁸ However, no study to date 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 findings are that in DRG neurons containing both TRPV1 and TRPA1, pretreatment with propofol or Allyl isothiocyanate (AITC), restores TRPV1 sensitivity to agonist stimulation in previously de-sensitized DRG neurons. In contrast, restoration of TRPV1 sensitivity to agonist stimulation by propofol or AITC was not observed in DRG neurons containing only TRPV1 receptors (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 (lacking TRPA1), neither propofol nor AITC restore 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 also 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—12 week old male C57BL/6 mice 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 (30–40g) were used in this study. The ganglia were dissected from the lumbar (L1–L6) segments of the spinal cord and incubated with collagenase (Type IV, 0.15%) at 37°C for 50 min and dissociated by gentle trituration. Neurons were cultured on cover glasses precoated with poly-D-lysine and laminin at 37°C in F-12/DMEM (50/50) medium supplemented with 10% fetal bovine serum, 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 μM) in the medium. Cells began to develop neurites within ~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 the 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 (PBS) without Ca²⁺ or Mg²⁺. The cells were resuspended in 100 μl of electrolytic Buffer R in a sterile eppendorff tube and 5 μg of 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. Following electroporation, the cells were then placed in F-12/DMEM (50/50) supplemented with 10% fetal bovine serum at 37°C. A proportion of the cells were seeded into a 6-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 immuno-blot 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 acetoxymethyl ester (fura-2/AM; 2 μM) 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). Cover slips containing the fura-2-loaded DRG neurons were placed in a temperature-regulated (30°C) chamber (Warner Instruments, Hamden, CT) mounted on the stage of an Olympus IX-81 inverted fluorescence microscope (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 Ca²⁺ concentration ([Ca²⁺]_i) measurements were simultaneously performed on multiple individual DRG neurons using a fluorescence imaging system (Easy Ratio Pro, Photon Technology International, Lawrenceville, NJ) equipped with a multi wavelength spectrofluorometer (DeltaRAM X) and a QuantEM 512SC electron multiplying Charged-Coupled Device camera (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. Due to the fact that calibration procedures rely on a number of assumptions, the ratio of the light intensities at the two wavelengths was used to measure qualitative changes in [Ca²⁺]_i. Just before data acquisition, background fluorescence was measured and automatically subtracted from the subsequent experimental measurement using software from Photon Technology International (Easy Ratio Pro).

Immuno-blot Analysis of TRPA1 and TRPV1—Immuno-blot analysis was carried out on whole F-11 cell lysates as previously described 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 SDS-PAGE on 12% polyacrylamide gels and transferred to nitrocellulose membranes. Nonspecific binding

was blocked with Tris-buffered saline solution (0.1% (v/v) Tween-20 in 20 mM Tris base, 137 mM NaCl adjusted to pH 7.6 with HCl), containing 3% (w/v) bovine serum albumin for 1 h at room temperature. Antibodies against total TRPV1 and TRPA1 were diluted 1:1000 in Tris-buffered saline containing 1% bovine serum albumin for immuno-blotting (2 h). After washing in Tris-buffered saline 3 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 1% bovine serum albumin). Membranes were again washed and bound antibody was detected by enhanced chemiluminescence. Immunoreactivity was quantified by scanning densitometry and analyzed using ImageJ software (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 cover-slips of F-11 cells. Results obtained from each animal (for DRG experiments) or cover-slip of cells (for F-11 cell experiments) were averaged so that all animals or cover-slips 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 the figures refer to the variability in the peak Ca^{2+} response compared to the control response or a specific intervention as noted in the legend. Statistical analysis was conducted using NCSS software (Kaysville, UT).

Experimental Protocols

DRG neurons were chosen for study by distinguishing small (less than 30 μm) nociceptive neurons (capsaicin-sensitive) from large (more than 40 μm) mechano-sensitive neurons (capsaicin-insensitive) using a precalibrated eyepiece reticle to measure the diameter of the neuronal cell body. The Diprivan® (Astra Zeneca, London, United Kingdom) lipid emulsion form of propofol (56 mM [10 mg/ml] propofol, 10% soybean oil, 2.25% glycerol, 1.2% purified phospholipid) was used in all protocols and will be referred to as propofol throughout the manuscript. 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 containing both TRPV1 and TRPA1 receptors were first identified 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, while 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 repeatedly exposed to capsaicin (100 nM) every 30 s to induce de-sensitization of the channel. Following de-sensitization, neurons were exposed to propofol (10 μM , 10 min) and capsaicin was then 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 percent 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 further determine 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 (lacking TRPA1). In addition, the effect of the specific TRPV1 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 percent 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 further determine 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 repeatedly exposed to capsaicin as described in Protocol 1. Following de-sensitization, 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 only functional TRPV1 receptors were first identified by treating the cells with a 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 percent of the response to the final application of capsaicin during the initial de-sensitization of TRPV1.

Protocol 4: Effect of Propofol on Restoration of TRPV1 Sensitivity to Agonist Stimulation in Cultured F-11 Cells Expressing TRPV1 Only

—To further determine 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 first identified 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 percent of the response to the final application of capsaicin during the initial de-sensitization of TRPV1.

Materials—F-11 cells were the kind gift of 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, CO). Fura-2/AM. was obtained from Texas Fluorescence Laboratories (Austin, TX). Anti-TRPV1 antibody, capsaicin and AITC were purchased from σ Chemical Co. (St. Louis, MO). HC-030031 was purchased from Enzo Life Sciences (Plymouth Meeting, PA). Propofol (Diprivan® formulation) and Intralipid were obtained from Cleveland Clinic pharmacy (Cleveland, OH). Collagenase was obtained from Worthington Biochemical Corp. (Lakewood, NJ). TRPV1 and TRPA1 cDNA were kindly provided by David Julius (Ph.D., Professor, Dept. of Physiology, University of California at San Francisco, San Francisco, California).

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 first identified 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 also determined *via* immuno-blot analysis of DRG cell lystates (fig. 1A, inset). TRPV1 was expressed in F-11 cells transfected with TRPV1 cDNA while TRPA1 was not present. Repetitive stimulation of the preselected DRG neurons with capsaicin resulted in a progressive decrease (de-sensitization) in peak $[Ca^{2+}]_i$ that was maintained following 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 DRG's 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 ($99.1 \pm 6\%$ 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 first identified 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 (de-sensitization) in peak $[Ca^{2+}]_i$ that was maintained following 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

Verification of TRPV1 and TRPA1 protein expression was first determined *via* immuno-blot analysis of F-11 cell lystates obtained from the same group of transfected cells that were 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) 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

Verification of TRPV1 protein expression was first determined *via* immuno-blot analysis of F-11 cell lysates obtained from the same group of TRPV1 transfected cells that were 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, 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 $[Ca^{2+}]_i$ while 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 is 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

This is the first study to assess the role of TRPA1 receptor activation on TRPV1 dependent Ca^{2+} signaling as well as mediating the propofol-induced restoration of TRPV1 sensitivity to agonist stimulation. Recent studies have shown that TRPA1 and TRPV1 exhibit cross-talk between each other resulting in modulation of receptor sensitivity to agonist stimulation.^{10,10–16} Recent studies have indicated that acute TRPA1 activation can de-sensitize TRPV1 receptors and *vice versa*.^{15,16} However, the role of TRPA1 activation on restoration of TRPV1 sensitivity in previously de-sensitized 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 sensitivity.¹⁸ Therefore, 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. 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 previously described by others.^{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 functional TRPV1 and TRPA1 receptors were treated with propofol following de-sensitization with capsaicin and $[Ca^{2+}]_i$ was measured in response to another challenge 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 de-sensitized 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 unexpected finding was that addition of propofol or AITC following TRPV1 de-sensitization did not elicit any transient increase in $[Ca^{2+}]_i$ (fig. 2C and fig. 2E) 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 due to the lack of TRPA1 receptors in the population of DRG neurons being studied. However, the population of DRG neurons utilized 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 following TRPV1 de-sensitization did not elicit any transient increase in $[Ca^{2+}]_i$ may be due to a TRPV1 de-sensitization-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(s) that can modulate TRPV1 activity. In fact, a recent study demonstrated that TRPA1 is regulated by TRPV1 independent of intracellular Ca^{2+} .²¹ In addition, we recently demonstrated that propofol restores TRPV1 sensitivity to agonist stimulation *via* activation of protein kinase C ϵ , which is a Ca^{2+} independent PKC 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 currently 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 indicating cross-talk between TRPV1 and TRPA1 receptors which regulates and modulates nociceptive ion channel function.^{10,10–16}

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 (lacking TRPA1) were treated with propofol or AITC following de-sensitization with capsaicin and $[Ca^{2+}]_i$ was measured in response to another challenge with capsaicin. Our findings indicate that in DRG neurons that contain only functional TRPV1 receptors (lacking TRPA1), neither propofol nor AITC were 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 further substantiate 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 further characterize the role of TRPA1 in the propofol-induced restoration of TRPV1 sensitivity. Subsequently, we next 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-induced 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 if the presence of TRPA1 receptors is required for propofol to restore TRPV1 sensitivity, we next 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 further substantiate the TRPA1 receptor as a key component of the signaling mechanism(s) 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 recent studies have indicated that TRPA1 activation *de-sensitizes* TRPV1 receptors.^{15,16} An important distinction that needs to be addressed is that these studies investigated the affect of acute 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.*, de-sensitized or not) to agonists could also affect how other receptors, most notably TRPA1, responds to particular regulatory pathways such as TRPV1 activation. One could surmise that the process of TRPV1 de-sensitization could initiate an intracellular signaling pathway that alters the functional characteristics of the TRPA1 receptor. Whether or not de-sensitization of TRPV1 alters TRPA1 sensitivity to agonists is currently being investigated by our laboratory. In addition, we also found it interesting that in DRG neurons, TRPV1 receptors appear to desensitize more rapidly when TRPA1 receptors are not present, as indicated by a reduced number of capsaicin applications needed to fully de-sensitize the receptor. This observation substantiates our finding that TRPA1 activation can restore the sensitivity of de-sensitized TRPV1 receptors. Moreover, propofol and AITC also failed to elicit any transient increase in $[Ca^{2+}]_i$ in the F-11 cells transfected with both TRPV1 and TRPA1 following de-sensitization, a finding identical to that observed in DRG neurons that contained both TRPV1 and TRPA1 receptors. Given that propofol has been shown to directly activate TRPA1 receptors,¹⁷ but did not cause an increase in $[Ca^{2+}]_i$ following capsaicin-induced de-sensitization 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 previously stated, these questions are currently being assessed. In addition, we acknowledge that 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 which is not an endogenous mediator of the receptor. Therefore, the translational impact of this investigation may be limited. Further studies are currently underway 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 is of great importance as knowledge of their interactions may prove vital to uncover novel

treatment modalities for various clinically-important pathologies such as diabetic neuropathic and inflammatory pain.^{23–25} Moreover, the current findings also implicate 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 metabolic syndrome,^{29,30} as well as explain some of the pathophysiology associated with autonomic system reflexes and potentially to vasovagal/neurocardiogenic syncope disorders.²⁷ Moreover, a recent review article 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 just to name a few.³¹

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Figure 1.

Figure 1A: Representative trace depicting the effect of a single application of capsaicin (Cap; 100 nM), the specific transient receptor potential ankrin receptor type 1 (TRPA1) activator Allyl isothiocyanate (AITC, 100 μ M), or propofol (10 μ M) on intracellular free calcium concentration in mouse dorsal root ganglion (DRG) neurons. Inset: Representative immuno-blot depicting TRPA1 and transient receptor potential vanilloid receptor type 1 (TRPV1) protein expression in cultured DRG neurons. Representative traces depicting the effect of time, propofol (10 μ M), propofol in the presence of the specific TRPA1 inhibitor HC-030031 (0.5 μ M), and AITC (100 μ M) following capsaicin-induced (100 nM) desensitization on restoration of transient receptor potential vanilloid receptor type 1 sensitivity in mouse DRG neurons that contain functional TRPV1 and TRPA1 receptors are depicted in Fig. 1B, 1C, 1D and 1E, respectively. Summarized data for Fig. 1B–E are depicted in Fig. 1F. Data are expressed as a percent of the response to the final application of capsaicin (Final Cap) in the untreated control (% of Control) and reported as the mean value $\pm$ SD * = P less than 0.05 compared to final capsaicin in the untreated control. # = P less than 0.05 compared to propofol (Pro) plus Capsaicin (Cap). n = DRG neurons from 5 different mice.

Figure 2.

Figure 2A: Representative trace depicting the effect of a single application of capsaicin (Cap; 100 nM), the specific transient receptor potential ankrin receptor type 1 (TRPA1) activator Allyl isothiocyanate (AITC, 100 μM), or propofol (Pro, 10 μM) on intracellular free calcium concentration in mouse dorsal root ganglion (DRG) neurons. Representative traces depicting the effect of time, propofol (10 μM) and AITC (100 μM) following capsaicin-induced (100 nM) de-sensitization on restoration of TRPV1 sensitivity in mouse DRG neurons that contain only functional TRPV1 receptors are depicted in Fig. 2B, 2C and 2D, respectively. Summarized data for Fig. 2B–D are depicted in Fig. 2E. Data are expressed as a percent of the response to the final application of capsaicin (Final Capsaicin) in the untreated control (% of Control) and reported as the mean value ± SD n = DRG neurons from 5 different mice.

Figure 3.

Figure 3A: Representative immuno-blot depicting transient receptor potential ankyrin receptor type 1 (TRPA1) and the transient receptor potential vanilloid receptor type 1 (TRPV1) protein expression in F-11 cells transfected with both TRPA1 and TRPV1 complementary DNA (cDNA). Fig. 3B: Representative trace depicting the effect of a single application of capsaicin (Cap; 100 nM), the specific TRPA1 activator Allyl isothiocyanate (AITC, 100 μ M), or propofol (10 μ M) on intracellular free calcium concentration in F-11 cells transfected with both TRPA1 and TRPV1 cDNA. Fig. 3C: Representative trace depicting the effect of propofol (5 μ M) following capsaicin-induced (100 nM) de-sensitization on restoration of TRPV1 sensitivity in F-11 cells transfected with both TRPA1 and TRPV1 cDNA. Dashed line indicates the statistical comparison that was made. Fig. 3D: Summarized data depicting the effect of propofol (Pro; 1, 5, 10 μ M) following capsaicin-induced de-sensitization on restoration of TRPV1 sensitivity in F-11 cells transfected with both TRPA1 and TRPV1 cDNA. Data are expressed as a percent of the response to the final application of capsaicin before propofol treatment (Control) and reported as the mean value $\pm$ SD * = P less than 0.05 compared to Control. n = 9 separate cover-slips of F-11 cells.

Figure 4.

Figure 4A: Representative trace depicting the effect of the specific transient receptor potential ankyrin receptor type 1 (TRPA1) activator Allyl isothiocyanate (AITC, 100 μ M) following capsaicin-induced (100 nM) de-sensitization on restoration of transient receptor potential vanilloid receptor type 1 (TRPV1) sensitivity in F-11 cells transfected with both TRPA1 and TRPV1 complementary DNA (cDNA). Fig. 4B: Summarized data depicting the effect of AITC following capsaicin-induced de-sensitization on restoration of TRPV1 sensitivity in F-11 cells transfected with both TRPA1 and TRPV1 cDNA. Data are expressed as a percent of the response to the final application of capsaicin before AITC treatment (Control) and reported as the mean value $\pm$ SD * = P less than 0.05 compared to Control. n = 4 separate cover-slips of F-11 cells.

Figure 5.

Figure 5A: Representative immuno-blot depicting transient receptor potential ankrin receptor type 1 (TRPA1) and transient receptor potential vanilloid receptor type 1 (TRPV1) protein expression in F-11 cells transfected with TRPV1 complementary DNA (cDNA) only. Fig. 5B: Representative trace depicting the effect of a single application of capsaicin (Cap; 100 nM), Allyl isothiocyanate (AITC, 100 μ M), or propofol (Pro, 10 μ M) on intracellular free calcium concentration in F-11 cells transfected with TRPV1 cDNA only. Fig. 5C: Representative trace depicting the effect of propofol (5 μ M) following capsaicin-induced (100 nM) de-sensitization on restoration of TRPV1 sensitivity in F-11 cells transfected with TRPV1 cDNA only. Dashed line indicates the statistical comparison that was made. Fig. 5D: Summarized data depicting the effect of propofol (Pro; 1, 5, 10 μ M) following capsaicin-induced de-sensitization on restoration of TRPV1 sensitivity in F-11 cells transfected with TRPV1 cDNA. Data are expressed as a percent of the response to the final application of capsaicin before propofol treatment (Control) and reported as the mean value $\pm$ SD. n = 9 separate cover-slips of F-11 cells.

Figure 6.

Figure 6A: Representative trace depicting the effect of the specific transient receptor potential ankrin receptor type 1 (TRPA1) activator Allyl isothiocyanate (AITC, 100 μ M) following capsaicin-induced (100 nM) de-sensitization on restoration of transient receptor potential vanilloid receptor type 1 (TRPV1) sensitivity in F-11 cells transfected with TRPV1 complementary DNA (cDNA) only. Dashed line indicates the statistical comparison that was made. Fig. 6B: Summarized data depicting the effect of AITC following capsaicin-induced de-sensitization on restoration of TRPV1 sensitivity in F-11 cells transfected with TRPV1 cDNA only. Data are expressed as a percent of the response to the final application of capsaicin before AITC treatment (Control) and reported as the mean value $\pm$ SD. $n = 4$ separate cover-slips of F-11 cells.